Liver fibrosis improved in 12 patients (30.7%), progressed in 11 patients (28.2%), and remained unchanged in 16 patients (41%). As shown in Table 1, fasting plasma glucose, A1C, insulin resistance indicators, and prevalence of metabolic disorders were not significantly different among the three liver fibrosis groups. In the Cox proportional hazard model (supplementary Table 3), although some of the confidence intervals were very wide because of the small sample size, improvement of liver fibrosis was significantly associated with changes in A1C between the initial and final liver biopsies (ΔA1C) ($P = 0.005$) and use of insulin for the treatment of diabetes ($P = 0.019$). Both ΔA1C and use of insulin were independently associated with the improvement of liver fibrosis after adjusted for each other. However, ΔA1C was more strongly associated with the improvement of liver fibrosis than use of insulin (χ^2; 7.97 vs. 4.58, respectively; supplementary Table 3).

CONCLUSIONS — In the present study, we showed that a change in glycemic control (ΔA1C), but not changes in insulin resistance indicators, was an independent predictor of the progression of liver fibrosis in Japanese patients with NAFLD. This is the first report identifying a change in A1C as a predictor of the histological course in the liver of patients with NAFLD. Two of five previous longitudinal studies have identified obesity, higher BMI, and homeostasis model assessment of insulin resistance (HOMA-IR) as predictors of liver fibrosis progression in Western populations (4,5). The difference between those results and the results of the present study may be due in part to differences in the assessed severity of obesity and insulin resistance between the populations. We previously demonstrated that diabetes is an independent risk factor for the progression of liver fibrosis in hepatitis C (6) and that diabetes accelerates the pathology of nonalcoholic steatohepatitis in the type 2 diabetic rat model OLETF (7).

Liver fibrosis is closely associated with two regulators of fibrosis: transforming growth factor (TGF)-β (8,9) and plasminogen activator inhibitor type 1 (PAI-1) (8,10). High glucose levels induce the expression of TGF-β (11) and PAI-1 (12). We previously reported that the expression of TGF family member genes, PAI-1, and genes involved in fibrogenesis are upregulated in the livers of patients with type 2 diabetes (13,14), suggesting that a diabetic state increases the risk for liver fibrosis.

In the present study, only ΔA1C was associated with the progression of liver fibrosis, but not liver inflammation (data not shown). We speculate that the reduction of A1C inhibits the expression of master genes such as *TGF-β* and *PAI-1* that are involved in the regulation of fibrogenesis, rather than genes involved in liver inflammation, and thereby improves liver fibrosis in NAFLD.

The major limitation of this study was small population size. We could not evaluate the changes of liver histology according to the difference in detail characteristics such as treatment of diabetes. Lower statistical power of this study should be consider when we evaluate the results.

In conclusion, our study suggested that ΔA1C could predict liver fibrosis progression in Japanese patients with NAFLD, and tight glycemic control may ameliorate liver fibrosis. Future large-scale prospective studies are needed to confirm our results.

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No potential conflicts of interest relevant to this article were reported.

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Altered Hepatic Gene Expression Profiles Associated With Myocardial Ischemia

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Background—Acute coronary syndrome is sometimes accompanied by accelerated coagulability, lipid metabolism, and inflammatory responses, which are not attributable to the cardiac events alone. We hypothesized that the liver plays a pivotal role in the pathophysiology of acute coronary syndrome. We simultaneously analyzed the gene expression profiles of the liver and heart during acute myocardial ischemia in mice.

Methods and Results—Mice were divided into 3 treatment groups: sham operation, ischemia/reperfusion, and myocardial infarction. Mice with liver ischemia/reperfusion were included as additional controls. Marked changes in hepatic gene expression were observed after 24 hours, despite the lack of histological changes in the liver. Genes related to tissue remodeling, adhesion molecules, and morphogenesis were significantly upregulated in the livers of mice with myocardial ischemia/reperfusion or infarction but not in those with liver ischemia/reperfusion. Myocardial ischemia, but not changes in the hemodynamic state, was postulated to significantly alter hepatic gene expression. Moreover, detailed analysis of the signaling pathway suggested the presence of humoral factors that intervened between the heart and liver. To address these points, we used isolated primary hepatocytes and showed that osteopontin released from the heart actually altered the signaling pathways of primary hepatocytes to those observed in the livers of mice under myocardial ischemia. Moreover, osteopontin stimulated primary hepatocytes to secrete vascular endothelial growth factor-A, which is important for tissue remodeling.

Conclusions—Hepatic gene expression is potentially regulated by cardiac humoral factors under myocardial ischemia. These results provide new insights into the pathophysiology of acute coronary syndrome. (***Circ Cardiovasc Genet.* 2010;3:68-77.**)

Key Words: coronary disease ■ genetics ■ liver ■ myocardial infarction

In addition to chest pain, acute coronary syndrome (ACS) is sometimes accompanied by systemic manifestations, such as proinflammatory responses, activation of the coagulation-fibrinolytic system, and lipid metabolism.[1–3] These are considered to be systemic reactions involving multiple organs, which exacerbate the cardiac events.

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C-reactive protein, coagulation factors, and protein C, the levels of which fluctuate in ACS, are liver-specific factors. Although these reports were based on a limited number of factors, the observations suggest a close relation between the liver and myocardial ischemia and imply that the liver plays a pivotal role in the pathophysiology of ACS.

cDNA microarray technology allows simultaneous analysis of the expression levels of thousands of genes. Genome-based expression profiling provides useful information on the molecular pathogenesis of various diseases as well as disease progression and prognosis.[4–7] Previous microarray studies have examined the molecular dynamics of the myocardium induced by myocardial ischemia.[8,9] However, global gene expression analyses applied to the liver affected by myocardial ischemia have not been reported.

In this study, we examined the responses of hepatic gene expression to myocardial ischemia. Given the systemic inflammation that characterizes ACSs, we postulated that regulation of hepatic genes occurs by inflammatory mediators and not by alterations in hemodynamics or hepatic perfusion. Therefore, we used whole-genome transcriptional profiling to identify hepatic genes selectively regulated in myocardial ischemia.

Methods

This study was approved by institutional and governmental animal research committees and was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). C57BL/6J mice (n=46; body weight, 24.1±1.4 g; 8 to 10

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Table 1. Biochemical Assessment

		6 h				24 h			
	Before Operation	Sham	I/R	Infarction	Liver I/R	Sham	I/R	Infarction	Liver I/R
No.	5	5	5	5	5	6	5	5	5
CPK, U/L	944±98	5031±646	11597±1272*	19830±1154*	8673±1379	1702±181	1913±184	2939±515†	1595±349
AST, U/L	94±4	674±41	899±21*	1858±59*	414±43	200±19	277±14	661±28*	163±22
ALT, U/L	58±4	119±9	115±6	153±7	143±18	46±3	64±6	107±11*	42±3
LDH, U/L	652±32	2684±206	3432±80†	5264±111*	2478±446	681±72	867±37	2095±164*	862±209

Values are presented as mean±SE. CPK indicates creatine kinase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; and LDH, lactate dehydrogenase.

*$P<0.01$ compared with sham.

†$P<0.05$ compared with sham.

weeks of age; Charles River Laboratories, Yokohama, Japan) were divided into the following treatment groups: sham operation (n=11), ischemia/reperfusion (I/R; n=10), myocardial infarction (MI; n=10), liver I/R (n=10), and sham operation plus hydralazine (n=5). Hepatic gene expression was evaluated among these groups, and the results were further investigated in primary mouse hepatocytes.

Additional Methods

An expanded Methods section containing details of animal surgery, hydralazine group, liver I/R group, blood sampling and analysis, histopathological analysis, blood pressure and heart rate measurements, microarray experiments, processing of cDNA microarray data, extraction of significantly upregulated cardiac and hepatic genes, pathway analysis, ELISA for secreted osteopontin and vascular endothelial growth factor (*VEGF*), primary hepatocyte experiments, and quantitative real-time detection polymerase chain reaction (RTD-PCR) is available in the online-only Data Supplement.

All microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database with the series accession number GSE14843.

Data Analysis

The data are presented as the mean±SEM for each group of mice and were analyzed by ANOVA with Bonferroni post hoc test for multiple comparisons. Statistical analyses of blood sampling, blood pressure, and heart rate were performed with the Steel (heterogeneity of variance) multicomparison test. Significance was set at $P<0.05$. Statistical analyses were performed with SAS statistical software (SAS Institute Japan, Tokyo, Japan).

Results

Establishment of Cardiac I/R or MI in Mice

Cardiac I/R or MI was successfully induced in normal C57BL/6J mice. The levels of cardiac enzymes, such as creatine kinase, aspartate aminotransferase, and lactate dehydrogenase, increased significantly after 6 hours in the I/R group and showed markedly greater increases in the infarction group compared with the sham group (Table 1). In addition, the normalization of these enzyme levels was reduced after 24 hours in the infarction group.

