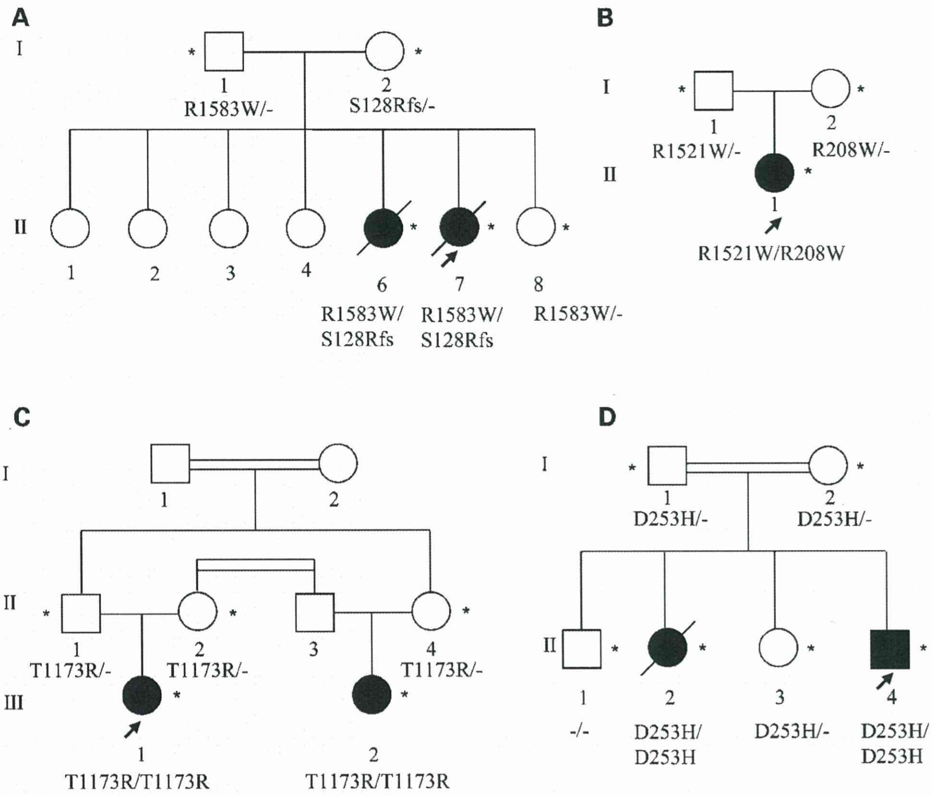

Figure 1. Pedigree of the families with the *ABCA3* mutations p.R1583W/p.S128Rfs (**A**), p.R1521W/R208W (**B**), p.T1173R/p.T1173R (**C**) and p.D253H/p.T1173R (**D**). Asterisks indicate family members with ABCA3 mutation analysis, and arrows indicate index patients.

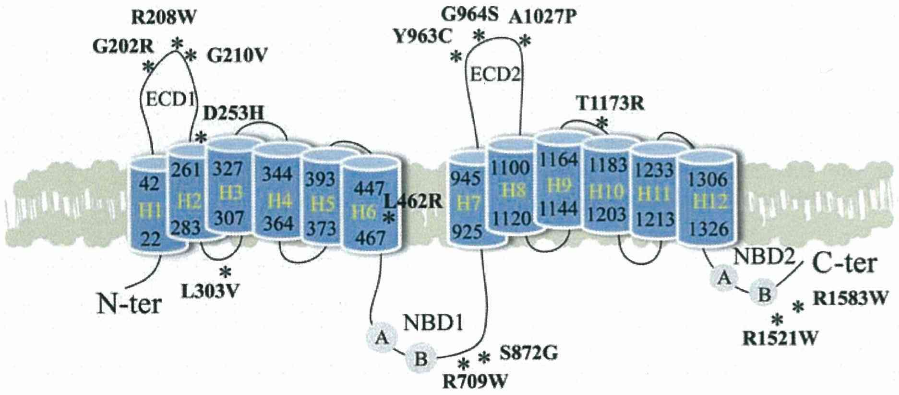

Figure 2. Schematic representation of the ABCA3 protein [N-terminal (N-ter) to C-terminal (C-ter) domains] and the location of the novel mutations (indicated by an asterisk). The 12 putative transmembrane helix (H1–H12) domains, two extracellular domains (ECD1 and ECD2) and two nucleotide-binding domains (NBD) (including the conserved motifs Walker A and B) are represented.

Characteristics of patients with *ABCA3* mutations

The characteristics of the 10 children with ABCA3 mutations are reported in Table 2. Among them, nine (90%) had NRD, five progressed to ILD and five died of respiratory failure (all during the first year of life).

Three children harboring homozygous and compound heterozygous mutations who developed ILD (patients 1, 2 and 6) were treated with methylprednisolone pulse for, respectively, 14 months (patient 1), 6 years (patient 2) and 11 years (patient 6, who is still on methylprednisolone pulse). They also received azithromycin for, respectively, 2 years (patient 1), 12 years (patient 2) and 2 years (patient 6), patients 2 and 6 still being treated. Two patients (patients 2 and 6) required prolonged oxygen supplementation for, respectively, 10 years (patient 2) and 11 years (patient 6, who is still on oxygen).

Two children harboring heterozygous mutations developed ILD (patients 10 and 9). The first child (patient 10) was

Table 2. Clinical characteristics of the 10 patients with *ABCA3* mutations

	ABCA3-mutated patients ($n = 10$)
Sex: male/female, *n* (%)	4 (40)/6 (60)
Median age at onset in months (range)	0 (0–6)
Neonatal respiratory distress, *n* (%)	9 (90)
Hypoxemia, *n* (%)	10 (100)
Physical findings, *n* (%)	
Tachypnea	10 (100)
Retractions	10 (100)
HRCT findings, *n* (%)	
Ground-glass opacities	8 (100)
Lung cysts	3 (38)
Interlobular septal thickening	2 (25)
Consolidation	4 (50)
Lung biopsy, *n* (%)	
Type-II cell hyperplasia	6 (100)
Septal thickening	6 (100)
Mild fibrosis	5 (83)
Intra-alveolar macrophages	6 (100)
Outcome *n* (%)	
ILD/death	5 (50)/5 (50)

ILD, interstitial lung disease; *n* corresponds to the number of available patients' clinical data.

treated by monthly methylprednisolone pulse for 3 years and required oxygen supplementation for 8 months. The second one (patient 9) did not receive any treatment during the study period.

All 10 patients had clinical symptoms of respiratory failure. High-resolution computed tomography (HRCT) scans were available for eight patients and predominantly showed ground-glass opacities (Fig. 3A and Supplementary Material, Supporting Information 2). Lung biopsy was performed in six patients, all of whom had alveolar septal thickening, a few interstitial inflammatory cells (polymorphonuclear neutrophils and lymphocytes), uniform prominent type-II cell hyperplasia and accumulation of intra-alveolar macrophages (Fig. 3B). Electron microscopy was performed on a lung biopsy from the patient harboring the p.D253H mutation and showed abnormal lamellar bodies with dense inclusions (Fig. 3C).

BALF analysis

Western blot analysis of surfactant proteins (Fig. 4) was performed in seven patients, who had the following *ABCA3* mutations: p.D253H (patient 2), p.T1173R (patient 1), p.L462R/p.G964S (patient 3), p.G202R/p.L303V (patient 7), p.Y963C (patient 8), p.R1583W/p.S128Rfs (patient 4) and p.S872G (patient 10), respectively. SP-C (Fig. 4A) and SP-B (Fig. 4B) were detected at a size of 3.7 and 8 kDa in variable amounts, the smallest amount being found in the patient with the p.G202R/p.L303V *ABCA3* mutation (patient 7) in whom SP-C and SP-B are only faintly visible. This patient was the only one who cannot be weaned off mechanical ventilation before he died of respiratory failure. Interestingly, the two children harboring homozygous mutations (patients 1 and 2) have small but detectable amounts, which were nonetheless compatible with life. In contrast, patient 4, who died within the first month of life, had amount of SP-B and SP-C similar to control.

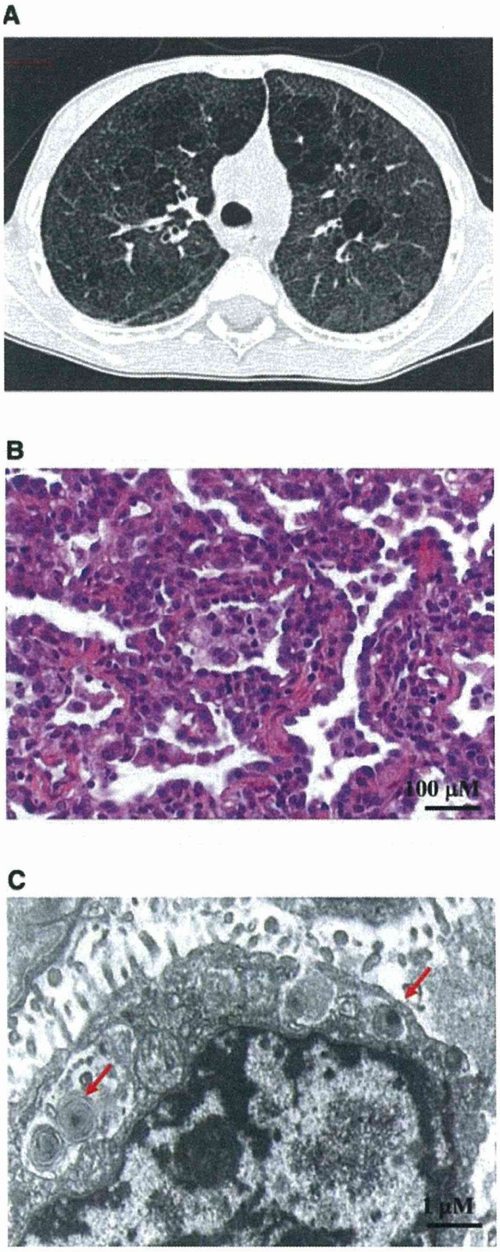

Figure 3. (**A**) HRCT scan, (**B**) hematoxylin and eosin (HE) staining of lung tissue and (**C**) electron microscopy of type-II cells from the patient (patient 2) harboring the *ABCA3* homozygous mutation p.D253H. Red arrows indicate lamellar bodies.

Characterization of ABCA3 mutations

The two mutations p.T1173R and p.D253H were deliberately chosen for subsequent functional studies because they were homozygous. Also, since past functional studies of ABCA3 focused mainly on NRD-associated mutations, it was crucial to study the consequences of these two mutations associated with progression towards ILD.

ABCA3 localization and processing. We first investigated the intracellular localization of the mutated ABCA3 protein.

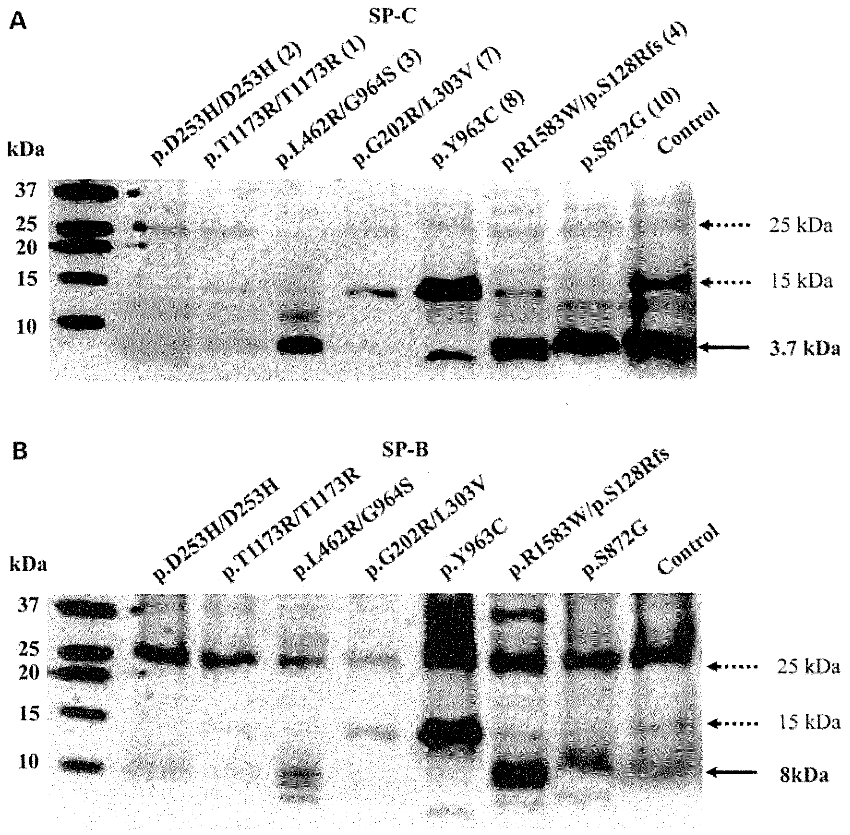

Figure 4. Western blot analysis of surfactant proteins B and C (SP-B and SP-C, respectively) in BALF from seven patients with *ABCA3* mutations and from a control without ILD. Dashed arrows indicate proSP-C (15 and 25 kDa) and proSP-B (15 and 25 kDa). Plain arrows indicate mature SP-C (3.7 kDa) and SP-B (8 kDa).