Histologically, azan or hematoxylin/eosin staining showed wall thinning, coagulation necrosis, and transmural fibrosis in the risk area in the infarction group but not in the I/R group (data not shown). As shown in Table 2, no significant differences were found in heart rate or blood pressure after 24 hours compared with the preoperative values in the sham and I/R groups, whereas a decrease in blood pressure was found in the infarction group.

Histological Assessment of the Liver After Cardiac I/R or MI

The I/R and infarction groups showed a minimal, but transient, increase in alanine aminotransferase ALT. Although alanine aminotransferase may be released from the myocardium[10] rather than from the liver, to exclude the effect of the transient change in hepatic venous pressure associated with cardiogenic shock, we examined histological changes in the liver after myocardial I/R or infarction. No histological abnormalities were observed in the shocked liver, as indicated by the lack of hepatocyte necrosis in acinar zone 3 in the sham, I/R, and infarction groups (Figure 1a, 1c, 1e, and 1g; hematoxylin/eosin staining). In addition, no signs of liver congestion were observed, as indicated by the lack of dilatation of the terminal hepatic venules and adjacent sinusoids in the sham, I/R, or infarction group (Figure 1b, 1d, 1f, and 1h; silver staining).

On transmission electron microscopy, no ischemic changes, such as swelling or loss of cristae in the mitochondria, a mixed irregular pattern or swelling of the rough endoplasmic reticulum, or dilatation or indistinct appearance of the sinusoids, were observed in the sham, I/R, or infarction group (Figure 2A through 2C). Based on these results, histological analysis did not demonstrate the presence of shock or congestive liver in the I/R or infarction group.

Changes in the Hepatic Gene Expression Profile After Cardiac I/R or MI

Although no histological changes were observed in the liver after cardiac I/R or MI, significant changes in gene expression were noted. Hierarchical clustering analysis, which is a nonsupervised learning method that includes 23 281 nonfil-

Table 2. HR, sBP, and mBP

		24 h		
	Before Operation	Sham	I/R	Infarction
HR, bpm	575±27	553±30	553±27	568±16
sBP, mm Hg	105±2	102±2	95±1	80±4*
mBP, mm Hg	78±3	74±2	63±2	56±4†

Values are presented as mean±SE. HR indicates heart rate; sBP, systolic blood pressure; and mBP, mean blood pressure.

*$P<0.01$ compared with sham.

†$P<0.05$ compared with sham.

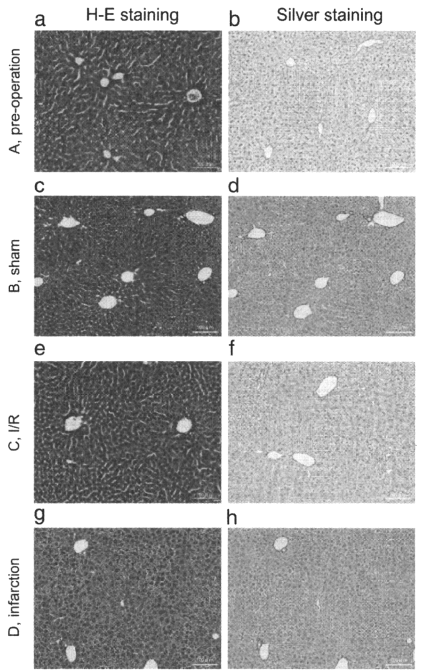

Figure 1. Histological comparison of hematoxylin/eosin staining and silver staining of the liver after 24 hours. Preoperation (A), sham (B), I/R (C), and infarction (D). Scale bars represent 100 μm. Hematoxylin/eosin staining (a, c, e, and g); silver staining (b, d, f, and h). No indication of shock or congestive liver was observed in any group (magnification, ×200).

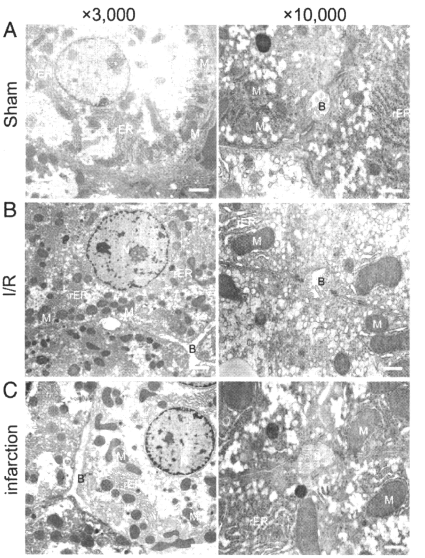

Figure 2. Representative electron microphotographs of the liver after 24 hours. Sham (A), I/R (B), and infarction (C). Scale bars represent 2 μm on the left (magnification, ×3000) and 500 nm on the right (magnification, ×10 000). No indication of shocked liver was observed in any group. M indicates mitochondria; rER, rough endoplasmic reticulum; B, bile canaliculi; N, nucleus.

tered genes, produced clusters for the I/R or infarction group and the sham-operated group (data not shown). Because nonfiltered genes may include those that are unchanged in all samples, which generated "noise" that prevented efficient gene clustering, we filtered out these genes with different stringency and performed hierarchical clustering. Hierarchical clustering with 9165 (log-ratio variations >40th percentile) or 5156 (log-ratio variations >50th percentile) filtered genes clearly demonstrated clusters for the I/R or infarction group after 24 hours, for the I/R or infarction group after 6 hours, and for the sham group after 6 and 24 hours (supplemental Figure I). Hierarchical clustering with 773 (log-ratio variations >80th percentile) or 96 (log-ratio variations >90th percentile) filtered genes showed more detailed and clearer clusters for the I/R group after 24 hours, for the infarction group after 24 hours, for the I/R or infarction group after 6 hours, and for the sham group after 6 and 24 hours (Figure 3). Thus, by filtering out "noise" genes, more detailed and clearer clustering could be obtained, thus addressing the reliability of the analysis.[11] The increased robustness (R-index) and decreased discrepancy (D-index) of clustering with filtering conditions supported this finding (supplemental Figure I; expanded Methods and Results).

Class prediction analysis, a supervised learning method based on the compound covariate predictor, was performed with various clinical parameters, including provocation (I/R or infarction), 6 hours (I/R or infarction after 6 hours), 24 hours (I/R or infarction after 24 hours), and time (sham or 6 hours, sham or 24 hours, and 6 or 24 hours). The results

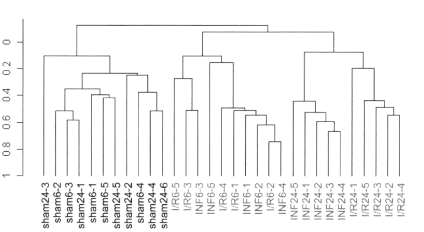

Figure 3. Hierarchical clustering analysis with 96 filtered genes (genes with log-ratio variation in the 90th percentile and data missing >5% were excluded). The resulting dendrogram shows clear clusters for the I/R group after 24 hours, the infarction group after 24 hours, and the sham group after 6 and 24 hours.

Table 3. Class Prediction Analysis (Supervised Learning Methods)

Classifier Category	Clinical Group	Total No. of Classes	No. of Cases Misclassified	Classifier *P*	Mean Percent of Correct Classification	No. of Genes in the Classifiers ($P<0.002$)
Provocation	I/R	10	2	0.02	80	85
	INF	10	2			
6 h	I/R	5	2	0.48	50	23
	INF	5	3			
24 h	I/R	5	0	0.015	100	218
	INF	5	0			
Time	Sham	11	0	0.001	90	644
	6 h	10	2			
	Sham	11	0	<0.0005	95	3380
	24 h	10	1			
	6 h	10	1	0.004	85	747
	24 h	10	2			

INF indicates infarction.

indicated that provocation, 24 hours, and time significantly classified these models (Table 3).

Both nonsupervised and supervised learning methods indicated differences in hepatic gene expression profiling among sham, 6 hours, and 24 hours after heart provocation, and different heart provocation (I/R or infarction) may generate differences in hepatic gene expression, especially 24 hours after provocation.

Identification of Genes Differentially Expressed Between I/R and Infarction

Because the filtering process may result in loss of important genes, for identification of differentially expressed genes among different groups, we used a class comparison analysis tool (http://linus.nci.nih.gov/BRB-ArrayTools.html). Class comparison analysis ($P<0.0005$) among the 5 groups (ie, sham, I/R-6, I/R-24, infarction-6, and infarction-24) was performed, and genes that were differentially expressed among the 5 groups were extracted. On 1-way hierarchical clustering analysis of the extracted genes and heat map, 6 gene clusters were assigned on the basis of the gene expression patterns (Figure 4). Of the 6 groups, group 2 showed significant upregulation for I/R and infarction after 24 hours compared with the other groups. Group 3 showed upregulation for I/R, but not for infarction, after 24 hours. Group 4 showed downregulation for I/R and infarction after 24 hours compared with the other groups. Group 5 showed downregulation for infarction after 24 hours compared with the other groups. Representative genes (>3-fold difference in *t* value) and frequent pathways observed in each group (based on the MetaCore database) are listed in supplemental Tables I through IV.

Interestingly, in group 2, genes related to tissue remodeling, adhesion molecules, and morphogenesis were significantly upregulated. This may be related to the induction of tissue repair factors, such as antigenic factor and myocardiogenic factors, associated with I/R or infarction. In addition, genes involved in the cell cycle and apoptosis and neuron-related genes, such as retinoblastoma 1, angiopoietin-like 4, apoptotic peptidase-activating factor 1, transformation-related protein 53 (*p53*), and Eph receptor B1, were preferentially expressed. The expression of group 2 genes was significantly correlated with serum creatine kinase levels, suggesting that these genes reflect the severity of cardiac damage. Especially, ($R=0.856$, $P<e^{-07}$) and apoptotic peptidase-activating factor 1 ($R=0.856$, $P<e^{-07}$) were highly correlated with creatine kinase (supplemental Table I).

In group 3, in addition to the genes described earlier, chemokine and hormone gene pathways involved in interleukin (IL)-8 and androgen or estrogen receptor signaling were upregulated, suggesting that more tissue repair and bioreactive signaling pathways were activated. This may reflect the presence of a living myocyte I/R condition. In group 4, genes involved in lipid catabolism, immune response, proteolysis, and oxidative stress, such as apolipoprotein A-II, CD7 antigen, and reduced nicotinamide-adenine dinucleotide phosphate oxidase 1, were downregulated in the infarction and I/R groups after 24 hours. In group 5, genes involved in muscle and neurite morphogenesis, such as myosin (heavy polypeptide 11, smooth muscle) and ephrin A5, were significantly downregulated in the infarction group after 24 hours.

Effects of Hemodynamic State on Hepatic Gene Expression Profile

To exclude the possibility that changes in hemodynamic state induced alterations in hepatic gene expression, we examined the livers of mice subjected to liver I/R. For liver I/R, gentle occlusion of the hepatic artery and portal vein was applied so that the extent of liver injury was comparable with those in the myocardial I/R and infarction models (Table 1).

We analyzed the gene expression profile of the liver I/R group by using the same extracted genes as shown in Figure 4. The gene expression patterns induced in the myocardial I/R and infarction groups are clearly different from those in the liver I/R group (Figure 5), except for the group 3 gene cluster in myocardial I/R. It should be noted that the group 3 gene cluster was upregulated in the myocardial I/R group at 24

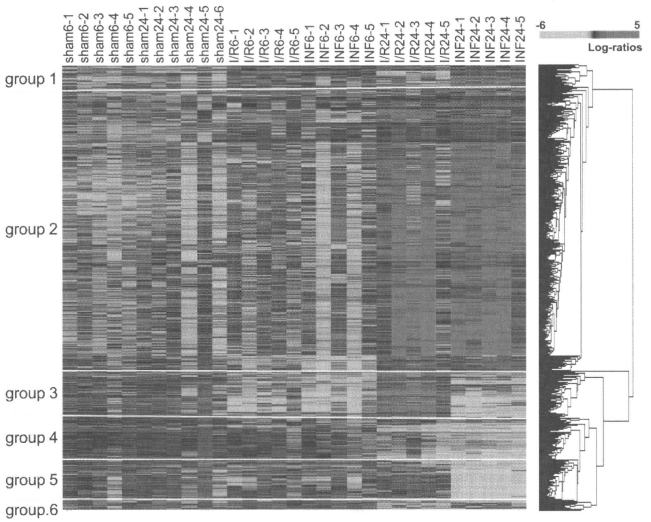

Figure 4. One-way hierarchical clustering and a heat map of 1166 genes that were extracted by class comparison analysis ($P<0.0005$). Each column corresponds to a sample, and each row represents a gene. The gene cluster data are graphically presented as colored images: red indicates upregulated genes, and green indicates downregulated genes. The genes with the most similar patterns of expression are adjacent to one another. Detailed definitions of each group are given in the text. Representative genes and frequently observed pathways are listed in supplemental Tables I through IV.

hours after provocation, whereas it was upregulated from 6 hours after provocation in the liver I/R group. Therefore, the delayed changes in hepatic gene expression in the myocardial I/R and infarction models may be due to different mechanisms resulting from liver I/R.

The assessment of liver weight revealed no differences between the myocardial I/R and infarction groups (supplemental Table V). This result supports our histological findings and indicates an absence of liver congestion in the myocardial I/R and infarction groups.

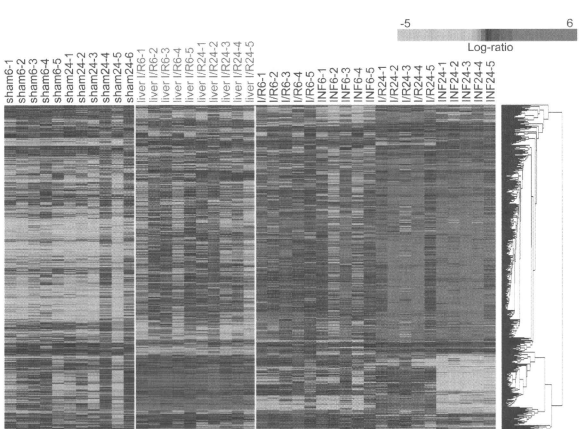

Figure 5. One-way hierarchical clustering and a heat map of the liver I/R group and others with the same extracted genes as shown in Figure 4. Each column corresponds to a sample, and each row represents a gene. The gene cluster data are graphically presented as colored images: red indicates upregulated genes, and green indicates downregulated genes. The genes with the most similar patterns of expression are adjacent to one another. Gene expression patterns induced in the liver I/R group clearly differed from those in the myocardial I/R and infarction groups.

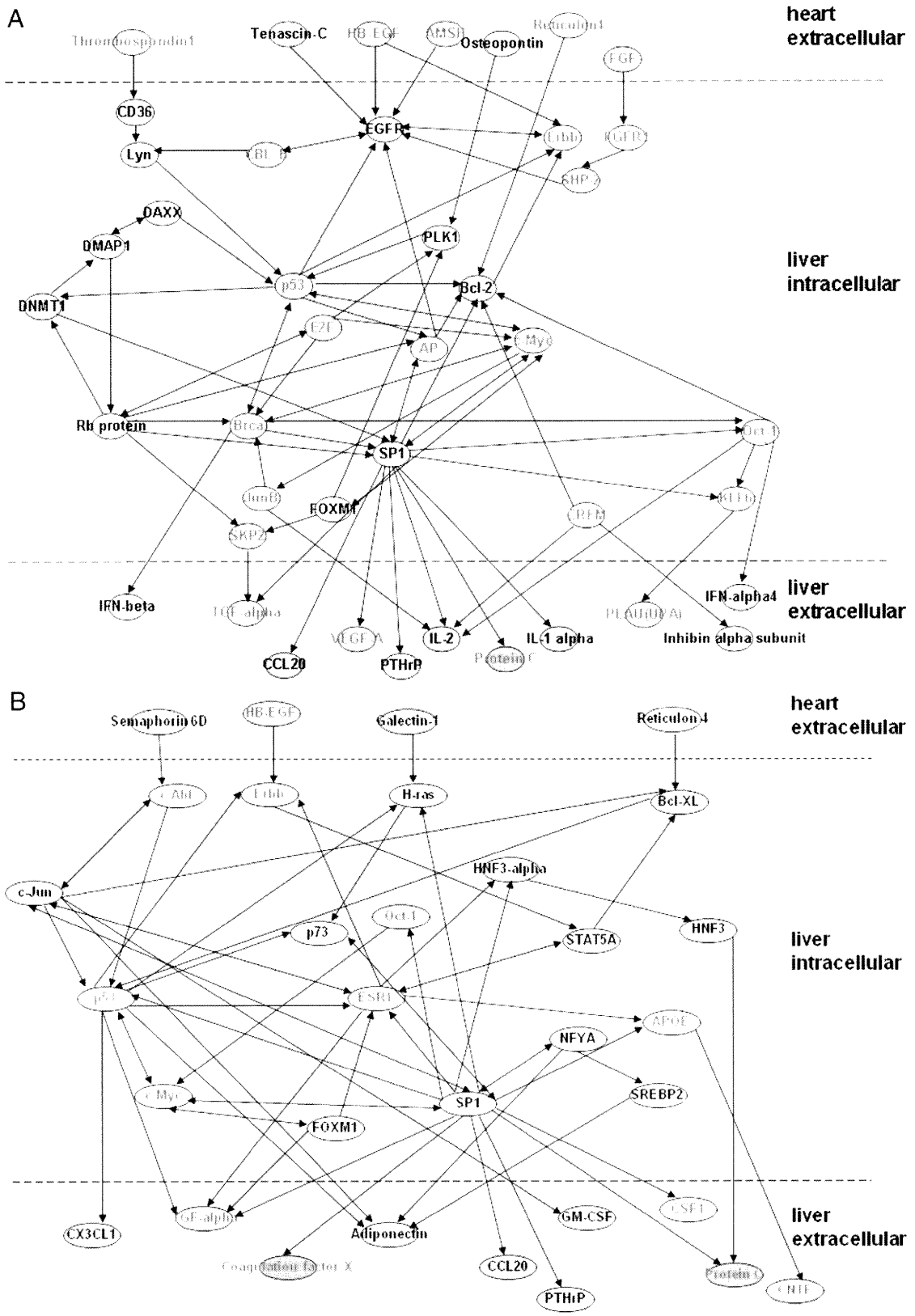

Figure 6. A, Postulated gene network of differentially expressed genes in infarction. B, Postulated gene network of differentially expressed genes in I/R. Detailed definitions of heart-extracellular, liver-intracellular, and liver-extracellular are given in the Methods. Yellow ovals indicate genes related to angiogenesis; green ovals, genes related to coagulation-fibrinolysis; blue ovals, genes related to inflammation; and red characters, genes upregulated in microarray analysis of primary hepatocytes treated with osteopontin. The network diagrams consist of representative genes. All abbreviations are defined in supplemental Tables VI through XI.