Similar to the WT protein, ABCA3 mutants co-localized with Lysotracker staining and partially with ERtracker staining (Fig. 5). ABCA3 might co-localize with ER during their folding. However, no accumulation in the ER was observed. The same results were obtained with transiently transfected cells (data not shown). Thus, p.D253H and p.T1173R mutants were not associated with a localization defect. ABCA3 expression was studied on protein extracts from A549-transfected cells. In WT, p.T1173R and p.D253H cells, anti-GFP antibody revealed two bands of 180 kDa (150 kDa ABCA3 + 30 kDa GFP) and 220 kDa (190 + 30 kDa GFP), respectively (Fig. 6). As previously suggested, these two bands might reflect two processing forms (15–18).

Lamellar bodies in ABCA3 WT, p.D253H and p.T1173R cells. Lamellar bodies were not observed in A549 cells (Fig. 7). As shown previously with HEK293 cells (15), transfection of ABCA3 in A549 cells is sufficient to induce lamellar body formation (Fig. 7). The p.D253H mutation induced abnormal lamellar bodies with electron-dense inclusions (Fig. 7, dashed arrows), consistent with the results of the patient's lung biopsy (Fig. 3C). In cells transfected with the p.T1173R mutation, abnormal lamellar bodies are the most frequently observed (irregularly arranged, phospholipid lamellae but eccentrically packed), even if some appeared almost normal.

Cytokine production by ABCA3 WT, p.D253H and p.T1173R cells. We next investigated whether ABCA3 mutants could induce inflammation. IL-8 was chosen as a well-known marker in inflammatory chronic lung disease. Using quantitative PCR (qPCR), we found that IL-8 mRNA levels were increased in p.T1173R cells (Fig. 8A). A significant, faint increase in IL-8 mRNA expression was also observed between WT and p.D253H cells. ABCA3 mRNA levels were similar in WT and mutated cells (data not shown), indicating that the increased IL-8 mRNA level in p.T1173R cells was not due to a transfection issue. At the protein level, ELISA results confirmed that A549 cells expressing the p.T1173R mutant produced more IL-8 than did WT cells (Fig. 8A). In contrast, IL-8 production by p.D253H cells was similar to that of WT cells (Fig. 8B). Finally, TGF-β and MCP-1secretions were similar between WT and transfected cells (data not shown).

IL-8 production is controlled chiefly by MAPK and NF-κB signaling (19). To determine whether these signaling pathways were involved in the observed IL-8 overproduction by p.T1173R cells, we used specific inhibitors. Treatment of cells with inhibitors of MAPK (p38, JNK, ERK1/2) and NF-κB showed that IL-8 production (in WT and mutant cells) was mainly ERK1/2 dependent. The lack of involvement of NF-κB was confirmed by measuring NF-κB promoter activity as done previously (20) (data not shown). However, with

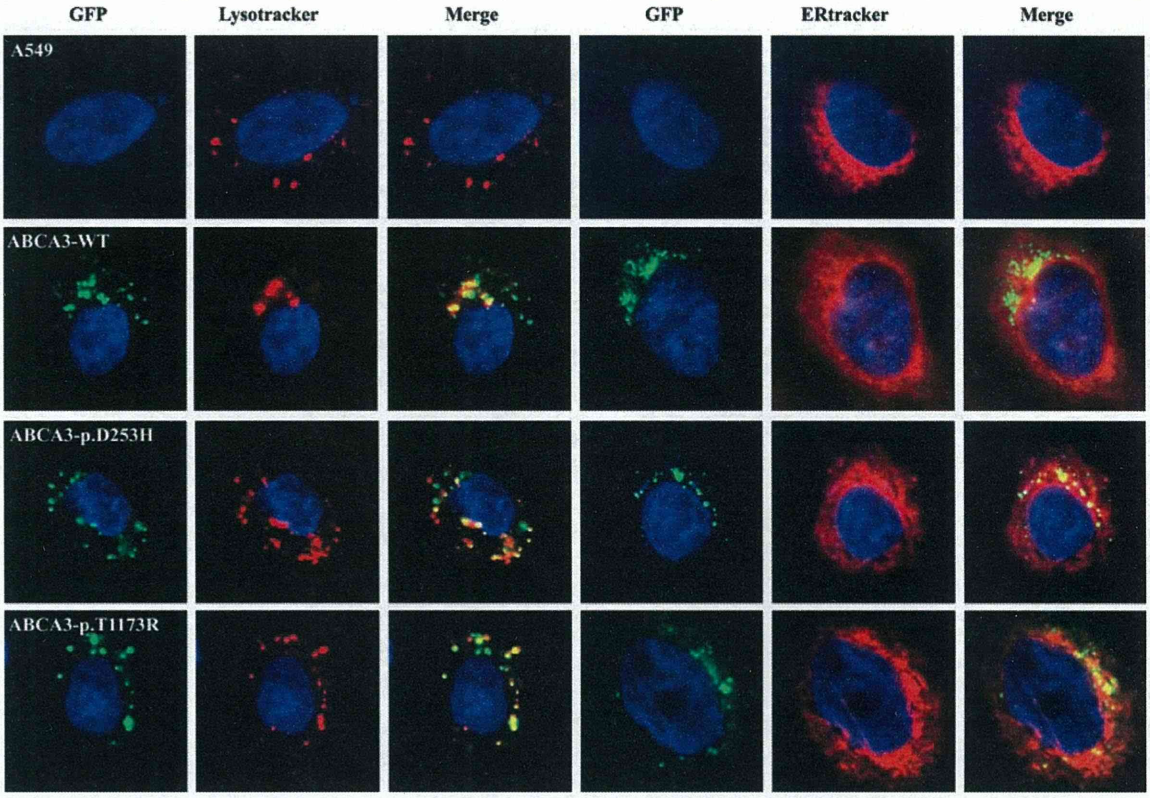

Figure 5. Intracellular localization of wild-type ABCA3 and of the p.D253H and p.T1173R mutants. A549 cells either non-transfected or transfected with mock vector, wild-type protein ABCA3-WT (**A**) or mutated proteins p.D253H (**B**) and p.T1173R (**C**) were analyzed using confocal microscopy. Lysotracker and ERtracker were used to stain lysosomes and the endoplasmic reticulum, respectively.

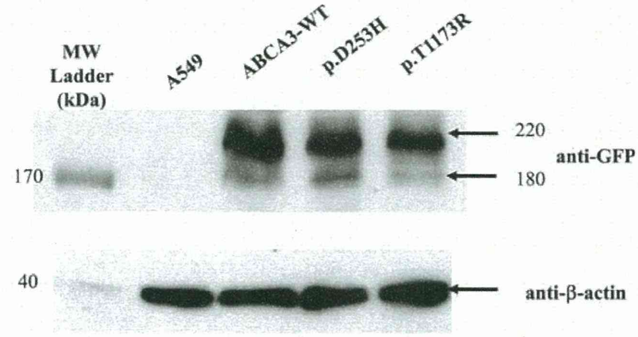

Figure 6. Western blot analysis of ABCA3 in cells transiently transfected with ABCA3-WT or the p.D253H or p.T1173R mutation. ABCA3 expression was detected using anti-GFP antibody (top panel). Equal loading was verified using anti-β actin antibody (bottom panel).

ERK1/2 inhibitor treatment, IL-8 production by p.T1173R cells remained increased compared with WT and p.D253H cells. These results suggest that, even if ERK1/2 signaling is involved in IL-8 production, another signaling pathway may be involved in the increased IL-8 production detected in p.T1173R cells.

Western blot and relative quantification of ERK1/2 phosphorylation confirmed that the observed IL-8 overproduction in p.T1173R cells was independent of ERK1/2 signaling. Finally, caspase 3/7 activity was similar in WT and mutant cells, indicating that these mutations did not induce apoptosis.

DISCUSSION

We identified 15 (13 novel) *ABCA3* mutations in 47 children (32%) who had NRD and/or ILD and no *SFTPB* or *SFTPC* mutations (5). None of these mutations was found in either the public polymorphism database or our controls. The amino acids affected by the mutations were conserved in mammalian *ABCA3* sequences. All 10 patients with *ABCA3* (21%) mutations had severe respiratory symptoms and abnormal chest imaging findings. Ninety percent of patients harboring *ABCA3* mutations had NRD. Finally, parents heterozygous for the p.R1583W, p.S128Rfs, p.R1521W or p.R208V mutations were not affected. In three patients, a mutation was found in a single allele but the clinical phenotype (NRD) may support the existence of a second mutation (in introns, deletions and so on) not detected by our sequencing method. Haploinsufficiency has been suggested as a mechanism leading to clinical phenotype emergence in patients with only one mutated allele (10,21). Similarly, *Abca3*$^{+/-}$ mice, despite normal respiratory function, had fewer lamellar bodies and altered surfactant lipid synthesis compared with wild-type mice, suggesting susceptibility to NRD or ILD (22). However, even if the observed clinical phenotypes are

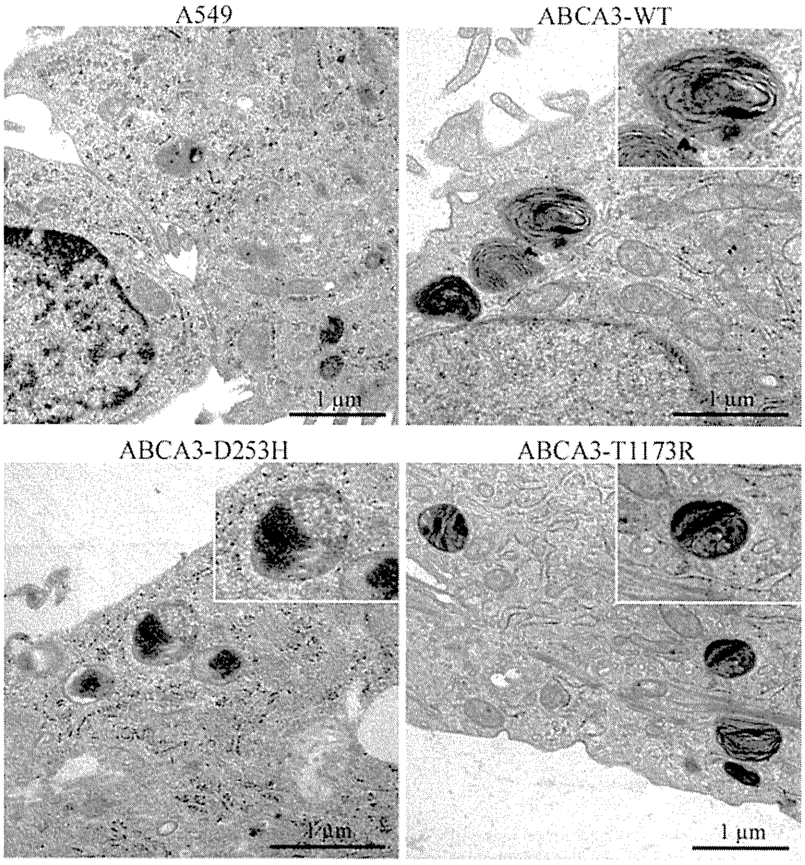

Figure 7. Lamellar body analysis. A549 cells transfected with mock vector (pEGFP-N1), ABCA3-WT (**A**), or mutated ABCA3-D253H (**B**) or ABCA3-T1173R-GFP were analyzed using electron microscopy.

compatible with *ABCA3* deficiency, we cannot conclude that *ABCA3* heterozygosis is responsible for this phenotype.

As previously described (1,11,13), some patients with *ABCA3* mutations had a less severe phenotype than that usually associated with *ABCA3* mutations (10). These variations in the clinical and radiological features may be related to the nature of the mutation (16). Previous studies showed that *ABCA3* mutations led to abnormal processing and/or trafficking of the ABCA3 protein (15), alterations in ABCA3 protein functions such as ATPase activity (16), or impaired lipid transport (23). Interactions with variants in other genes and/or with external factors such as viral infections may also influence the observed phenotype (24).

ABCA3 deficiency in full-term patients with NRD was shown previously to be associated with abnormal processing of SP-B and SP-C with an accumulation of precursors of SP-B and absence of mature SP-C (11). In our patients with *ABCA3* deficiency, we observed that SP-C and SP-B expression levels varied considerably across patients. Indeed, less severe patients had decreased amounts of SP-B and SP-C, whereas patient who died of respiratory failure showed normal expression of both proteins. This discrepancy may be explained by our technique of western blot, which is performed on lyophilized supernatant and improved the level of surfactant protein detection. It may also be explained by the function of ABCA3, which is critical for the proper formation of lamellar bodies and surfactant function but is not responsible for SP-B or SP-C synthesis. Hence, the pattern of SPs may be independent of the clinical status. To confirm the hypothesis, it would have been interesting to compare the patterns of siblings harboring the p.D253H mutation. Unfortunately, BALF from this patient was not available. Finally, as reported previously for *SFTPC* mutations (25), the presence or absence of SP-B and SP-C might be neither sensitive nor specific for *ABCA3*-related diseases.

Electron microscopy examination of a lung biopsy specimen is the reference standard for evaluating lamellar body characteristics and for providing a preliminary diagnosis prior to *ABCA3* gene analysis. Indeed, abnormal lamellar bodies with electron-dense inclusions have been described in association with *ABCA3* mutations in previous studies (10,21,26). However, electron microscopy cannot be performed routinely. The presence of abnormal lamellar bodies in the patient harboring the p.D253H mutation supports a pathogenic effect of this mutation. These abnormalities were also observed *in vitro* in p.D253H- and p.T1173R-transfected cells, suggesting that *ABCA3* abnormalities may consistently induce abnormal lamellar bodies. However, since we do not have the corresponding biopsy from the patient harboring the p.T1173R, we cannot draw a firm conclusion on this point.

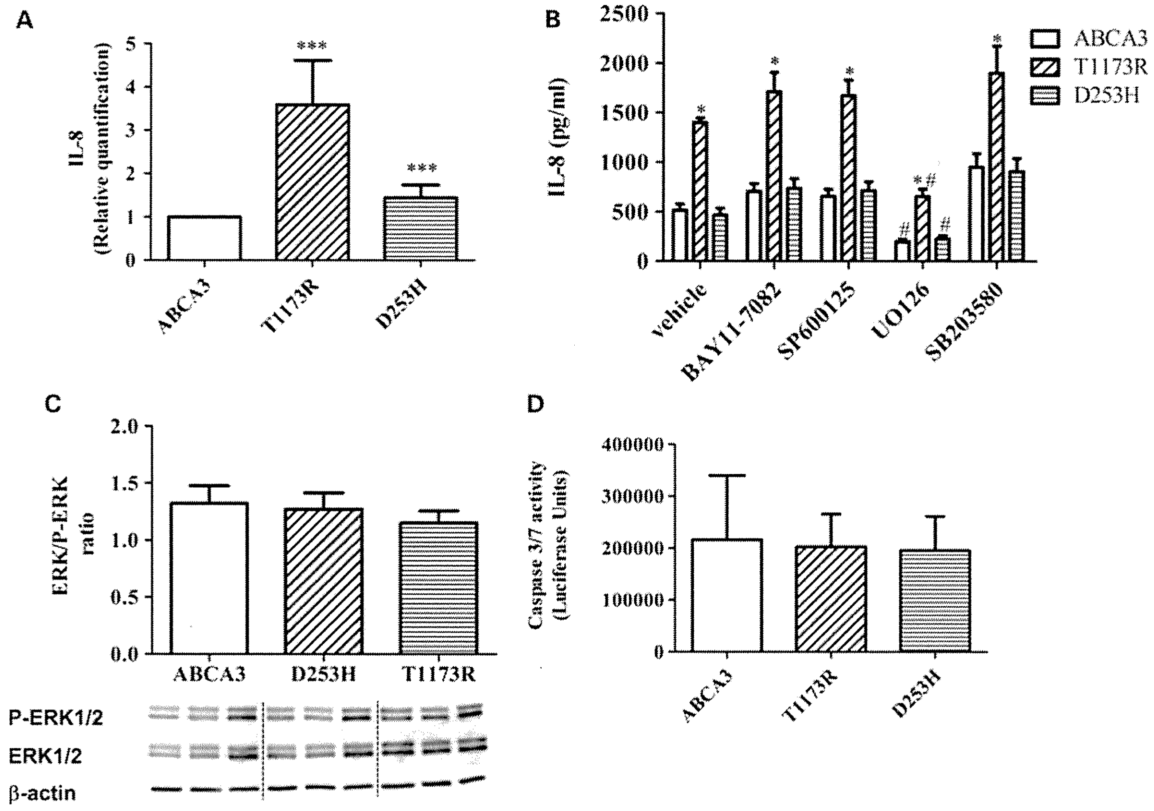

Figure 8. (**A**) IL-8 mRNA relative quantification was performed using qPCR. Results are representative of the mean ± SD of three experiments performed in triplicate. $***P \leq 0.001$. (**B**) Role for MAPK and NF-κB-dependent signaling in IL-8 secretion by ABCA3-WT, D253H and T1173R cells. Cells were treated with 10 μM inhibitors of ERK1/2 (U0126), p38 (SB203580), JNK (SP600125) or NF-κB (BAY11-7082) for 24 h; $*P \leq 0.05$: ABCA3 versus T1173R; $\#P \leq 0.05$: vehicle versus U0126. Supernatants were tested for IL-8 by ELISA. Data are means ± SD of three experiments performed in triplicate. (**C**) ERK1/2 phosphorylation measurement. Data are means ± SD of four experiments performed in triplicate. Results are expressed as P-ERK/ERK ratio (top panel). Western blot analysis of phospho-ERK1/2, total ERK1/2 and β-actin (bottom panel). (**D**) Caspase 3/7 activity. Data are means ± SD of three experiments performed in triplicate.

We performed *in vitro* experiments to elucidate the pathophysiological effects of two mutations associated with progression towards ILD, p.D253H and T1173R. These mutations did not alter the localization or maturation of the protein. Past functional studies on other *ABCA3* mutations showed localization/folding defects or functional defects (15,16,18,21,23,27). As pointed out recently, the effect of *ABCA3* mutations on lung epithelial cells depends on the ABCA3 protein defects (18). We found that the functional abnormalities differed between the two mutations. The p.D253H and p.T1173R mutations induced abnormal lamellar bodies. ABCA3 being a major transporter of phosphatidylcholine and phosphatidylglycerol into lamellar bodies, the lamellar body alterations suggest abnormalities in phospholipid trafficking that need to be characterized. The recent proteomic characterization of lamellar bodies may help to achieve this challenging objective (28). The p.T1173R mutation was also associated with increased production of IL-8, a well-known chemotactic molecule for neutrophils. Interestingly, increased IL-8 production was also detected in cells expressing *SFTPC* (Δexon 4 and the common p.I73T) mutations (29). In contrast, we do not find differences in TGF-β production, a cytokine that has been extensively studied in adults with idiopathic pulmonary fibrosis (30). Also, no differences in MCP-1 production were observed between WT and mutated cells. MCP-1 has been shown previously to contribute to the pathogenesis of pediatric ILD (31). However, in this study, none of the children had familial ILD, and surfactant genetic screening was not done, thus we do not know whether MCP-1 production is related to surfactant-associated disorders. In fact, surfactant genetic disorders are a subclass of pediatric ILD (2), which include various clinical phenotypes associated with specific clinical and biological features. Altered intercellular signaling was also shown recently in cells expressing *SFTPC* variants. CXCR1 and CCR2 expression by lymphocytes and neutrophils is probably dependent on an unidentified soluble mediator secreted by p.I73T cells (32). These studies, combined with our data, suggest that inflammatory pathways are involved in genetic surfactant disorders. However, better characterization of these pathways is required if specific treatments are to be sought.

In conclusion, although rare, ABCA3 deficiency should be considered in full-term newborns with severe respiratory distress and in older patients with ILD. Since *ABCA3* mutations lead to distinct functional defects, functional analysis of each *ABCA3* mutation is necessary to identify specific molecular targets that could be modulated or corrected by therapeutic agents.

MATERIALS AND METHODS

Patients

Through a national program on rare lung diseases, which has been described elsewhere (5), we recruited 121 pediatric patients with diffuse lung disease, over a 5-year period (2002–2007). Among the 121 patients with diffuse lung disease, 86 had respiratory distress, 59 presenting with neonatal onset (NRD) and 18 patients died. *ABCA3* genetic screening was performed in 47 children with severe respiratory distress or familial history compatible with autosomal recessive inheritance. Patients with presence of *SFTPC* or *SFTPB* mutations or insufficient information from medical records were excluded.

For each patient, we retrieved the following information from the medical records: family history, clinical presentation, findings by radiography and HRCT of the chest and lung biopsy findings (including those obtained by electron microscopy). The control for the BALF study was one child with uveitis who underwent bronchoscopy because of suspected sarcoidosis. Bronchoscopy showed no evidence of sarcoidosis or ILD. The control population for the genetic tests consisted of 23 individuals of European descent who had no history of lung disease. The protocol was accepted by the appropriate Committee for the Protection of Individuals in Biochemical Research, as required by French legislation. Written informed consent was obtained from the patients or their next of kin before study inclusion (5).

Genetic analysis

Genomic DNA was extracted from blood samples using an automated BioRobot EZ1 workstation (Qiagen, Hilden, Germany). Parental DNA was sequenced when samples were available. *ABCA3* primers were designed to amplify the 30 coding exons and their respective splice junctions (10). Primers were purchased from Sigma-Aldrich (Lyon, France) and Taq polymerase from Applied Biosystems (Foster City, CA, USA). Sequencing reactions were performed as described previously (5). Identified mutations were verified on two PCR products. Nucleotide numbering reflected *ABCA3* cDNA numbering, with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence NM_001089.2. The reference sequence NP_001080.2 of ABCA3 protein was used for amino acid numbering.

Histological examination of lung tissue

Lung tissue obtained by surgical biopsy was examined by light microscopy using a standard hematoxylin and eosin staining protocol. Electron microscopy was conducted using standard protocols.

Collection of BALF

We retrospectively analyzed BALF from seven children with *ABCA3* mutations. Fiber-optic bronchoscopy with bronchoalveolar lavage was performed under sedation, as previously described (33).

ABCA3 vectors

The pEGFPN1-ABCA3 plasmid, called ABCA3-WT hereafter, was obtained as described previously (16). Mutagenesis was induced using PCR-based site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA, USA). Mutagenesis primers (Sigma) were as follows: *D253H-For*-5′-ACCCGCCGTTCATCGCACACCCCTTCC-3′, *D253H-Rev*-5′-GGAAGGGGTGTGCGATGAACGGCGGGT-3′; *T1173R-For*-5′-ACGTGCGTGCCTTCAGGCGGGACG-3′, and *T1173R-Rev*-5′-CGTCCCGCCTGAAGGCACGCACGT-3′. Mutagenesis was confirmed by sequencing.

Cell culture and transfection

A549 cells were cultured as described previously (34). Cells (1×10^6) were transfected with 1 µg of ABCA3-WT, ABCA3-D253H or ABCA3-T1173R plasmid using a nucleofector device (Lonza, Cologne, Germany) as recommended by the manufacturer. For stable transfection, GFP-positive cells were selected using a FACSAria cell sorter (BD, Le Pont-De-Claix, France) and plated with 0.5 mg/ml of Geneticin (Invitrogen, Paisley, UK). Three weeks after selection, stably transfected cells were examined by immunofluorescence and maintained with 0.3 mg/ml of Geneticin. Experiments with transiently transfected cells (Lipofectamine, Invitrogen) were performed 24 h post-transfection. Analysis of NF-κB activation was done with NF-κB luciferase plasmid (20).

Fluorescence microscopy

Cells transfected transiently or stably with A549 were plated in 35 mm Petri dishes (iBidi, Martinsried, Germany). Living cells were stained with either Lysotracker red (lysosome probe) or ERtracker red (endoplasmic reticulum probe) (Invitrogen, Paisley, UK). DAPI (Sigma-Aldrich, Lyon, France) was used to stain the nucleus. Fluorescence microscopy was achieved using a Zeiss Axiovert 200 microscope (Zeiss, Le Pecq, France).

Cytokine/ERK ELISA and caspase 3/7

Cells stably transfected with A549 (1×10^5) were seeded in 96-well plates (TPP, Trasadingen, Switzerland). After 24 h, the cells were incubated with vehicle (DMSO) or 10 µM inhibitors of ERK1/2 (U0126), p38 (SB203580), JNK (SP600125) (Sigma-Aldrich) or NF-κB (BAY11-7082) (Calbiochem, San Diego, CA, USA). Human IL-8, MCP-1 and TGF-β concentrations in cell culture supernatants were determined 24 h later using the DuoSet enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA). Relative ERK1/2 phosphorylation was measured using a cell-based ERK1/2 ELISA kit (RayBiotech, Norcross, GA, USA) following the manufacturer's instructions. Caspase 3/7 activity (Promega, Madison, WI, USA) was measured as recommended by the manufacturer.

IL8 real-time qPCR

Total RNA was extracted using a nucleospin extract II kit (Macherey Nagel, Duren, Germany). Reverse transcription was performed with 0.8 μg of total extracted RNA, using the ABI high-capacity cDNA archive kit (Applied Biosystems). RT-PCR was performed using an ABI StepOnePlus^{TM}. Each reaction contained 10 μl of 2× TaqMan® Fast Universal PCR Master Mix (Applied Biosystems), 1 μl of IL-8 (Hs00174103_m1), ABCA3 (Hs00975518_m1) or GAPDH (Hs03929097_g1) TaqMan® probe and 40 ng of cDNA as the template in a final volume of 20 μl. Data were analyzed using the comparative C_t method ($\Delta\Delta C_t$). For relative quantification, the amount of IL-8 was normalized for GAPDH (endogenous gene) relative to wild-type cells (ABCA3-WT) used as the calibrator and was calculated using the $2^{-\Delta\Delta C_t}$method as published previously (35). Each point corresponds to the mean ± SD of three experiments performed in triplicate.

Western blot

BALF proteins were accurately quantified using a Qubit fluorometer (Invitrogen). Then, 24 μg of protein was fractionated using SDS–PAGE on 16% Tris–tricine gels, electrotransferred and probed by immunoblotting using antibodies to surfactant proteins SP-B and SP-C (Seven Hills Bioreagents, Cincinnati, OH, USA), as described previously (33).