Detailed Gene Network Analysis Between the Liver and Heart in Myocardial Ischemia

Several factors can affect the liver, including humoral factors released from the ischemic myocardium, the hemodynamic state, or the autonomic nervous system. We focused on the possibility that humoral factors released from the heart may affect the liver. Cardiac gene expression profiles induced by myocardial ischemia were investigated to identify cardiac genes affecting the liver. To obtain a detailed and comprehensive gene network for the liver and heart, individual data from the liver after 24 hours were integrated with pooled data from the risk area and nonrisk area of the heart. Initially, we divided the heart and liver genes into 3 groups: heart-extracellular, liver-intracellular, and liver-extracellular. To find the network among these induced genes, published results for the interactions of individual genes were integrated with these results by using MetaCore software (GeneGo, St. Joseph, Mich). Direct interactions between individual genes were sought. Genes were excluded according to the following criteria: (1) heart-extracellular, no output signal into liver-intracellular; (2) liver-intracellular, no bidirectional signals; and (3) liver-extracellular, no input signal from liver-intracellular. As expected, the network of these differentially expressed genes involved complex interactions of individual genes; however, representative signaling pathways for MI or I/R injury were identified (Figure 6).

During MI, fibroblast growth factor, osteopontin, and heparin-binding epidermal growth factor-like growth factor (*HB-EGF*) were upregulated in the heart and may have been systemically secreted. Endothelial growth factor receptor and fibroblast growth factor receptor-1 may play important roles in receiving these signals in the liver. Transcription factors such as *p53*, myelocytomatosis oncogene, *trans*-acting transcription factor 1, and octamer-binding transcription factor 1 are important molecules in the regulation of these signaling pathways. Protein C, VEGF-A, and urokinase were expected to be systemically secreted from the liver (supplemental Tables VI through VIII). After infarction, genes involved in inflammation, the coagulation-fibrinolytic system, and angiogenesis showed preferential expression. After I/R, heparin-binding epidermal growth factor was upregulated in the heart and was expected to be systemically secreted. V-*erb*-a erythroblastic leukemia viral oncogene homolog 4 (avian) may play an important role in receiving these signals in the liver. Transcription factors such as *trans*-acting transcription factor 1, *p53*, estrogen receptor-1α, and signal transducer and activator of transcription 5A are potentially important molecules for regulation of these signaling pathways. Protein C, coagulation factor X, ciliary neurotrophic factor, and colony-stimulating factor-1 (macrophage) (*CSF-1*) were expected to be systemically secreted from the liver. In I/R, angiogenesis-related genes were preferentially upregulated (supplemental Tables IX through XI). On comparison of the expression profiles of the heart and liver, genes expressed at significantly higher levels in the heart than in the liver were designated as He, and those expressed at significantly higher levels in the liver than in the heart were designated as Li. Genes expressed in both the heart and liver were described as He/Li (supplemental Tables VIII and XI). In this analysis, most of the factors that were expected to be secreted from the liver induced by I/R and infarction were liver-specific. Most of these genes were not significantly upregulated in the liver I/R groups.

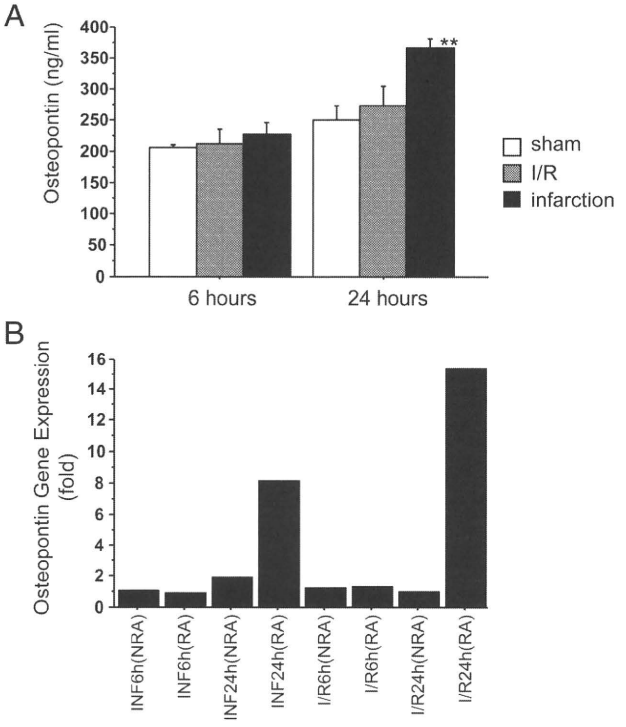

Figure 7. A, The time course of the serum osteopontin concentrations in sham, I/R, and infarction groups. The assessment of serum osteopontin by ELISA in the sham, I/R, and infarction groups after 6 and 24 hours. The serum concentrations of osteopontin after 24 hours were 365.7±14.6 ng/mL, 273.0±30.6 ng/mL, and 249.2±23.4 ng/mL in infarction, I/R, and sham groups, respectively. Error bars represent the SEM. $^{**}P<0.01$ compared with sham. B, The changes in osteopontin gene expression in the infarcted and reperfused heart. INF indicates infarction; NRA, nonrisk area; RA, risk area.

Serum Osteopontin Concentrations in Mice

Of the infarction-induced, cardiac-secreted factors that were expected to stimulate multiple liver genes, we quantified the serum levels of osteopontin by ELISA. Serum osteopontin concentration was significantly increased in the infarction group compared with the sham group ($P=0.0012$) after 24 hours (Figure 7A). In addition, the changes in osteopontin gene expression in the infarcted and reperfused heart are shown in Figure 7B.

Signaling Pathway in Primary Hepatocytes Treated With Osteopontin

To determine whether ischemia-induced, cardiac-secreted factors affected hepatic gene expression, we investigated the effects of osteopontin on primary mouse hepatocytes (supplemental Materials and Methods); 979 genes were upregulated and 734 genes were downregulated ($P<0.05$ and fold change >2.0 determined by class comparison analysis) by osteopontin in primary hepatocytes (GSE14843). The most frequent pathway processes observed among upregulated genes as determined with the use of MetaCore software are

shown in supplemental Table XII. Osteopontin upregulated signaling pathways of protein C, angiogenesis, cell adhesion, etc, which were observed in groups 2 and 3 gene clusters in the mouse liver under conditions of myocardial ischemia (Figure 4; supplemental Tables I and II). The role of osteopontin in the postulated gene network connecting the liver and heart in myocardial ischemia is shown in Figure 6. Interestingly, many of the genes included in the postulated gene network were actually activated by osteopontin ($P<0.05$ or fold change >2.0 by class comparison analysis) in primary hepatocytes. Unexpectedly, osteopontin activated *HB-EGF*, thrombospondin 1, and fibroblast growth factor, which were released from the ischemic heart (Figure 6; supplemental Table VI) in primary hepatocytes. These results indicated that these proteins were released from the liver and from the heart under conditions of myocardial ischemia through osteopontin, and an autocrine signaling pathway may exist in the liver.

Among the candidate hepatic-secreted factors under conditions of myocardial ischemia (Figure 6A; supplemental Table VIII), we quantified the levels of *VEGF-A* in the supernatants of primary hepatocytes treated with osteopontin. The concentration of *VEGF-A* measured by ELISA was significantly increased in the supernatants of primary hepatocytes treated with osteopontin (n=6) compared with the mock group (n=7; $P=0.0042$; supplemental Figure II). Thus, important factors for tissue remodeling could be released from the liver through humoral factors, such as osteopontin, that are released from the heart under conditions of myocardial ischemia.