A549 cell extracts were prepared from 3×10^5 cells and solubilized as described previously (34). An equal amount of protein (10 μg) from each sample was size-separated on 10% SDS–polyacrylamide gel and electrotransferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Immunodetection was performed with antibodies specific for the total and phosphorylated forms of ERK1/2 (Cell Signaling Technology, Beverly, MA, USA) and β-actin (Sigma-Aldrich). Secondary antibodies were from Cell Signaling Technology. Bound antibodies were detected using SuperSignal West Femto chemiluminescent substrate (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Between successive probes, membranes were treated with Restore Western Blot Stripping Reagent (Pierce). Molecular masses were determined using the SeeBlue® Plus2 Pre-Stained Standard (Invitrogen). Images were recorded with a Fujifilm LAS-3000 bioimaging system (Fujifilm, Stamford, CT, USA).

For the study of ABCA3 expression, 35 μg of transiently transfected cells (Lipofectamin, 48 h) was used. Immunoblotting was performed with an anti-eGFP antibody (Clontech, Mountain View, CA, USA).

Statistics

The statistical significance of differences between groups was tested using the unpaired Student's *t*-test with a threshold of $P < 0.05$.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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REFERENCES

1. Bullard, J.E., Wert, S.E., Whitsett, J.A., Dean, M. and Nogee, L.M. (2005) ABCA3 mutations associated with pediatric interstitial lung disease. *Am. J. Respir. Crit. Care. Med.*, **172**, 1026–1031.
2. Deutsch, G.H., Young, L.R., Deterding, R.R., Fan, L.L., Dell, S.D., Bean, J.A., Brody, A.S., Nogee, L.M., Trapnell, B.C., Langston, C. *et al.* (2007) Diffuse lung disease in young children: application of a novel classification scheme. *Am. J. Respir. Crit. Care. Med.*, **176**, 1120–1128.
3. Hartl, D. and Griese, M. (2005) Interstitial lung disease in children—genetic background and associated phenotypes. *Respir. Res.*, **6**, 32.
4. Nogee, L.M., Garnier, G., Dietz, H.C., Singer, L., Murphy, A.M., deMello, D.E. and Colten, H.R. (1994) A mutation in the surfactant protein B gene responsible for fatal neonatal respiratory disease in multiple kindreds. *J. Clin. Invest.*, **93**, 1860–1863.
5. Guillot, L., Epaud, R., Thouvenin, G., Jonard, L., Mohsni, A., Couderc, R., Counil, F., de Blic, J., Taam, R.A., Le Bourgeois, M. *et al.* (2009) New surfactant protein C gene mutations associated with diffuse lung disease. *J. Med. Genet.*, **46**, 490–494.
6. Stahlman, M.T., Besnard, V., Wert, S.E., Weaver, T.E., Dingle, S., Xu, Y., von Zychlin, K., Olson, S.J. and Whitsett, J.A. (2007) Expression of ABCA3 in developing lung and other tissues. *J. Histochem. Cytochem.*, **55**, 71–83.
7. Mulugeta, S., Gray, J.M., Notarfrancesco, K.L., Gonzales, L.W., Koval, M., Feinstein, S.I., Ballard, P.L., Fisher, A.B. and Shuman, H. (2002) Identification of LBM180, a lamellar body limiting membrane protein of alveolar type II cells, as the ABC transporter protein ABCA3. *J. Biol. Chem.*, **277**, 22147–22155.
8. Connors, T.D., Van Raay, T.J., Petry, L.R., Klinger, K.W., Landes, G.M. and Burn, T.C. (1997) The cloning of a human ABC gene (ABC3) mapping to chromosome 16p13.3. *Genomics*, **39**, 231–234.
9. Klugbauer, N. and Hofmann, F. (1996) Primary structure of a novel ABC transporter with a chromosomal localization on the band encoding the multidrug resistance-associated protein. *FEBS Lett.*, **391**, 61–65.
10. Shulenin, S., Nogee, L.M., Annilo, T., Wert, S.E., Whitsett, J.A. and Dean, M. (2004) ABCA3 gene mutations in newborns with fatal surfactant deficiency. *N. Engl. J. Med.*, **350**, 1296–1303.
11. Brasch, F., Schimanski, S., Muhlfeld, C., Barlage, S., Langmann, T., Aslanidis, C., Boettcher, A., Dada, A., Schroten, H., Mildenberger, E. *et al.* (2006) Alteration of the pulmonary surfactant system in full-term infants with hereditary ABCA3 deficiency. *Am. J. Respir. Crit. Care. Med.*, **174**, 571–580.
12. Whitsett, J.A., Wert, S.E. and Xu, Y. (2005) Genetic disorders of surfactant homeostasis. *Biol. Neonate.*, **87**, 283–287.
13. Doan, M.L., Guillerman, R.P., Dishop, M.K., Nogee, L.M., Langston, C., Mallory, G.B., Sockrider, M.M. and Fan, L.L. (2008) Clinical,

radiological and pathological features of ABCA3 mutations in children. *Thorax*, **63**, 366–373.
14. Somaschini, M., Nogee, L.M., Sassi, I., Danhaive, O., Presi, S., Boldrini, R., Montrasio, C., Ferrari, M., Wert, S.E. and Carrera, P. (2007) Unexplained neonatal respiratory distress due to congenital surfactant deficiency. *J. Pediatr.*, **150**, 649–653, 653 e641.
15. Cheong, N., Madesh, M., Gonzales, L.W., Zhao, M., Yu, K., Ballard, P.L. and Shuman, H. (2006) Functional and trafficking defects in ATP binding cassette A3 mutants associated with respiratory distress syndrome. *J. Biol. Chem.*, **281**, 9791–9800.
16. Matsumura, Y., Ban, N., Ueda, K. and Inagaki, N. (2006) Characterization and classification of ATP-binding cassette transporter ABCA3 mutants in fatal surfactant deficiency. *J. Biol. Chem.*, **281**, 34503–34514.
17. Nagata, K., Yamamoto, A., Ban, N., Tanaka, A.R., Matsuo, M., Kioka, N., Inagaki, N. and Ueda, K. (2004) Human ABCA3, a product of a responsible gene for abca3 for fatal surfactant deficiency in newborns, exhibits unique ATP hydrolysis activity and generates intracellular multilamellar vesicles. *Biochem. Biophys. Res. Commun.*, **324**, 262–268.
18. Weichert, N., Kaltenborn, E., Hector, A., Woischnik, M., Schams, A., Holzinger, A., Kern, S. and Griese, M. (2011) Some ABCA3 mutations elevate ER stress and initiate apoptosis of lung epithelial cells. *Respir. Res.*, **12**, 4.
19. Hoffmann, E., Dittrich-Breiholz, O., Holtmann, H. and Kracht, M. (2002) Multiple control of interleukin-8 gene expression. *J. Leukoc. Biol.*, **72**, 847–855.
20. Muselet-Charlier, C., Roque, T., Boncoeur, E., Chadelat, K., Clement, A., Jacquot, J. and Tabary, O. (2007) Enhanced IL-1beta-induced IL-8 production in cystic fibrosis lung epithelial cells is dependent of both mitogen-activated protein kinases and NF-kappaB signaling. *Biochem. Biophys. Res. Commun.*, **357**, 402–407.
21. Park, S.K., Amos, L., Rao, A., Quasney, M.W., Matsumura, Y., Inagaki, N. and Dahmer, M.K. (2010) Identification and characterization of a novel ABCA3 mutation. *Physiol. Genomics*, **40**, 94–99.
22. Cheong, N., Zhang, H., Madesh, M., Zhao, M., Yu, K., Dodia, C., Fisher, A.B., Savani, R.C. and Shuman, H. (2007) ABCA3 is critical for lamellar body biogenesis in vivo. *J. Biol. Chem.*, **282**, 23811–23817.
23. Matsumura, Y., Ban, N. and Inagaki, N. (2008) Aberrant catalytic cycle and impaired lipid transport into intracellular vesicles in ABCA3 mutants associated with nonfatal pediatric interstitial lung disease. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **295**, L698–707.
24. Bullard, J.E. and Nogee, L.M. (2007) Heterozygosity for ABCA3 mutations modifies the severity of lung disease associated with a surfactant protein C gene (SFTPC) mutation. *Pediatr. Res.*, **62**, 176–179.
25. Hamvas, A. (2006) Inherited surfactant protein-B deficiency and surfactant protein-C associated disease: clinical features and evaluation. *Semin. Perinatol.*, **30**, 316–326.
26. Edwards, V., Cutz, E., Viero, S., Moore, A.M. and Nogee, L. (2005) Ultrastructure of lamellar bodies in congenital surfactant deficiency. *Ultrastruct. Pathol.*, **29**, 503–509.
27. Matsumura, Y., Sakai, H., Sasaki, M., Ban, N. and Inagaki, N. (2007) ABCA3-mediated choline-phospholipids uptake into intracellular vesicles in A549 cells. *FEBS Lett.*, **581**, 3139–3144.
28. Ridsdale, R., Na, C.L., Xu, Y., Greis, K.D. and Weaver, T. (2010) Comparative proteomic analysis of lung lamellar bodies and lysosome-related organelles. *PLoS One*, **6**, e16482.
29. Maguire, J.A., Mulugeta, S. and Beers, M.F. (2011) Endoplasmic reticulum stress induced by surfactant protein C BRICHOS mutants promotes proinflammatory signaling by epithelial cells. *Am. J. Respir. Cell Mol. Biol.*, **44**, 404–414.
30. Willis, B.C. and Borok, Z. (2007) TGF-beta-induced EMT: mechanisms and implications for fibrotic lung disease. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **293**, L525–534.
31. Hartl, D., Griese, M., Nicolai, T., Zissel, G., Prell, C., Reinhardt, D., Schendel, D.J. and Krauss-Etschmann, S. (2005) A role for MCP-1/CCR2 in interstitial lung disease in children. *Respir. Res.*, **6**, 93.
32. Woischnik, M., Sparr, C., Kern, S., Thurm, T., Hector, A., Hartl, D., Liebisch, G., Mulugeta, S., Beers, M.F., Schmitz, G. *et al.* (2010) A non-BRICHOS surfactant protein c mutation disrupts epithelial cell function and intercellular signaling. *BMC Cell Biol.*, **11**, 88.
33. Guillot, L., Carre, A., Szinnai, G., Castanet, M., Tron, E., Jaubert, F., Broutin, I., Counil, F., Feldmann, D., Clement, A. *et al.* (2010) NKX2-1 mutations leading to surfactant protein promoter dysregulation cause interstitial lung disease in 'brain-lung-thyroid syndrome'. *Hum. Mutat.*, **31**, E1146–E1162.
34. Guillot, L., Medjane, S., Le-Barillec, K., Balloy, V., Danel, C., Chignard, M. and Si-Tahar, M. (2004) Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like receptor 4 (TLR4)-dependent signaling pathways: evidence for an intracellular compartmentalization of TLR4. *J. Biol. Chem.*, **279**, 2712–2718.
35. Guillot, L., Carroll, S.F., Badawy, M. and Qureshi, S.T. (2008) Cryptococcus neoformans induces IL-8 secretion and CXCL1 expression by human bronchial epithelial cells. *Respir. Res.*, **9**, 9.

Analysis of factors influencing postprandial C-peptide levels in Japanese patients with type 2 diabetes: Comparison with C-peptide levels after glucagon load

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ABSTRACT

Aims/Introduction: Postprandial serum C-peptide levels are readily determined in clinical practice and have a good correlation with serum C-peptide levels after glucagon load; the measurement is often used as an index of endogenous insulin secretion. However, the factors affecting postprandial serum C-peptide levels remain to be evaluated.

Materials and Methods: To investigate the clinical factors affecting postprandial serum C-peptide, 2-h postprandial C-peptide levels after breakfast (PPCPR) were analyzed retrospectively for comparison with glucagon-stimulated C-peptide (CPR-6min) levels measured during hospital admission in 273 Japanese patients with type 2 diabetes.

Results: Multiple regression analysis showed that years from diagnosis, body mass index (BMI) and HbA_{1c} were the major independent variables predicting PPCPR ($R^2 = 0.315$). HbA_{1c} was a major factor predicting PPCPR, but did not predict CPR-6min. In addition, HbA_{1c} was negatively correlated with PPCPR ($r = -0.410$, $P < 0.0001$) and PPCPR/CPR-6min ($r = -0.313$, $P < 0.0001$).

Conclusions: PPCPR was correlated with common factors predicting CPR, including years from diagnosis and BMI, but also was negatively correlated with HbA_{1c}, a unique factor. These results show that chronic elevation of the glucose level might impair endogenous insulin secretion after meal load, but might have little effect on endogenous insulin secretion after glucagon load.

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KEY WORDS: C-peptide, Meal load, HbA_{1c}

INTRODUCTION

Type 2 diabetes is a heterogeneous disease characterized by insulin resistance and defective insulin secretion[1], and is progressive in that the mode of therapy must be altered over the decades of diabetes; diet and exercise therapy alone might be adequate initially, but secondary oral hypoglycemic agent (OHA) treatment and insulin treatment are eventually required[2,3]. This is, at least in part, as a result of progressive loss of pancreatic β-cell function. The results of the United Kingdom Progressive Diabetes Study (UKPDS) show that pancreatic β-cell function (%β), assessed by Homeostasis Model Assessment (HOMA) in patients allocated to diet or OHA decreased approximately 25% in 5 years[4]. In addition, a decline in endogenous insulin secretion over more than several decades of diabetes in patients including insulin-treated patients was observed in a cross-sectional study[5].

Determination of fasting serum C-peptide level and stimulated serum C-peptide level by intravenous glucagon is used widely to assess endogenous insulin secretory reserves[6–9], and the utility of the indices using C-peptide level in choosing insulin therapy has been shown[10]. The postprandial serum C-peptide level can easily be measured in clinical practice and has a good correlation with the serum C-peptide level after glucagon load[11]; it is often used as an index of endogenous insulin secretion, and can be used for both non-insulin-treated and insulin-treated patients[11–13]. Duration of diabetes and body mass index (BMI) are the major factors in serum fasting and glucagon-stimulated C-peptide levels[5,14], but the factors affecting postprandial serum C-peptide levels remain to be evaluated.

In the present study of Japanese patients with type 2 diabetes, to evaluate the clinical factors affecting postprandial serum C-peptide by cross-sectional study, 2-h postprandial C-peptide levels after breakfast were analyzed and compared with glucagon-stimulated C-peptide levels.

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SUBJECTS AND METHODS

Subjects

A total of 388 Japanese patients with type 2 diabetes who were admitted to Kyoto University Hospital between 1997 and 2002 for poor glycemic control were enrolled in the study. Patients with pancreatic or liver disease, taking diabetogenic medications, pregnant or with serum creatinine ≥1.3 mg/dL were excluded from the study. Type 2 diabetes mellitus was diagnosed based on the criteria of the American Diabetes Association (ADA)[15]. Patients with serum creatinine ≥1.3 mg/dL were excluded, as serum C-peptide immunoreactivity (CPR) is elevated by decreased renal function[16]. Of these patients, 115 were excluded as a result of incomplete clinical examinations and the remaining 273 patients, including patients without diabetic medication, oral hypoglycemic agent-treated patients and insulin-treated patients, were analyzed. The clinical profiles of the patients are shown in Table 1.

Methods

On the first day in hospital, medical history, physical examination and laboratory evaluation including glycosylated hemoglobin were carried out. HbA$_{1c}$ was measured using high performance liquid chromatography (HA-8180; Arcray, Kyoto, Japan). The HbA$_{1c}$ (%) value was estimated as a National Glycohemoglobin Standardization Program (NGSP) equivalent (%) calculated by the formula HbA$_{1c}$ (%) = HbA$_{1c}$ (Japan Diabetes Society [JDS]) (%) + 0.4%, considering the relational expression of HbA$_{1c}$ (JDS) (%) measured by the previous Japanese standard substance and measurement methods and HbA$_{1c}$ (NGSP)[17]. β-Cell function was evaluated within 1 week after an overnight fast by measuring fasting CPR (FCPR), CPR 6min after intravenous injection of 1 mg glucagon (CPR-6min)[6] and postprandial CPR. Serum CPR was measured by radioimmunoassay (Daiichi III; Daiichi Radioisotope Laboratories, Osaka, Japan). Postprandial CPR 2 h after breakfast (PPCPR) was determined. The meal at breakfast was prescribed as nutritional therapy according to the treatment guide for diabetes of the JDS[18], which included 516.6 ± 67.7 kcal (mean ± SD) energy consisting of 49% carbohydrate, 16% protein and 35% fat. In patients taking OHA, medication was stopped for measurement of CPR, but was maintained until 1 day before to prevent hyperglycemia during the test[5]. Plasma glucose was measured by the glucose oxidase method.

Table 1 | Clinical profiles of patients

No. patients	273
Male/female	158/115
Age (years)	61.2 ± 12.2
Years from diagnosis	9.6 ± 9.6
Systolic blood pressure (mmHg)	121.8 ± 12.9
Diastolic blood pressure (mmHg)	73.6 ± 9.6
BMI (kg/m^2)	23.9 ± 3.7
HbA$_{1c}$ at admission (%)	9.7 ± 2.0
sCre (mg/dL)	0.69 ± 0.18
Glucagon load: FPG/PG-6min (mg/dL)	164.1 ± 47.9/180.6 ± 49.1
Glucagon load: FCPR/CPR-6min (ng/mL)	1.80 ± 0.97/3.83 ± 1.76
Meal load: FPG/PPPG (mg/dL)	167.0 ± 54.8/271.5 ± 83.5
Meal load: FCPR/PPCPR (ng/mL)	1.76 ± 0.94/4.87 ± 2.41

BMI, body mass index; CPR-6min, C-peptide immunoreactivity 6 min after intravenous injection of 1 mg glucagon; FCPR, fasting CPR; FPG, fasting plasma glucose; OHA, oral hypoglycemic agents; PG-6min, plasma glucose 6 min after glucagon load; PPCPR, postprandial CPR; PPPG, postprandial plasma glucose; sCre, serum creatinine.

The study protocol was approved by the ethics committee of Kyoto University.

Statistical Analysis

Statistical analysis was carried out with the Stat View 5.0 system (SAS institute Inc., Cary, NC, USA). Data are presented as mean ± SD, unless otherwise noted. The relationship between the parametric clinical data and CPR values was investigated by Pearson's analysis. The relationship between the non-parametric clinical data and CPR values was investigated by Spearman's analysis. Clinical parameters among three groups were compared by analysis of variance (ANOVA). For comparison of two groups, Scheffé's test was carried out. *P*-values < 0.05 were considered statistically significant.

RESULTS

Simple correlation coefficients between FCPR, CPR-6min and PPCPR, and measures of variables (age, years from diagnosis, sex, BMI, systolic and diastolic blood pressure, HbA$_{1c}$, serum creatinine and plasma glucose [PG]) were calculated and are shown in Table 2. Years from diagnosis and BMI were significantly correlated with all three measures of CPR. PG and HbA$_{1c}$ were significantly correlated with PPCPR ($P < 0.0001$, $r = -0.410$), but not with CPR-6min (Figure 1).

Stepwise multiple regression analysis was carried out using the independent variables in Table 2 to predict CPR as a dependent variable (Table 3). FCPR was independently predicted by years from diagnosis, BMI and serum creatinine, accounting for 22.4% of the variability of FCPR. CPR-6min was independently predicted by years from diagnosis and BMI, accounting for 17.9% of the variability of the dependent variables. PPCPR was independently predicted by years from diagnosis, BMI and HbA$_{1c}$, accounting for 31.5% of the variability of the dependent variables. Thus, HbA$_{1c}$ is an important independent variable predicting PPCPR, but not FCPR or CPR-6min.

Because HbA$_{1c}$ might be involved in decreased PPCPR, the clinical data among three groups of increased HbA$_{1c}$ (≤8.5%, 8.6–10.3%, ≥10.4%) were compared, as shown in Table 4. Although there was no significant difference among these groups in FCPR and CPR-6min, PPCRP was significantly reduced with increasing levels of HbA$_{1c}$. CPR-6min was significantly correlated with PPCPR ($P < 0.0001$, $r = 0.564$, PPCPR = 0.774 × CPR-6min + 1.913; Figure 2a). PPCPR was correlated with CPR-6min in each tertile group of HbA$_{1c}$ (HbA$_{1c}$ ≤ 8.5:

Table 2 | *P*-values and *r*-values of correlation between C-peptide immunoreactivity and measures of variables

	FCPR (ng/mL)	CPR-6min (ng/mL)	PPCPR (ng/mL)
Age (years)	0.4257 (ND)	0.0456 (−0.121)	0.3896 (ND)
Years from diagnosis	0.0024 (−0.182)	<0.0001 (−0.246)	0.0007 (−0.205)
Sex	0.0709 (ND)	0.1879 (ND)	0.8321 (ND)
BMI (kg/m^2)	<0.0001 (0.435)	<0.0001 (0.367)	<0.0001 (0.311)
Systolic blood pressure (mmHg)	0.5551 (ND)	0.9388 (ND)	0.0865 (ND)
Diastolic blood pressure (mmHg)	0.5739 (ND)	0.0327 (0.130)	0.0705 (ND)
HbA$_{1c}$ (%)	0.0443 (−0.122)	0.1507 (ND)	<0.0001 (−0.410)
sCre (mg/dL)	0.0104 (0.155)	0.1641 (ND)	0.0140 (0.148)
FPG (mg/dL)	0.3764 (ND)	ND	ND
PG-6min (mg/dL)	ND	0.7333 (ND)	ND
PPPG (mg/dL)	ND	ND	<0.0001 (−0.285)

All correlations except correlations between sex and C-peptide immunoreactivity (CPR) were analyzed by Pearson's analysis. Correlations between sex and CPR were analyzed by Spearman's analysis. *P*-values are shown. In parenthesis, *r*-values are shown.
BMI, body mass index; CPR-6min, C-peptide immunoreactivity 6 min after intravenous injection of 1 mg glucagon; FCPR, fasting CPR; FPG, fasting plasma glucose; ND, not determined; PG-6min, plasma glucose 6 min after glucagon load; PPCPR: postprandial CPR; PPPG: postprandial plasma glucose; sCre: serum creatinine.

$P < 0.0001$, $r = 0.595$, $y = 2.159 + 0.970x$, $n = 90$; $8.6\% \leq \text{HbA}_{1c} \leq 10.3\%$: $P < 0.0001$, $r = 0.674$, $y = 1.587 + 0.829x$, $n = 92$; $10.4\% \leq \text{HbA}_{1c}$: $P < 0.0001$, $r = 0.494$, $y = 2.091 + 0.482x$, $n = 91$). Because the higher HbA$_{1c}$ group was distributed mainly below the regression line of total patients and the lower HbA$_{1c}$ group above the line in the scattergram, and the increase in PPCPR per CPR-6min in the regression line of each tertile group was lower in the higher HbA$_{1c}$ group, we examined the correlation between the ratio of PPCPR to CPR-6min (PPCPR/CPR-6min) and HbA$_{1c}$. PPCPR/CPR-6min was negatively correlated with HbA$_{1c}$ ($P < 0.0001$, $r = -0.313$; Figure 2b).

DISCUSSION

In the present study, HbA$_{1c}$ was negatively correlated with PPCPR, but not with FCPR or CPR-6min, which suggests that chronic elevation of the glucose level might impair endogenous insulin secretion after a meal load.

Although meal load is not equivalent to glucose load, as it contains nutrients other than carbohydrates that modulate glucose-induced insulin secretion, elevated glucose in plasma might play an important role in meal-stimulated insulin secretion. Indeed, the plasma glucose level after a meal load was increased considerably to more than 100 mg/dL in average. In contrast, the increment of glucose after glucagon load was only approximately 15 mg/dL, indicating a small contribution of glucose elevation to increased insulin secretion by glucagon loading.

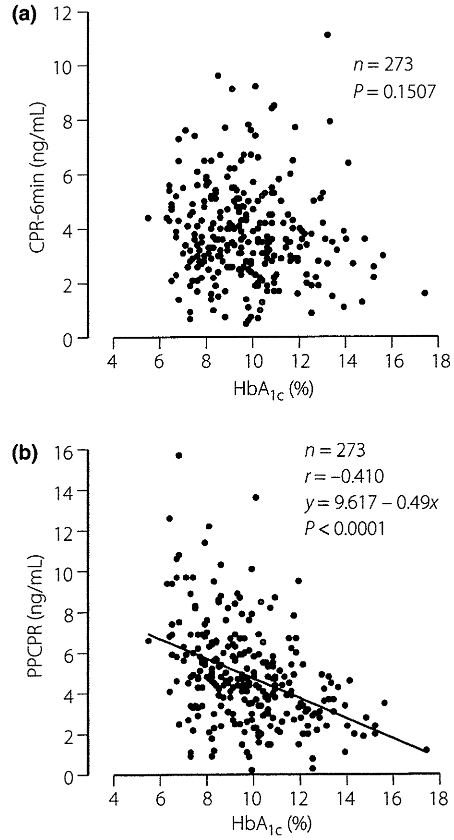

Figure 1 | The relationship between HbA$_{1c}$ and (a) C-peptide immunoreactivity 6 min after intravenous injection of 1 mg glucagon (CPR-6min) and (b) 2-h postprandial C-peptide levels after breakfast (PPCPR).

Because HbA$_{1c}$ was positively correlated with PPPG in the present study ($P < 0.0001$, $r = 0.570$), HbA$_{1c}$ reflects postprandial glucose level. In simple correlation, both HbA$_{1c}$ and PPPG were significantly correlated with PPCPR; whereas in stepwise regression analysis, HbA$_{1c}$ was important to predict PPCPR, but PPPG was not. In addition, in simple correlation to PPCPR, the *r*-value for HbA$_{1c}$ (0.410) was larger compared with that for PPPG (0.285; Table 2). These results show that PPCPR is more strongly affected by chronic elevation of glucose levels than by transient elevation of glucose levels.

Multiple regression analysis showed that years from diagnosis, BMI and HbA$_{1c}$ were the major independent variables predicting PPCPR. This shows that years from diagnosis and BMI are common major factors predicting CPR. In contrast, HbA$_{1c}$ was the major factor predicting PPCPR, but not FCPR or CPR-6min, and was negatively correlated with PPCPR. We hypothesized that CPR-6min reflects reserve capacity of endogenous insulin secretion independent of glycemic control and that PPCPR is predicted by a fundamental factor independent of glycemic control and by a variable factor dependent of glycemic control. CPR-6min predicted 31.8% of the variability of PPCPR as shown in Figure 2a. When a regression model using CPR-6min

Table 3 | Stepwise multiple regression analysis for predictors of C-peptide immunoreactivity

	F-value	Partial regression coefficient	Standard partial regression coefficient	R^2 (*R*)
FCPR (ng/mL)				
Years from diagnosis	9.4	−0.017	−0.170	0.224 (0.473)
BMI (kg/m^2)	55.2	0.108	0.406	
sCre (mg/dL)	7.3	0.823	0.149	
CPR-6min (ng/mL)				
Years from diagnosis	14.6	−0.039	−0.214	0.179 (0.423)
BMI (kg/m^2)	38.9	0.170	0.349	
PPCPR (ng/mL)				
Years from diagnosis	23.4	−0.063	−0.252	0.315 (0.561)
BMI (kg/m^2)	27.5	0.178	0.270	
HbA_{1c} (%)	68.7	−0.516	−0.431	

BMI, body mass index; CPR-6min, C-peptide immunoreactivity 6 min after intravenous injection of 1 mg glucagon; FCPR, fasting CPR; PPCPR, postprandial CPR; sCre, serum creatinine.