Quantitative RTD-PCR

We performed a quantitative RTD-PCR with TaqMan probes. In the I/R group, protein C, coagulation factor X, *CNTF*, and *CSF-1* were upregulated in the liver. In the infarction group, protein C, urokinase, and *VEGF-A* were upregulated in the liver (supplemental Figure IIIA). In the hepatocytes treated with osteopontin, protein C, coagulation factor X, ciliary neurotrophic factor, *CSF-1*, urokinase, and *VEGF-A* were upregulated compared with the mock group (supplemental Figure IIIB). These results were consistent with those of cDNA microarray analyses performed in this study.

Discussion

The liver is an essential organ that synthesizes many bioactive proteins, including acute-phase inflammatory proteins (eg, C-reactive protein and IL-6) and coagulation factors. Therefore, it has been speculated that the liver may be involved in systemic reactions that modify the pathophysiology of ACS, although this possibility has not been addressed in detail.

In this study, we examined the gene expression profiles of the livers of mice affected by myocardial I/R or infarction. Marked changes in hepatic gene expression were observed after 24 hours, despite the lack of histological changes in the liver. These changes were essentially restored to normal after 3 to 7 days (data not shown). These findings may not be due to hemodynamic changes during myocardial I/R or infarction. Instead, inflammatory mediators or humoral factors released from the affected heart may be responsible for the observed alterations in hepatic gene expression. This was further confirmed by investigation of signaling pathways in primary hepatocytes induced by osteopontin, a candidate humoral factor released from the ischemic myocardium in vitro.

To exclude the possibility that these changes in gene expression were due to systemic hypotension during I/R or infarction, we performed an additional experiment involving liver I/R to examine whether a pattern of gene expression similar to that in the myocardial I/R and infarction groups could be observed in the liver. Hepatic gene expression in the liver I/R group was completely different from those in the myocardial I/R and infarction groups, with the exception of a small gene cluster (group 3). Although the group 3 gene cluster was upregulated in both the liver I/R and myocardial I/R groups at 24 hours after provocation, peak expression was delayed in the myocardial I/R group compared with the liver I/R group. A recent report of extended observations of cytokine expression in murine hepatic I/R injury indicated that the levels of expression of tumor necrosis factor-α, IL-1β, and IL-6 peaked within 4 hours and returned to baseline at 24 hours.[12] In contrast, in the myocardial I/R and infarction models, these cytokines peaked $\approx$24 to 48 hours and decreased at 7 days.[13] These findings were consistent with those of this study (data not shown). Therefore, the delayed peak of hepatic gene expression observed in this study may be correlated with the extent of inflammation in the myocardium after destruction of myocytes, rather than changes in the hemodynamic state of the liver. The lack of histological changes in the liver in the myocardial I/R and infarction models supported these suggestions, although the influence of hemodynamic state on hepatic gene expression should be carefully considered.

Interestingly, genes related to tissue remodeling, adhesion molecules, and morphogenesis were significantly upregulated in the livers of mice that were subjected to I/R or infarction. This may be related to the induction of tissue repair factors such as angiogenic or myocardiogenic factors in the heart undergoing I/R or infarction. In support of this notion, in addition to the genes upregulated during infarction, chemokines and hormonal factors, including IL-8, androgen, and estrogen receptor genes, were upregulated during I/R. These findings may reflect the presence of living myocytes and the greater release of tissue repair and bioreactive factors during I/R than during infarction.

A recent study that included a sequential analysis of ischemic mouse heart with quantitative RT-PCR demonstrated expression of IL-1β, IL-6, monocyte chemoattractant protein-1, macrophage inflammatory protein-1, and granulocyte-CSF at 6 and 24 hours.[13] These results were essentially consistent with those of our microarray analysis of pooled RNA extracted from heart specimens (data not shown).

In this study, the hepatic RNA samples were not pooled but were used to analyze the hepatic gene expression profiles individually. This strategy was successful, in that our microarray results were consistent with those produced from pooled or nonpooled liver specimens. Moreover, it facilitated the statistical evaluation of differentially expressed genes

among the various groups and revealed dynamic changes in hepatic gene expression through clustering analysis.

We analyzed the network connecting the heart-extracellular genes and liver-intracellular genes induced after I/R injury or infarction by using expression data from pooled heart samples and averaged the expression data for individual liver samples. The results suggested that factors secreted from the heart altered gene expression in the liver. By detailed analysis of signaling pathways, we identified 9 candidate genes (eg, *Osteopontin*, *HB-EGF*, *Reticulon 4*) that were upregulated in the heart and were expected to be systemically secreted and to regulate gene expression in the liver (Figure 6). Moreover, we identified the factors that were expected to be secreted from the liver induced by these signaling pathways, such as protein C, coagulation factor X, *CNTF*, *CSF-1*, and angiogenesis-related genes. These factors were expected to be systemically secreted from the liver and to modulate the pathophysiology and outcome of ACS. It has been reported that protein C prevents myocardial I/R injury,[14] *VEGF* enhances capillary density and improves cardiac function,[15] and urokinase is essential for cardiac functional recovery after acute myocardial infarction.[16]

Of the factors that were expected to be secreted from the heart, we confirmed that infarction increased the serum osteopontin concentration after 24 hours. Osteopontin is essential for the development of myocytes, tissue repair, and angiogenesis, and its downstream products, eg, polo-like kinase, were upregulated in the liver. To confirm these findings, we examined the signaling pathways in primary hepatocytes treated with osteopontin. Osteopontin activated signaling pathways of protein C, angiogenesis, and cell adhesion (supplemental Table XII) by inducing the expression of protein C, urokinase, *VEGF-A*, *CSF-1*, factor X, and ciliary neurotrophic factor (*CNTF*) in primary hepatocytes, which was confirmed by RTD-PCR or ELISA (supplemental Figures II and IIIB). Moreover, many other genes involved in the postulated gene network associating the liver and heart (Figure 6A and 6B) were actually activated in primary hepatocytes treated with osteopontin, confirming this signaling pathway. These results suggest that humoral factors play important roles in signal transduction from the ischemic myocardium to the liver.

Although our results addressed humoral factors from the heart that may affect hepatic gene expression, the effects of other factors, such as autonomic nerves, should also be considered. Because the liver has rich sympathetic and parasympathetic innervation,[17–19] it is possible that sympathetic hyperactivity affects hepatic gene expression. Although hydralazine has been reported to activate sympathetic nerves,[20,21] we observed no differences in gene expression in the hydralazine-treated group compared with the sham-operated group (data not shown). Therefore, autonomic nerves seemed to have little effect on hepatic gene expression determined in this study.

In conclusion, we reported new insights into the pathophysiology of ACS, which may facilitate identification of the mechanisms by which an acute coronary event causes systemic reactions. Further studies are needed to determine whether early therapeutic targeting of the liver during an acute coronary event has any beneficial effect on the clinical outcome in these patients.

Study Limitations

Although we confirmed that the serum osteopontin concentration was increased during myocardial ischemia, other proteins that could potentially be secreted from the heart and liver were not assayed. Further studies are needed to determine whether these proteins, including osteopontin, actually affect hepatic gene expression as observed in this study.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Acute coronary syndrome (ACS) is accompanied by systemic changes in inflammation, coagulation, and metabolism, which may affect the outcome and prognosis of ACS. These systemic reactions are not explained by cardiac events alone. Several lines of evidence suggest that patients with fatty liver disease have a high risk of developing cardiovascular diseases, and it is possible to speculate that the liver is involved in a systemic reaction that modifies the pathogenesis of ACS. However, the relation between liver and myocardial ischemia in the acute ischemic phase has not been elucidated so far. In this investigation, we simultaneously analyzed the gene expression profiles of the liver and heart during acute myocardial ischemia in mice and observed the presence of humoral factors that intervened between the heart and liver. These humoral factors were released from the heart and influenced the liver to secrete important tissue remodeling factors. One of these humoral factors, osteopontin, a widely expressed glycoprotein, was increased in the ischemic heart and altered the gene expression of hepatocytes to produce important tissue remodeling factors (such as vascular endothelial growth factor-A). Our observations suggest that hepatic gene expression is potentially regulated by humoral factors of cardiac origin provoked by myocardial ischemia, and we provide direct evidence that the liver is involved in a systemic reaction that accompanies ACS. Our findings provide potential new insights into the pathophysiology of ACS.

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Influence of Age on Clock Gene Expression in Peripheral Blood Cells of Healthy Women

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Recent studies have demonstrated a close relationship between circadian clock function and the development of obesity and various age-related diseases. In this study, we investigated whether messenger RNA (mRNA) levels of clock genes are associated with age, body mass index, blood pressures, fasting plasma glucose, or shift work. Peripheral blood cells were obtained from 70 healthy women, including 25 shift workers, at approximately 9:00 AM. Transcript levels of clock genes (*CLOCK*, *BMAL1*, *PER1*, and *PER3*) were determined by real-time quantitative polymerase chain reaction. Stepwise multiple regression analysis demonstrated that *BMAL1* mRNA levels were correlated only with age ($\beta = -.50$, $p < .001$). In contrast, *PER3* levels were correlated with fasting plasma glucose ($\beta = -.29$, $p < .05$) and shift work ($\beta = .31$, $p < .05$). These results suggest that increased age, glucose intolerance, and irregular hours independently affect the intracellular clock in humans.