Table 4 | Comparison of clinical characteristics and clinical profile among groups according to HbA_{1c} at admission

Groups (HbA_{1c} at admission)	I (≤8.5%)	II (8.6–10.3%)	III (≥10.4%)	*P*
No. patients	90	92	91	
HbA_{1c} (%)	7.6 ± 0.1	9.5 ± 0.1*	12.0 ± 0.1*†	<0.0001
Sex (male/female)	53/37	55/37	50/41	
Age (years)	64.2 ± 1.2	61.6 ± 1.3	57.6 ± 1.3*	0.0011
BMI (kg/m^2)	24.2 ± 0.3	24.1 ± 0.4	23.5 ± 0.4	0.3579
Years from diagnosis	11.7 ± 1.2	9.7 ± 0.8	7.4 ± 0.8*	0.0088
bSBP (mmHg)	122.9 ± 1.4	120.6 ± 1.3	121.3 ± 1.4	0.4746
DBP (mmHg)	72.9 ± 1.2	72.7 ± 1.0	75.3 ± 0.9	0.1302
sCre (mg/dL)	0.74 ± 0.02	0.70 ± 0.02	0.63 ± 0.02*†	<0.0001
FPG (mg/dL)	134.2 ± 3.7	163.4 ± 3.9*	195.0 ± 5.4*†	<0.0001
PG-6min (mg/dL)	152.4 ± 3.9	178.7 ± 4.0*	211.1 ± 5.6*†	<0.0001
PPPG (mg/dL)	223.3 ± 6.4	268.2 ± 7.7*	323.7 ± 8.9*†	<0.0001
FCPR (ng/mL)	1.92 ± 0.10	1.84 ± 0.10	1.66 ± 0.11	0.2004
CPR-6min (ng/mL)	3.85 ± 0.18	3.97 ± 0.19	3.66 ± 0.18	0.5467
PPCPR (ng/mL)	5.90 ± 0.29	4.88 ± 0.24*	3.86 ± 0.18*†	<0.0001

Data are presented as mean ± SE.

*$P < 0.01$ vs group I, †$P < 0.01$ vs group II.

BMI, body mass index; CPR-6min, C-peptide immunoreactivity 6 min after intravenous injection of 1 mg glucagon; DBP, diastolic blood pressure; FCPR, fasting CPR; FPG, fasting plasma glucose; PG-6min, plasma glucose 6 min after glucagon load; PPCPR, postprandial CPR; PPPG, postprandial plasma glucose; SBP, systolic blood pressure; sCre, serum creatinine. FPG and FCPR are values when meal load was carried out.

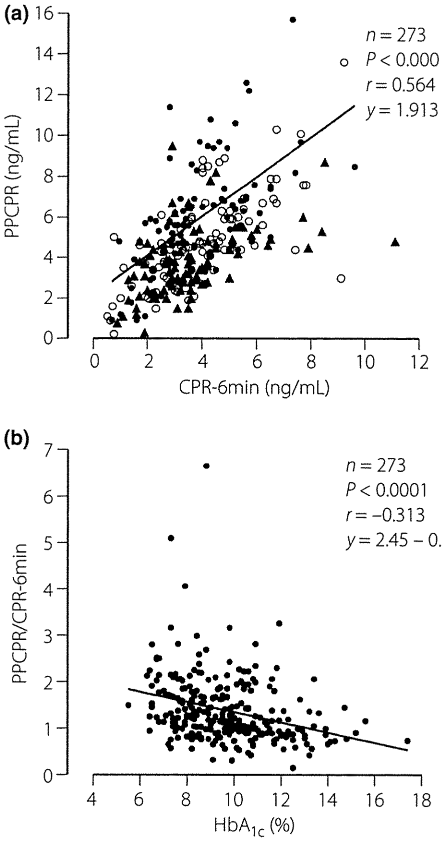

Figure 2 | Relationship between (a) C-peptide immunoreactivity 6 min after intravenous injection of 1 mg glucagon (CPR-6min) and 2-h postprandial C-peptide levels after breakfast (PPCPR) and (b) PPCPR/CPR-6min and HbA_{1c}. Black circles, $HbA_{1c} \leq 8.5\%$; white circles, $8.6\% \leq HbA_{1c} \leq 10.3\%$, black triangles, $10.4\% \leq HbA_{1c}$

and HbA_{1c} as independent variables to predict PPCPR as a dependent variable was used, CPR-6min and HbA_{1c} predicted 44.9% of the variability of PPCPR ($P < 0.0001$, $R = 0.670$, PPCPR = 6.286 + 0.730 × CPR-6min − 0.434 × HbA_{1c}). The addition of HbA_{1c} as an independent variable increased the prediction of the variability of PPCPR by 13.1%. In the present study, PPCPR/CPR-6min was used as a putative index of variability dependent of glycemic control and was found to be correlated with HbA_{1c} in the present study (Figure 2b). Furthermore, improvement of glycemic control by treatment ameliorates the CPR response after oral glucose load[19–21]. In addition, the CPR response after glucagon load is affected little by treatment to improve hyperglycemia and it is not correlated with the CPR response after oral glucose load before treatment, whereas it is well-correlated with improved CPR response after oral glucose load after treatment[21]. Reversible impairment of endogenous insulin response after glucose load is explained by glucose toxicity, in which chronic hyperglycemia deteriorates meal-induced and glucose-induced insulin secretion and insulin-sensitive glucose disposal[22]. Therefore, the chronic high glucose level shown by high HbA_{1c} might impair endogenous insulin secretion after meal load, but has little effect on endogenous insulin secretion after glucagon load. The lack of influence of HbA_{1c} on CPR-6min might be helpful to evaluate reserve capacity of endogenous insulin secretion, even when glycemic control is poor enough to deteriorate postprandial insulin secretion. In contrast, PPCPR is affected by HbA_{1c} and might reflect the state of deteriorated insulin secretion by glucose toxicity that may be recovered by improved glycemic control.

In stepwise regression analysis, HbA_{1c} was not important to predict FCPR, but was important to predict PPCPR. In simple correlation, HbA_{1c} was significantly negatively correlated not only with PPCPR, but also with FCPR, whereas the P-value and r-value for FCPR were larger and smaller, respectively, compared with those for PPCPR (Table 2). Taken together, these findings suggest that glucose toxicity might deteriorate not only postprandial insulin secretion, but also fasting insulin secretion, whereas postprandial insulin secretion might be more vulnerable to glucose toxicity than to fasting insulin secretion.

The suppressive effect of glucose toxicity on insulin secretion *in vivo* might be attributable to impairment of β-cell responsiveness to glucose[22] and to impairment of incretin effect[23,24]. However, it is important to understand why glucagon-stimulated CPR is preserved despite severe impairment of glucose-stimulated CPR before treatment to improve hyperglycemia[21]. This remains largely unknown, but our hypothesis based on an *in vitro* study is that deteriorated intracellular glucose metabolism plays an important role in impaired glucose-induced insulin secretion[25] and that increased intracellular cyclic adenosine 3′,5′-monophosphate concentration derived from glucagon stimulation ameliorates impaired intracellular glucose metabolism to improve suppressed insulin secretion[26].

A recent study showed that indices using CPR correlate well with β-cell mass by analysis of β-cell areas of samples obtained during pancreatectomy and serum levels of CPR before operation[27]. Thus, PPCPR might reflect not only β-cell mass, but also reversible impairment of endogenous secretion as a result of chronic glucose elevation.

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REFERENCES

1. DeFronzo RA. Lilly lecture 1987. The triumvirate: β-cell, muscle, liver. A collusion responsible for NIDDM. *Diabetes* 1988; 37: 667–687.
2. Yki-Järvinen H, Kauppila M, Kujansuu E, *et al.* Comparison of insulin regimens in patients with non-insulin-dependent diabetes mellitus. *N Engl J Med* 1992; 327: 1426–1433.
3. Turner RC, Cull CA, Frighi V, *et al.* Glycemic control with diet, sulfonylurea, metformin, or insulin in patients with type 2 diabetes mellitus: progressive requirement for multiple therapies (UKPDS 49). UK Prospective Diabetes Study (UKPDS) Group. *JAMA* 1999; 281: 2005–2012.
4. U.K. Prospective Diabetes Study Group. U.K. prospective diabetes study 16. Overview of 6 years' therapy of type II diabetes: a progressive disease. *Diabetes* 1995; 44: 1249–1258.
5. Funakoshi S, Fujimoto S, Hamasaki A, *et al.* Analysis of factors influencing pancreatic β-cell function in Japanese patients with type 2 diabetes: association with body mass index and duration of diabetic exposure. *Diabetes Res Clin Pract* 2008; 82: 353–358.
6. Faber OK, Binder C. C-peptide response to glucagon. A test for the residual β-cell function in diabetes mellitus. *Diabetes* 1977; 26: 605–610.
7. Hendriksen C, Faber OK, Drejer J, *et al.* Prevalence of residual B-cell function in insulin-treated diabetics evaluated by the plasma C-peptide response to intravenous glucagon. *Diabetologia* 1977; 13: 615–619.
8. Jayyab AK, Heding LG, Czyzyk A, *et al.* Serum C-peptide and IRI levels after administration of glucagon and glucose in non-insulin-dependent diabetics. *Horm Metab Res* 1982; 14: 112–116.
9. Gjessing HJ, Damsgaard EM, Matzen LE, *et al.* Reproducibility of β-cell function estimates in non-insulin-dependent diabetes mellitus. *Diabetes Care* 1987; 10: 558–562.
10. Funakoshi S, Fujimoto S, Hamasaki A, *et al.* Utility of indices using C-peptide levels for indication of insulin therapy to achieve good glycemic control in Japanese patients with type 2 diabetes. *J Diabetes Invest* 2011. doi:10.1111/j.2040-1124.2010.00096.x.
11. Koskinen PJ, Viikari JS, Irjala KM. Glucagon-stimulated and postprandial plasma C-peptide values as measures of insulin secretory capacity. *Diabetes Care* 1988; 11: 318–322.
12. Aoki Y. Variation of endogenous insulin secretion in association with treatment status: assessment by serum C-peptide and modified urinary C-peptide. *Diabetes Res Clin Pract* 1991; 14: 165–173.
13. Aoki Y, Yanagisawa Y, Yazaki K, *et al.* Clinical significance of the two-hour postprandial serum C-peptide level in patients with diabetes mellitus. *J Jpn Diabetes Soc* 1992; 35: 811–818 (Japanese).
14. Chung JO, Cho DH, Chung DJ, *et al.* Plasma C-peptide level is inversely associated with family history of type 2 diabetes in Korean type 2 diabetic patients. *Endocr J* 2010; 57: 931–938.
15. American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care* 2010; 33: S62–S69.
16. Kajinuma H, Tanabashi S, Ishiwata K, *et al.* Urinary excretion of C-peptide in relation to renal function. In: Baba S (ed.). *Proinsulin, Insulin, C-peptide*. Excerpta Medica, Amsterdam, 1979; 183–189.
17. The Committee of Japan Diabetes Society on the diagnostic criteria of diabetes mellitus. Report of the Committee on the classification and diagnostic criteria of diabetes mellitus. *J Diabetes Invest* 2010; 1: 212–228.
18. Japan Diabetes Society. (ed.). *Treatment Guide for Diabetes 2007*. Japan Diabetes Society, Bunkodo, Tokyo, Japan, 2007.

19. Seino Y, Ikeda M, Kurahachi H, *et al.* Failure of suppress plasma glucagon concentrations by orally administered glucose in diabetic patients after treatment. *Diabetes* 1978; 27: 1145–1150.
20. Kosaka K, Kuzuya T, Akanuma Y, *et al.* Increase in insulin response after treatment of overt maturity-onset diabetes is independent of the mode of treatment. *Diabetologia* 1980; 18: 23–28.
21. Iwasaki Y, Kondo K, Hasegawa H, *et al.* C-peptide response to glucagon in type 2 diabetes mellitus: a comparison with oral glucose tolerance test. *Diabetes Res* 1994; 25: 129–137.
22. Rossetti L, Giaccari A, DeFronzo RA. Glucose toxicity. *Diabetes Care* 1990; 13: 610–630.
23. Knop FK, Vilsbøll T, Højberg PV, *et al.* Reduced incretin effect in type 2 diabetes: cause or consequence of the diabetic state? *Diabetes* 2007; 56: 1951–1959.
24. Højberg PV, Vilsbøll T, Zander M, *et al.* Four weeks of near-normalization of blood glucose has no effect on postprandial GLP-1 and GIP secretion, but augments pancreatic B-cell responsiveness to a meal in patients with Type 2 diabetes. *Diabet Med* 2008; 25: 1268–1275.
25. Fujimoto S, Nabe K, Takehiro M, *et al.* Impaired metabolism-secretion coupling in pancreatic β-cells: role of determinants of mitochondrial ATP production. *Diabetes Res Clin Pract* 2007; 77(Suppl. 1): S2–S10.
26. Mukai E, Fujimoto S, Sato H, *et al.* Exendin-4 suppresses Src activation and reactive oxygen species production in diabetic GK rat islets in an Epac-dependent manner. *Diabetes* 2011; 60: 218–226.
27. Meier JJ, Menge BA, Breuer TG, *et al.* Functional assessment of pancreatic β-cell area in humans. *Diabetes* 2009; 58: 1595–1603.