Key Words: Aging—Biological clock—Circadian rhythm—Shift work.

BIOLOGICAL clocks represent an adaptation to daily 24-hour changes in the environment and enable organisms to maintain physiological homeostasis (1,2). The circadian clock resides in the hypothalamic suprachiasmatic nucleus (SCN), which is recognized as being the master clock. The same clock also exists in almost all peripheral tissues, including liver, heart, kidney (3,4), and blood cells (5,6). Although the SCN is not essential for driving peripheral oscillations, it appears to coordinate peripheral clocks (3).

The intracellular clock consists of autoregulatory transcriptional–translational feedback loops that have both positive and negative elements (1,2). The positive components are two basic helix–loop–helix transcription factors called CLOCK and brain and muscle Arnt-like protein 1 (BMAL1) (7,8). The heterodimer activates the transcription of several other clock genes, including Period (*PER*) and Cryptochrome (*CRY*) (9–11). The resultant PER and CRY proteins form a heterodimer, translocate to the nucleus, and inhibit the activity of CLOCK–BMAL1, thus forming a negative feedback loop. The intracellular clock is thought to directly and/or indirectly regulate the expression of numerous genes (1,2).

It has become evident that circadian clock function is important to the preservation of health. For example, a certain mutation in the *CLOCK* gene induces obesity and metabolic syndrome in mice (12), and genetic variations in the *BMAL1* and *CLOCK* genes are associated with susceptibility to obesity, hypertension, and type 2 diabetes in humans (13–15). Additionally, the *Per2* gene plays an important role in tumor suppression and DNA damage responses: *Per2* mutant mice are known to be cancer prone (16). Interestingly, several epidemiological studies have shown that shift work is associated with obesity, hypertriglyceridemia, glucose intolerance, hypertension, and breast cancer (17–20). Moreover, aging is known to be associated with a variety of alterations in circadian rhythms, which may contribute to various age-related diseases (21,22). It was previously shown that cellular senescence impairs circadian expression of clock genes in vitro (22). These findings raise the possibility that shift work and aging may cause various pathological conditions (e.g., obesity and cancer) through the impairment of biological clock function.

To date, the effects of aging and shift work on intracellular clocks remain to be determined. To address this issue, we measured the messenger RNA (mRNA) levels of *BMAL1*, *PER1*, and *PER3* in peripheral blood cells obtained from healthy women at approximately 9:00 AM, as indicators of overall clock function (6). We investigated the associations between the mRNA levels and age, shift work, body mass index (BMI), blood pressure, and fasting plasma glucose concentration.

Methods

Participants and Sampling

We recruited 70 healthy women (age range 20–79 years, median 53.5 years; BMI range 17.1–31.5 kg/m^2, median 22.2 kg/m^2) from the registered healthy volunteers at the clinical trial unit of LTA PS Clinic (Fukuoka, Japan) between December 2007 and April 2008 and the staff of Moka Hospital (Moka, Japan) between September and November 2008. Because mRNA levels of a subset of clock genes in

Table 1. Characteristics of Participants

	All Participants		Participants Who Kept Regular Hours		Shift Workers	
	n	*M* ± *SD* or %	*n*	*M* ± *SD* or %	*n*	*M* ± *SD* or %
Total	70		45		25	
Age (y)	70	52 ± 14	45	59 ± 11	25	40 ± 12**
Participants aged 65 y or older	15	21	14	31	1	4**
Postmenopausal women	40	57	36	80	4	16**
BMI (kg/m^2)	70	22.5 ± 3.0	45	22.4 ± 2.9	25	22.6 ± 3.2
Participants with BMI ≥25 kg/m^2	14	20	10	22	4	16
Systolic blood pressure (mmHg)	69	120 ± 12	44	121 ± 13	25	118 ± 10
Diastolic blood pressure (mmHg)	69	70 ± 9	44	71 ± 8	25	68 ± 9
Participants with blood pressure ≥140/90 mmHg	3	4	2	5	1	4
Fasting plasma glucose (mg/dL)	59	93 ± 9	44	94 ± 9	15	93 ± 10
Relative messenger RNA levels of clock genes						
CLOCK	70	1 ± 0.20	45	1.04 ± 0.21	25	0.93 ± 0.16*
BMAL1	70	1 ± 0.17	45	0.98 ± 0.16	25	1.04 ± 0.18
PER1	70	1 ± 0.51	45	0.92 ± 0.44	25	1.15 ± 0.58
PER3	70	1 ± 0.33	45	0.89 ± 0.23	25	1.19 ± 0.40**

Notes: BMI = body mass index.
*$p < .05$, **$p < .01$ versus participants who kept regular hours.

peripheral blood cells differ between men and women (unpublished data, 2009), only women were included. The following participants were excluded from the study: those who took any medications during the 2 weeks preceding the study; those who experienced jet lag in the preceding month; and those with a sleep disorder, inflammatory disease, malignancy, severe anemia (hemoglobin level <8.0 g/dL), diagnosed hypertension, or diabetes. This information was obtained by interviewing participants and by reviewing the medical records if available. Additional information about the lifestyles (sleep time and mealtimes) was also collected from all participants on the day of the study.

Physical measurements and venous blood sampling were performed between 8:30 and 9:30 AM following an overnight fast. Systolic and diastolic blood pressures were measured in a standardized manner. The samples for RNA isolation were collected into PAXgene Blood RNA tubes (Becton, Dickinson and Company Japan, Tokyo, Japan), and the tubes were incubated at room temperature for at least 4 hours and then stored at −80°C until RNA extraction. In all the 56 participants aged 40 years and older and 3 of 14 participants younger than 40 years, plasma glucose levels were measured using a commercial kit.

As shown in Table 1, 40 of the 70 participants were postmenopausal women. Fourteen participants were overweight or obese, three participants had mild hypertension (systolic and diastolic blood pressures: 143–145 and 86–87 mmHg, respectively), but none of the participants had diabetes (fasting plasma glucose ≥126 mg/dL). Twenty-five participants at Moka Hospital were shift workers and had worked through the night several times during the preceding month. The other 45 women had kept regular hours for at least 2 weeks prior to the beginning of the study, usually waking between 5:00 and 8:00 AM and retiring between 9:00 PM and 1:00 AM. Four participants did not eat breakfast, and three participants did not eat lunch. The other 38 typically consumed three meals per day.

This study was approved by the ethics committees of Jichi Medical University (Shimotsuke, Japan) and Medical Co. LTA (Fukuoka) and was conducted in accordance with the Declaration of Helsinki. Participants participated in the study only after providing their written informed consent.

RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction

Total RNA isolation was achieved using a PAXgene Blood RNA kit (Qiagen Japan, Tokyo, Japan) according to the manufacturer's instruction. Complementary DNA (cDNA) was synthesized from 300 ng of total RNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems Japan, Tokyo, Japan). Gene expression was analyzed by real-time quantitative polymerase chain reaction (PCR) using an Applied Biosystems' StepOnePlus Real-Time PCR system. All specific sets of primers and TaqMan probes (TaqMan Gene Expression Assays) were obtained from Applied Biosystems. To control for variation in the amount of cDNA available for PCR in the different samples, gene expression levels of the target sequences were normalized to the expression of an endogenous control, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The GenBank accession numbers, assay ID, and target exons were NM_004898.2, Hs00231857_m1, and 18-19 for *CLOCK*; NM_001178.4, Hs00154147_m1, and 9-10 for *BMAL1*; NM_002616.1, Hs00242988_m1, and 22-23 for *PER1*; NM_016831.1, Hs00213466_m1, and 15-16 for *PER3*; and NM_002046.3, Hs99999905_m1, and 3-3 for *GAPDH*, respectively. Data were analyzed using the comparative threshold cycle method.

Table 2. Correlation Coefficients Between Clock Gene Messenger RNA Levels and Variables

	CLOCK	*BMAL1*	*PER1*	*PER3*
All participants				
Age	0.15	−0.29*	−0.13	−0.41**
BMI	0.16	−0.05	−0.06	0.21
Systolic blood pressure	−0.03	−0.03	−0.07	−0.02
Diastolic blood pressure	0.19	0.18	−0.04	0.05
Fasting plasma glucose	−0.25	−0.10	0.01	−0.31*
Participants who kept regular hours				
Age	−0.06	−0.46**	0.24	−0.11
BMI	0.20	−0.05	0.05	0.26
Systolic blood pressure	−0.04	−0.12	−0.11	0.10
Diastolic blood pressure	0.19	0.12	−0.04	0.22
Fasting plasma glucose	−0.25	−0.07	0.17	−0.28
Shift workers				
Age	0.01	0.07	−0.27	−0.29
BMI	0.15	−0.06	−0.22	0.20
Systolic blood pressure	−0.12	0.19	0.06	−0.06
Diastolic blood pressure	0.07	0.35	0.04	0.06
Fasting plasma glucose	−0.42	−0.16	−0.49	−0.39

Notes: BMI = body mass index.
*$p < .05$, **$p < .01$.