Impact of endogenous and exogenous insulin on basal energy expenditure in patients with type 2 diabetes under standard treatment[1–3]

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ABSTRACT

Background: Factors that affect resting energy expenditure or basal energy expenditure (BEE) in patients with type 2 diabetes under standard treatment have not been evaluated in detail.

Objective: We determined the clinical factors that affected BEE in addition to body composition in patients with type 2 diabetes under standard treatment.

Design: BEE was measured by using indirect calorimetry under a strict basal condition in 58 Japanese patients with type 2 diabetes after >7 d as inpatients under management of diabetes with medical nutrition therapy and medications. Insulin secretion was measured with a glucagon test. Stepwise regression was applied to explore determinants of BEE.

Results: In the stepwise estimation, insulin secretion ($P = 0.015$), insulin therapy ($P = 0.012$), and pulse rate ($P = 0.011$) were selected in addition to fat-free mass (FFM) ($P < 0.001$) and fat mass ($P = 0.006$) as significant independent determinants of BEE. Standardized partial regression coefficients of the additional 3 factors were −0.16, −0.15, and 0.15, respectively, whereas those for FFM and fat mass were 0.82 and 0.19, respectively. The additional 3 factors explained another 3.9% of the variability of BEE, and the adjusted coefficient of determination was 83.4%. Age, sex, other medications, and parameters of glycemic control were not significant determinants beyond the combined contribution of body composition, endogenous and exogenous insulin, and pulse rate.

Conclusion: Endogenous insulin secretion and exogenous insulin administered in treatment have significant independent effects in the lowering of BEE in patients with diabetes under standard management with medical nutrition therapy and medications. *Am J Clin Nutr* 2011;94:1513–8.

INTRODUCTION

MNT[4] is the basis and starting point of treatment of all patients with diabetes, and the failure of MNT alone may predict the inability to attain optimal glycemic control. In addition, in obese patients with diabetes, MNT aims at weight control to improve insulin resistance and avoid obesity-related health problems. Therefore, estimation of the daily energy expenditure of patients is necessary for individualized diabetic meal plans. For example, MNT for obese patients provides a diet of 500–1000 kcal less energy than the estimated energy expenditure (1). REE or BEE is defined as the minimal amount of energy expended to maintain metabolic activities of cells, tissues, and organs and represents a large component of daily energy expenditure. REE or BEE is used to estimate total energy expenditure as a multiple of REE or BEE according to daily activity.

In healthy subjects, 65–90% of the interindividual variation in REE is explained by FFM or high-metabolic-rate organ mass (2, 3). In patients with diabetes, FFM is also assumed to be a main factor that affects REE and BEE. The influence of the diabetic condition on energy expenditure has also been examined. After adjustment for FFM, REE and BEE in patients with diabetes were shown to be greater than those in healthy control (4, 5). In contrast, no difference was reported in FFM-adjusted REE between mildly hyperglycemic patients and control subjects (6). In addition, a treatment-induced reduction of REE has been reported in type 1 and type 2 diabetes (7, 8). However, the factors that affect REE or BEE in patients with diabetes under standard treatment, which are the more important in clinical practice, have not been precisely evaluated.

In most studies that evaluated energy expenditure, REE has been measured rather than BEE. However, resting conditions are defined less rigorously than are basal conditions, and REE can include components that involve physical or psychological stress and variations in ambient and body temperature (9–11). BEE is measured early in the morning before the subject has engaged in any physical activity and ≥10 h after ingestion of any food, drink, or nicotine and remains remarkably constant on a daily basis (9, 11). We applied the measurement of BEE under such

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[4] Abbreviations used: A_{1c}, glycohemoglobin; BEE, basal energy expenditure; CPR6′, C-peptide immunoreactivity 6 min after intravenous glucagon injection; EGO, endogenous glucose output; FFM, fat-free mass; FPG, fasting plasma glucose; Hb A_{1c}, glycated hemoglobin; MNT, medical nutrition therapy; PG, plasma glucose; PPPG, mean preprandial plasma glucose for 3 consecutive days before the measurement of BEE; REE, resting energy expenditure.

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strict basal conditions to minimize the interindividual variation derived from methodologic or external unknown factors.

In the current study, clinical factors that might affect BEE in patients with diabetes under standard treatment were evaluated.

SUBJECTS AND METHODS

Subjects

Japanese patients with type 2 diabetes who were admitted to the Department of Diabetes and Clinical Nutrition, Kyoto University Hospital, Kyoto, Japan, for diabetes self-management education during the period of December 2007 through September 2009 were recruited. The study protocol was approved by the ethics committee of Kyoto University. Written informed consent was obtained from all participants.

During admission, which averaged ~18 d, participants took MNT with or without medications, including oral hypoglycemic agents and insulin, according to the treatment guide for diabetes of the Japan Diabetes Society (12). The physical activity of subjects was not restricted, but participants did not engage in intensive exercise. Participants were screened by a medical history, physical examination, and laboratory examination to ensure the absence of hepatic, renal (including macroalbuminurea), pulmonary, thyroid, and cardiac dysfunction, inflammation, and malignant tumors. Participants who took steroids or β-blockers and subjects who had physical disabilities were excluded.

Energy expenditure

BEE was measured in the morning >7 d after admission under glycemic control with MNT (29.0 ± 2.6 kcal/kg of standard body weight/d that consisted of 52% carbohydrate, 20% protein, and 28% fat in energy-intake percentages) and with without prescribed medications. Standard body weight (in kg) was calculated by multiplying 22 (kg/m^2) by square of height (in m). Premenopausal women were studied during their follicular phase within 7 d after the last day of menstruation.

Whole-body oxygen consumption and carbon dioxide production were measured for >10 min with indirect calorimetry (AE300S; Minato Medical Science) by one investigator at the bedside of each participant under the strict condition of the methods described previously (9, 10, 13). Briefly, afebrile patients in a postabsorptive state after an overnight fast (14 h) with <10 mmol capillary PG/L before the measurement remained in a supine position after waking on the bed in the ward without smoking or taking caffeine, and measurements were performed at room temperature between 22°C and 27°C. After discarding the initial 5 min of data, we adopted 5 consecutive minutes of data from the rest in accord with the steady state definition (10) during which the CV for oxygen consumption and carbon dioxide production was achieved at ≤10% and applied them to the Weir formula together with 24-h urinary urea nitrogen (14).

Body composition

Height was measured on the day of admission, and body weight was measured immediately after measurement of BEE. FFM and fat mass were measured with a dual energy X-ray absorptiometry scanner (Discovery; Hologic) within 3 d before or after the measurement of BEE.

Clinical and metabolic factors

The duration of diabetes was determined from medical records, medical histories, and previous clinical data according to the criteria for the diagnosis of diabetes proposed by the American Diabetes Association (15). Hb A_{1c} was measured by using HPLC (ADAMS A1C HA8180; Arcray) and expressed as a National Glycohemoglobin Standardization Program equivalent value (A_{1c}; percentage) calculated by the formula

$$\text{Percentage of } A_{1c} = \text{percentage of Hb } A_{1c} \text{ (Japan Diabetes Society)} + 0.4\% \qquad (1)$$

which considers the relational expression of Hb A_{1c} (Japan Diabetes Society) (percentage) measured by using the previous Japanese standard substance and measurement methods and Hb A_{1c} (National Glycohemoglobin Standardization Program) (16). Thyroid stimulating hormone was measured by using electrochemiluminescence immunoassay (Elecsys Anti-Tg; Roche Diagnostics GmbH) to confirm the absence of thyroid dysfunction. Capillary glucose before each meal was measured with a glucose meter (One Touch Ultra; Johnson & Johnson) and expressed as capillary PG. As a variable of glycemic control, the mean preprandial PG for 3 consecutive days before the measurement of BEE and FPG just before the measurement of BEE was used. The mean pulse rate was calculated from the records of checkups by nurses on 3 consecutive days including the day of BEE measurement.

Insulin secretion

β cell function was evaluated after an overnight fast by measuring CPR6′ (17) because the test is valid in patients who take insulin therapy. The serum C-peptide immunoreactivity was measured by using an immunoenzymometric assay (ST AIA-PACK C-peptide; Toso). On the morning of the glucagon test, participants took their medication after the test.

Statistical analysis

Descriptive data were expressed as means ± SDs. Information of medications, including insulin, sulfonylurea, and metformin, was coded as use = 1 and nonuse = 0. Sex was coded as male = 1 and female = 0. The Mann-Whitney U and Fisher's exact tests were performed to identify differences in the characteristics of men and women. The interrelation between BEE and clinical factors was investigated by means of Kendall's rank correlation coefficients. Clinical factors include body composition (FFM and fat mass), FPG, mean preprandial PG, A_{1c}, insulin secretion, mean pulse rate, dietary energy, and medications (ie, insulin, sulfonylurea, and metformin). A multiple linear regression analysis was performed to evaluate the contribution of each determinant to BEE. Variables were selected by stepwise estimation from age, sex, and the clinical factors previously described. When P was <0.05 and ≥ 0.15, the variable was added and removed, respectively. Data were analyzed by use of Stata 11.0 software (StataCorp). Statistical significance was set at $P < 0.05$ (2-tailed).

TABLE 1
Characteristics of participants[1]

	All	Men	Women
Patients (n)	58	35	23
Age (y)[2]	60.2 ± 9.2[3]	57.6 ± 9.7	64.0 ± 7.1
Body weight (kg)[2]	63.7 ± 15.6	68.3 ± 16.5	56.8 ± 11.2
BMI (kg/m^2)	24.2 ± 5.0	24.1 ± 5.6	24.4 ± 4.1
FFM (kg)[2]	48.4 ± 11.1	54.1 ± 9.4	39.7 ± 7.1
BEE (kcal/d)[2]	1294 ± 227	1397 ± 217	1136 ± 135
CPR6′ (ng/mL)	3.4 ± 1.8	3.4 ± 2.0	3.3 ± 1.6
FPG (mg/dL)	114.5 ± 25.2	113.7 ± 25.1	115.6 ± 25.9
PPPG (mg/dL)	143.9 ± 35.5	147.5 ± 37.6	138.5 ± 32.0
A_{1c} (%)	10.5 ± 2.5	10.3 ± 2.4	10.9 ± 2.7
Pulse rate (beats/min)	72.7 ± 10.1	75.0 ± 10.8	69.2 ± 7.8
Duration of diabetes (y)	8.4 ± 7.0	10.0 ± 7.9	5.9 ± 4.4
Treatment			
Diet (kcal · SBW^{-1} · d^{-1})	28.8 ± 2.4	28.7 ± 2.0	29.0 ± 3.0
Medications (n)			
Insulin only	24	17	7
Insulin + metformin	10	4	6
Insulin + sulfonylurea	3	2	1
Insulin + sulfonylurea + metformin	1	0	1
Sulfonylurea only	8	5	3
Sulfonylurea + metformin	5	2	3
Metformin only	4	3	1
None	3	2	1

[1] A_{1c}, glycohemoglobin; BEE, basal energy expenditure; CPR6′, C-peptide immunoreactivity 6 min after intravenous glucagon injection; FFM, fat-free mass; FPG, fasting plasma glucose just before the measurement of BEE; PPPG, mean preprandial plasma glucose for 3 consecutive days before the measurement of BEE; SBW, standard body weight.

[2] Mann-Whitney U test revealed significant ($P < 0.05$) differences between men and women.

[3] Mean ± SD (all such data).

RESULTS

Participant characteristics and results of measurement are shown in **Table 1**. BMI (in kg/m^2), CPR6′, FPG, PPPG, A_{1c}, pulse rate, duration of diabetes, and treatment did not differ significantly between men and women, whereas body weight, FFM, and BEE were significantly higher in men, and age was higher in women.