Statistical Analysis

Associations between clock gene mRNA levels and age, BMI, and shift work were assessed using Pearson's correlation coefficient and stepwise multiple regression analysis. Differences between participants who kept regular hours and shift workers were assessed by chi-square and *t* tests. Statistical significance was defined as $p < .05$. All calculations were performed using SPSS version 11 for Windows (SPSS Japan, Tokyo, Japan).

Results

As shown in Table 1, the shift workers were significantly younger than the participants who kept regular hours. Consequently, there were fewer postmenopausal women in the shift worker group. However, BMI, blood pressures, and fasting plasma glucose concentration did not differ between the groups. The mRNA levels of *CLOCK* were slightly but significantly lower in the shift workers, whereas their *PER3* levels were higher than that in the other participants.

In the 45 women who were not shift workers, there was significant correlation between the mRNA levels of *BMAL1* and age (Table 2; $r = -.46$, $p < .01$). This negative correlation was also detected in all participants (Table 2 and Figure 1A; $r = -.29$, $p = .01$). *PER3*, but not the other clock genes examined, also correlated with age ($r = -.41$, $p < .001$). In addition, *PER3* negatively correlated with fasting plasma glucose ($r = -.31$, $p < .05$) in accord with our previous results (6). However, these correlations were not observed in the shift workers (Table 2). Contrary to expectation, none of the clock genes examined correlated with BMI and blood pressures in either the 45 participants who kept regular hours or the entire group of 70 participants (Table 2 and Figure 1B).

Because the shift workers were significantly younger than the others, we next performed stepwise multiple regression analysis with age, BMI, blood pressures, fasting plasma glucose, and shift work as independent variables and clock gene transcript levels as dependent variables. This analysis further demonstrated that *BMAL1* was correlated only with age ($R = .50$, $p < .001$; age, $\beta = -.50$, $p < .001$). Moreover, *PER3* mRNA levels were correlated with fasting

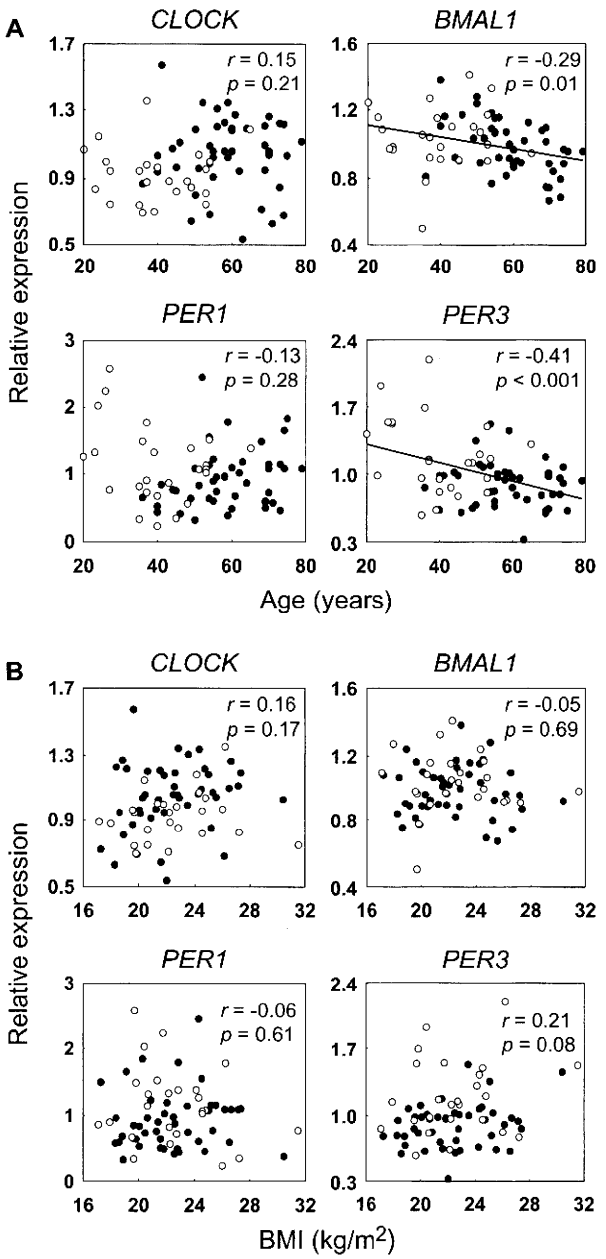

Figure 1. Relationship between messenger RNA levels of clock genes (*CLOCK*, *BMAL1*, *PER1*, and *PER3*) and age (**A**) or body mass index (BMI) (**B**) assessed using Pearson's correlation coefficient. Peripheral blood cells were obtained from 70 healthy women at approximately 9:00 AM. Twenty-five participants (white circles) were shift workers, and the other 45 women (black circles) kept regular hours for at least 2 weeks prior to the study. The transcript levels of clock genes were determined by real-time quantitative polymerase chain reaction. The mean value of all participants was set to 1 for each gene.

plasma glucose and shift work ($R = .44$, $p < .01$; fasting plasma glucose, $\beta = -.29$, $p < .05$; shift work, $\beta = .31$, $p < .05$). These results suggest that both age and shift work affect mRNA expression of a subset of clock genes.

Discussion

Here, we report for the first time a clear association between age and mRNA levels of *BMAL1*, which is a core element of the circadian clock, in humans. Although the rhythm of *BMAL1* expression in peripheral blood cells varies among individuals, *BMAL1* transcript levels often reach a trough level at the time specifically investigated in this study (5,6). Therefore, aging appears to affect *BMAL1* levels per se, rather than the phase of expression rhythms. Aging is known to influence the endocrine circadian system and the amplitudes of rhythms in particular. For example, secretion levels of various hormones, including cortisol, generally decrease in the elderly participants (23). It is speculated that the glucocorticoid signal is important for the oscillation of peripheral clocks as dexamethasone can reset them (24). In addition, age-associated decreases in nitric oxide production may also cause reversible impairment of the circadian clock (25). Furthermore, in this study, *BMAL1* levels in the postmenopausal participants were significantly lower than those in the menstruating women (data not shown). Thus, the age-related alterations in the endocrine circadian system may cause a reduction in the clock gene levels. Because at least part of the age-dependent disturbances of the circadian system can be reversed (26), the molecular machinery of intracellular clocks appears to be maintained even in advanced age. Further studies are needed to determine whether *BMAL1* levels correlate with circulating levels of some humoral factors including cortisol and nitric oxide or whether susceptibility to humoral cues decreases in the peripheral clocks of the elderly participants.

Previous studies from our laboratory have shown that the rhythmic expression of clock genes is blunted in both the liver and the visceral adipose tissues of obese diabetic mice (27) and in the peripheral leukocytes of patients with type 2 diabetes (6). In human leukocytes, the transcript levels of *BMAL1*, *PER1*, and *PER3* are inversely correlated with glycosylated hemoglobin levels (6). Given that several obesity-related humoral factors, including glucose (28), tumor necrosis factor α (29), and angiotensin II (30), can affect the circadian clock in vitro, it is possible that obesity alters the rhythmic expression of clock genes. Nevertheless, a significant association between BMI and clock gene expression was not detected in this study. The effect of mild simple obesity (without diabetes or hypertension) on circadian clock function in peripheral blood cells appears to be negligible.

Findings from recent studies suggest that circadian clock function plays a role in the development of obesity (12,14,15). However, the biological systems involved in body weight regulation are extremely complex, and many genes and chromosomal regions may contribute to defining the common obese phenotype (31). These genes are implicated in a wide variety of biological functions, including the regulation of food intake, energy expenditure, lipid and glucose metabolisms, and adipose tissue development (31). Therefore, it is not surprising that the association between BMI and clock gene expression was not detected in our small-scale study, even though such functions may be regulated by the biological clock.

Sleep–wake cycles have been shown to influence the rhythmic mRNA expression of clock genes in peripheral blood cells of healthy participants (32,33). Archer and colleagues (33) reported that *PER3* and *BMAL1* mRNA levels in peripheral blood cells of healthy participants were positively and negatively correlated with sleep time, respectively. The present study identified similar effects in people performing shift work. Several epidemiological studies suggest that shift work increases the risk of obesity, hypertriglyceridemia, glucose intolerance, hypertension, and breast cancer (17–20). As clock gene dysfunction is thought to cause these pathological conditions (12–16), impairment of the circadian clock may contribute to their subsequent development in shift workers. Further studies are needed to determine the degree of impairment in the clock oscillation system in shift workers.