Significant positive correlations were observed between BEE and FFM (r = 0.72) and dietary energy (r = 0.33), but dietary energy also had a significant positive correlation with FFM (r = 0.37) and a significant negative correlation with fat mass (r = −0.38) (**Table 2**). Fat mass had a significant negative correlation with mean preprandial PG (r = −0.30) and a significant positive correlation with insulin secretion (CPR6′) (r = 0.43)

TABLE 2
Correlations between BEE and possible determinants of BEE[1]

	BEE	FFM	FM	FPG	PPPG	A_{1c}	CPR6′	PR	Diet	Insulin[2]	Sulfonylurea[2]
BEE	1.00	—	—	—	—	—	—	—	—	—	—
FFM	0.72*	1.00	—	—	—	—	—	—	—	—	—
FM	0.18†	0.16	1.00	—	—	—	—	—	—	—	—
FPG	−0.05	−0.08	−0.13	1.00	—	—	—	—	—	—	—
PPPG	−0.08	−0.08	−0.30*	0.42*	1.00	—	—	—	—	—	—
A_{1c}	−0.07	−0.03	−0.14	0.16	0.18†	1.00	—	—	—	—	—
CPR6′	0.19†	0.19†	0.43*	0.03	−0.23†	−0.09	1.00	—	—	—	—
PR	0.21†	0.14	0.05	0.07	0.08	−0.06	0.07	1.00	—	—	—
Diet	0.33*	0.37*	−0.38*	0.07	0.20†	0.06	−0.14	0.09	1.00	—	—
Insulin[2]	−0.12	−0.06	−0.18	0.28†	0.37*	0.27†	−0.24†	0.13	0.00	1.00	—
Sulfonylurea[2]	0.01	−0.00	0.12	−0.36‡	−0.28†	−0.11	0.07	−0.15	−0.06	−0.57*	1.00
Metformin[2]	0.18	0.15	0.40*	−0.13	−0.38*	−0.01	0.29‡	0.05	−0.22	−0.16	0.01

[1] A_{1c}, glycohemoglobin; BEE, basal energy expenditure (kcal/d); CPR6′, C-peptide immunoreactivity 6 min after intravenous glucagon injection (ng/mL); Diet, energy of diet (kcal/d); FFM, fat-free mass (kg); FM, fat mass (kg); FPG, fasting plasma glucose just before the measurement of BEE (mg/dL); PPPG, mean preprandial plasma glucose for 3 consecutive days before the measurement of BEE (mg/dL); PR, pulse rate (beats/min). *,†,‡Kendall's correlation coefficients (n = 58): *$P < 0.001$, †$P < 0.05$, ‡$P < 0.01$.

[2] Use = 1; nonuse = 0.

TABLE 3
Regression models for BEE and body composition and other clinical factors[1]

	Coef (95% CI)	SE	Std coef	P	Adjusted R^2
Model 1					
BEE (FFM + FM)					0.795
Intercept	400.0 (277.1, 523.1)	61.4	—	<0.001	
FFM	17.0 (14.3, 19.7)	1.3	0.83	<0.001	
FM	4.3 (0.3, 8.4)	2.0	0.14	0.038	
Model 2[2]					
BEE (FFM + FM + CPR6′ + INS + PR)					0.834
Intercept	256.2 (59.3, 453.0)	98.1	—	0.012	
FFM	16.9 (14.3, 19.4)	1.3	0.82	<0.001	
FM	5.8 (1.8, 9.8)	2.0	0.19	0.006	
CPR6′	−19.9 (−35.6, −4.1)	7.9	−0.16	0.015	
Insulin[2]	−69.9 (−124.0, −15.7)	27.0	−0.15	0.012	
PR	3.3 (0.8, 5.9)	1.3	0.15	0.011	

[1] Model 1 predicted BEE only from body composition by linear regression analysis (n = 58). The final regression model (model 2) used independent variables selected from FFM, FM, age, sex, CPR6′, fasting plasma glucose, mean preprandial plasma glucose, A_{1c}, dietary energy, use of insulin, use of metformin, use of sulfonylurea, and pulse rate by stepwise estimation (n = 58). A_{1c}, glycohemoglobin (%); BEE, basal energy expenditure (kcal/d); Coef, partial regression coefficient; CPR6′, C-peptide immunoreactivity 6 min after intravenous glucagon injection (ng/mL); FFM, fat-free mass (kg) measured by using dual energy X-ray absorptiometry; FM, fat mass (kg) measured by using dual energy X-ray absorptiometry (kg); INS, insulin; PR, pulse rate (beats/min); Std coef, standardized coefficient.

[2] Use = 1; nonuse = 0.

and use of metformin (r = 0.40). FPG had a negative correlation with the use of sulfonylurea (r = −0.36). Mean preprandial PG had a positive correlation with the use of insulin (r = 0.37) and a negative correlation with the use of metformin (r = −0.38). Insulin secretion (CPR6′) had a negative correlation with the use of insulin (r = −0.24) and a positive correlation with the use of metformin (r = 0.29).

Because clinical factors were naturally correlated with each other, possible spurious correlation and suppressor variables were investigated by means of multiple regression analysis. The model that predicted BEE from body composition alone is shown in **Table 3** (model 1). Another model included selected variables by stepwise estimation (model 2). Insulin secretion (CPR6′; P = 0.015), use of insulin (P = 0.012) and pulse rate (P = 0.011) were significant determinants of BEE in addition to FFM (P < 0.001) and fat mass (P = 0.006). These additional factors explained another 3.9% of the variability of BEE, and the adjusted coefficient of determination was 83.4%. Standardized coefficients of FFM, fat mass, insulin secretion (CPR6′), use of insulin, and pulse rate in model 2 were 0.82, 0.19, −0.16, −0.15, and 0.15, respectively. Variance inflation factors of variables in model 2 were all <1.5. Other plausible determinants (ie, FPG, mean preprandial PG, A_{1c}, dietary energy, use of sulfonylurea, use of metformin, age, and sex) were not selected as significant contributors by stepwise estimation. The effect of insulin secretion (CPR6′) in BEE adjusted for FFM, fat mass, use of insulin, and pulse rate is illustrated in **Figure 1**.

DISCUSSION

In the current study, endogenous insulin secretion and exogenous insulin administered in treatment were both significant independent variables that predicted BEE in Japanese patients with type 2 diabetes under MNT and medications. In addition, both factors were negatively correlated with BEE. The current results suggested that the effect of insulin, regardless of the endogenous or exogenous source, had a significant negative impact on energy expenditure in patients with fair glycemic control under medical care.

In the current study, clinical factors regarding the effects of insulin predicted BEE, whereas factors regarding glycemic control did not predict BEE. The cause of these differing results is not clear, but the present results indicated that the effects of insulin affected BEE independently of glycemia. In a previous report, EGO was shown to be a significant predictor of elevated REE of patients with diabetes after adjustment for body composition, but the glucose concentration was not (18). Although FPG was correlated with EGO, it accounted for only 21% of its variability (19). Gluconeogenesis is an energy-consuming metabolic pathway and is thought to be a major source of increased EGO in diabetes (20–22). Because hepatic insulin deficiency plays an important role in increased gluconeogenesis in diabetes

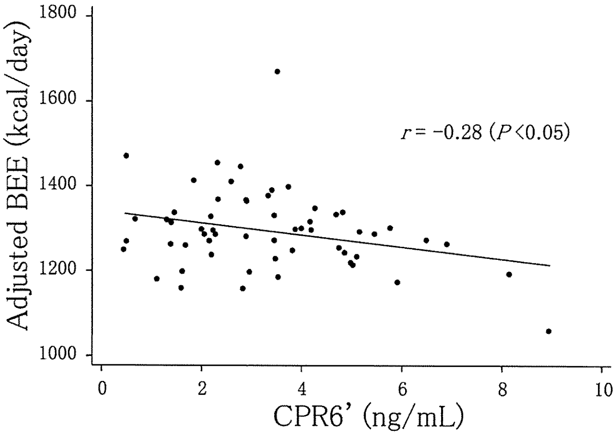

FIGURE 1. Relation between CPR6′ and BEE after adjustment for fat-free mass, fat mass, pulse rate, and insulin use (n = 58). BEE, basal energy expenditure; CPR6′, C-peptide immunoreactivity 6 min after intravenous glucagon injection.

(19), insulin, both endogenous and exogenous, may suppress BEE by reducing gluconeogenesis.

Protein turnover is also involved in energy expenditure. Protein turnover is often elevated in diabetes and has been shown to be correlated with REE in type 2 diabetes (23, 24). Because the treatment-induced reduction of nitrogen flux, which is an index of protein turnover, has been shown to be correlated with the C-peptide response to an oral glucose challenge (8), protein turnover may well play a role in the alteration of energy expenditure by insulin. In addition, impairment of insulin release is often accompanied by hyperglucagonemia in fasting and postprandial states in type 2 diabetes (25). Hyperglucagonemia is responsible for increased leucine oxidation and REE by insulin deprivation in type 1 diabetes (26). In addition, thermogenesis, especially via β-3 adrenergic receptors, has another impact on energy expenditure (20, 27). Considered together, these findings suggested that glucagon and catecholamine may also affect BEE in type 2 diabetes. However, we did not measure glucagon and catecholamine, which was a limitation of the current study.

Contrary to our results, the fasting insulin concentration has been reported to be positively correlated with energy expenditure (18). In the report (18), the positive correlation between insulin and energy expenditure was speculated to derive from the activation of the sympathetic nervous system by insulin (28). We showed that CPR6′ and insulin use were negatively correlated with BEE independently of the pulse rate, which is an indicator of the sympathetic nerve system activity, and was positively correlated with BEE independently of CPR6′ and insulin use. These findings suggested that the effect of insulin on BEE may be independent of the sympathetic nerve system activity at least in patients under treatment.

The difference in insulin sensitivity between lean Japanese subjects and obese American subjects may account for the difference in association of insulin with BEE. In general, a greater attribution of impaired insulin secretion and a lesser contribution of insulin resistance to glucose intolerance were shown in Japanese patients with type 2 diabetes, whose average BMI is ~25 but which is >30 in European and American populations (29). This difference may be attributable, in part, to the difference in BMI; in the Swedish population, the average CPR6′ is lower (~2 ng/mL) in lean subjects with BMI of ~25 than it is in obese subjects (~8 ng/mL) with BMI of ~30. Thus, our findings are in accord with those in Swedish lean subjects (30). Our previous finding of a positive correlation between BMI and CPR6′ also supported this attribution of difference in insulin secretion to difference in BMI (31).

Glycemia was shown to not be an independent determinant of BEE in this study. Although elevated BEE or REE after adjustment for body composition in patients with diabetes compared with in healthy subjects has been reported, the glucose concentration was not described (4, 5). Another study reported a significantly greater REE in patients with high glucose values (>10 mmol/L) compared with in patients with low glucose values (<10 mmol/L) (32). In addition, no increase in BEE was shown in patients with glycemic concentrations of ~9.5 mmol/L (6). Blood glucose concentrations of patients in the current study were improved by treatment, and FPG concentrations and mean preprandial PG concentrations were ~115 mg/dL (6.4 mmol/L) and 144 mg/dL (8.0 mmol/L) respectively. Therefore, the finding of no association between BEE and PG concentrations in the current study was not discrepant with previous reports and was in accord with the finding that REE did not significantly correlate with fasting glucose in type 2 diabetes in which the fasting glucose concentration was ~7.4 mmol/L (18). Together, these data suggested that glycemia is not an important factor in the prediction of BEE in patients with diabetes under treatment.

Dietary energy, treatment with sulfonylurea or metformin, age, and sex were not significant variables that predicted BEE. Sex itself was not a significant determinant but affected coefficients of FFM and fat mass when added to model 2. Researchers have noted a contribution of sex to BEE in addition to FFM and fat mass, although information of menstruation at the measurement of BEE was absent (4, 5). In the current study, BEE was measured during the follicular phase in accordance with a strict protocol for premenopausal women to avoid the influence of progesterone, which was supposed to increase energy expenditure. Another possibility is that our sample size was not large enough to show the contribution of sex to BEE. Last, with consideration of our previous finding that CPR6′ was independently influenced by BMI and the duration of diabetes in type 2 diabetes (31), we performed a regression analysis by adding these 2 putative covariates to model 2, which showed the insignificance of these determinants.

In conclusion, the current study shows that endogenous and exogenous insulin both have a significant impact on lowering BEE in patients with type 2 diabetes under standard treatment with MNT and medications. Longitudinal studies are required to elucidate the impact of the increment of C-peptide concentrations by insulinotropic agents and the administration of insulin on energy expenditure and gluconeogenesis during fair glycemic control.

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REFERENCES

1. American Diabetes Association, Bantle JP, Wylie-Rosett J, Albright AL, Apovian CM, Clark NG, Franz MJ, Hoogwerf BJ, Lichtenstein AH, Mayer-Davis E, et al. Nutrition recommendations and interventions for diabetes: a position statement of the American Diabetes Association. Diabetes Care 2008;31(suppl 1):S61–78.
2. Cunningham JJ. Body composition as a determinant of energy expenditure: a synthetic review and a proposed general prediction equation. Am J Clin Nutr 1991;54:963–9.
3. Javed F, He Q, Davidson LE, Thornton JC, Albu J, Boxt L, Krasnow N, Elia M, Kang P, Heshka S, Gallagher D. Brain and high metabolic rate organ mass: contributions to resting energy expenditure beyond fat-free mass. Am J Clin Nutr 2010;91:907–12.
4. Bitz C, Toubro S, Larsen TM, Harder H, Rennie KL, Jebb SA, Astrup A. Increased 24-h energy expenditure in type 2 diabetes. Diabetes Care 2004;27:2416–21.
5. Martin K, Wallace P, Rust PF, Garvey WT. Estimation of resting energy expenditure considering effects of race and diabetes status. Diabetes Care 2004;27:1405–11.
6. Ryan M, Sallé A, Guilloteau G, Genaitay M, Livingstone MB, Ritz P. Resting energy expenditure is not increased in mildly hyperglycaemic obese diabetic patients. Br J Nutr 2006;96:945–8.
7. Nair KS, Halliday D, Garrow JS. Increased energy expenditure in poorly controlled Type 1 (insulin-dependent) diabetic patients. Diabetologia 1984;27:13–6.