In this study, we measured the transcript levels of clock genes at only one time point. In addition, daily rhythms of biological functions, such as core body temperature, and rhythmic expression of clock-controlled genes were not investigated. Therefore, it is not known exactly what the altered levels of clock genes indicate. However, the fact that altered mRNA levels of clock genes in peripheral blood cells obtained at an appropriate time point often indicate impaired expression rhythms of them has been revealed in patients with various diseases, including type 2 diabetes (6), circadian rhythm sleep disorder (34), and obstructive sleep apnea syndrome (35). In addition, the clock gene transcript levels in peripheral blood cells (36) and adipose tissues (37) obtained at a time point were reported to be correlated with the metabolic syndrome parameters. Therefore, the alternate measurement approach used in this study may be useful to assess the rhythm and/or function of circadian clock at least in peripheral tissues. Whether the altered clock gene levels in peripheral blood cells are associated with the development of various diseases should be determined in future studies.

In summary, the mRNA levels of a subset of clock genes in the peripheral blood cells of healthy women were found to correlate with age, fasting plasma glucose, and shift work. These results suggest that aging, like glucose intolerance and irregular hours, affects the rhythmic expression of clock genes in humans.

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Effect of pioglitazone on muscle sympathetic nerve activity in type 2 diabetes mellitus with alpha-glucosidase inhibitor

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ABSTRACT

Activation of the sympathetic nervous system is augmented in patients with type 2 diabetes mellitus (DM). Pioglitazone, an anti-diabetic drug, improves insulin resistance, but its influence on sympathetic nerve activity is not clear. To identify the relationship between insulin resistance and sympathetic activity, we examined muscle sympathetic nerve activity (MSNA) in controlled type 2 DM patients with alpha-glucosidase inhibitor (GI). We measured MSNA and calculated homeostasis model assessment of insulin resistance index (HOMA-IR) in twelve DM patients treated with alpha-GI and thirteen age-matched healthy subjects. In DM patients with alpha-GI, all parameters were reexamined after three months of treatment with pioglitazone. MSNA and HOMA-IR were significantly greater in DM patients with alpha-GI compared to healthy subjects. Hemoglobin A1c did not differ in DM patients before and after pioglitazone. However, pioglitazone significantly decreased MSNA in DM patients compared with alpha-GI (21.7 ± 5.2 vs. 32.0 ± 6.8 burst/min, $p<0.01$). Furthermore, MSNA level in pioglitazone was similar to that in healthy subjects. HOMA-IR significantly decreased after pioglitazone, and a significant relationship was found between the absolute change in MSNA and HOMA-IR ($r=0.65$, $p<0.05$). These results suggest that improved insulin resistance with pioglitazone provides an additional effect on inhibition of sympathetic nerve activity.

1. Introduction

Type 2 diabetes mellitus (DM) is increasing worldwide with changes in life style and dietary habits (Pereira et al., 2005; World Health Organnization, 2007). Type 2 DM causes cardiovascular complications that are related to mortality and morbidity. Previous studies have not shown a beneficial effect of insulin and conventional sulfonylurea therapy on cardiovascular mortality and morbidity (Finfer et al., 2009; UK Prospective Diabetes Study (UKPDS), 1998). The results indicate that tight glycemic control mainly affects the cardiovascular outcome in DM patients without established macrovascular disease. Therefore, additional therapeutic strategy is warranted to prevent major cardiovascular events in type 2 DM.

Recently, augmented sympathetic nerve activity in uncomplicated DM has been identified using microneurography (Huggett et al., 2003). Underlying mechanisms of disturbed autonomic nervous system in DM are not understood clearly. The increased sympathetic outflow in DM patients has been partly assumed to result from serum insulin and glucose levels (Scherrer and Sartori, 1997; Straznicky et al., 2009; Fagius et al., 1996; Fagius, 2003). The exaggerated sympathetic outflow increases cardiovascular mortality and morbidity in humans via beta-receptor downregulation (Bohm et al., 1997), cardiac myocyte apoptosis (Communal et al., 1998), and arrhythmogenesis (Chaudhri et al., 2002). The relationship between sympathetic nerve activity and mortality in type 2 DM remains unclear, but reducing the sympathetic activation might be a potential therapeutic target in type 2 DM.

STOP-NIDDM trial showed that acarbose treatment in patients with impaired glucose tolerance was associated with a significant reduction in the risk of developing diabetes, hypertension, and cardiovascular complications (Chiasson et al., 2003). Acarbose acts as an alpha-glucosidase inhibitor (GI) and slows the digestion of starch in the small intestine without reducing insulin release (Meneilly et al., 2000) or insulin sensitivity (Matsumoto et al., 1998). On the other hand, PROactive study showed that pioglitazone, which acts on peroxisome proliferator responsive elements, reduced the composite of all-cause mortality, non-fatal myocardial infarction, and stroke in patients with type 2 diabetes at a high risk of macrovascular events (Dormandy et al., 2005). One possible explanation for the reduction in cardiovascular events is thought to be the improved serum insulin level and decreased insulin resistance. However, it is unclear whether these improvements affect sympathetic nerve activity. These observations suggest that

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pioglitazone might confer additional beneficial effect on the sympathetic nerve system in type 2 DM patients.

In this study, we hypothesized that 1) changing alpha-GI to pioglitazone would decrease sympathetic nerve activity and 2) improved insulin resistance with pioglitazone would contribute to reduction of sympathetic nerve activity, independent of lowering blood glucose. To identify the relationship between insulin resistance and sympathetic activity without blood glucose effects, we examined muscle sympathetic nerve activity (MSNA) with microneurography in controlled type 2 DM patient with alpha-GI before and after changing to pioglitazone.

2. Materials and methods

2.1. Subjects

Data were obtained from twelve controlled type 2 DM (6 men and 6 women) ranging in age from 49 to 72 years (mean 64.0 ± 6.5), who had been treated with one of alpha-GIs for more than 3 months prior to study recruitment (five with voglibose (0.9 mg/day), four with miglitol (150 mg/day), and three with acarbose (150 mg/day)). Patients with type 2 DM were diagnosed 3.8 ± 0.4 years ago and mean hemoglobin A1c (HbA1c) before medication was 7.6 ± 0.6%. The diagnosis of DM2 was confirmed as recommended by the Japan Diabetic Society (The Committee of Japan Diabetes Society for the Diagnostic Criteria of Diabetes Mellitus, 1999). Subjects had uncomplicated DM, defined as no clinical evidence of macrovascular disease and no retinopathy (normal fundoscopy), nephropathy (albumin excretion ratio <20 μg/min and normal renal function), or neuropathy (normal monofilament testing, normal nerve conductance velocity, normal vibration and reflex test, and normal Valsalva maneuver response). We excluded DM patients on combined treatment with insulin therapy and complicated with uncontrolled hypertension and/or hyperlipidemia. Of twelve patients, six were diagnosed with hypertension, and received long acting calcium channel blockade, four also received angiotensin II receptor blockade, and three were treated with statin therapy.

We recruited and screened thirteen healthy volunteers (7 men and 6 women) aged 53 to 71 years (mean 61.8 ± 4.6). None of the healthy subjects was taking medication. The screening included a physical examination, baseline electrocardiogram evaluation, echocardiography, and complete blood work examination including a 75 g oral glucose tolerance test to exclude diseases that might affect cardiovascular status, such as cardiac dysfunction, metabolic syndrome, diabetes mellitus, impaired glucose tolerance, chronic kidney disease, and hypertension.

All subjects gave written informed consent to participate in the study. The study protocol was approved by the ethics review panel of the Graduate School of Medical Science at Kanazawa University, Japan.

2.2. Measurements

Heart rate was determined from a continuous electrocardiogram. Arterial pressure was recorded continuously from the radial artery using a noninvasive tonometry monitoring system (JENTOW-7700; Nihon Colin, Komaki, Japan). The postganglionic MSNA was recorded from the left peroneal nerve, as described previously (Murai et al., 2006; Otowa et al., 2008; Murai et al., 2009). Briefly, with the subject in a comfortable supine position, the common peroneal nerve was located by palpation and stimulated electrically via the skin surface. A tungsten micro-electrode was inserted percutaneously into a motor fascicle of the peroneal nerve. The electrode was adjusted until spontaneous pulse-synchronous sympathetic burst activities could be recorded.

The electrodes were connected to a preamplifier at a gain of 1000 and to an amplifier at a gain of 70. The signal was fed through a band-pass filter (500–3000 kHz) and a resistance-capacitance integrated circuit with a time constant of 0.1 s to produce a mean voltage neurogram. During off-line analysis, multi-unit MSNA was identified based on its relationship to cardiac activity in the integrated nerve recording in a blinded fashion by an experienced investigator. Within mean voltage neurograms, the burst activity was identified as over 3 of signal to noise ratio. For each subject, multi-unit MSNA was expressed as the number per minute (burst frequency) and the number per 100 heartbeats (burst incidence).

Fasting blood glucose (FBG), immunoreactive insulin (IRI), HbA1c, brain natriuretic peptide (BNP), and lipids were measured on the same study days. The homeostasis model assessment of the insulin resistance index was calculated as HOMA-IR = fasting glucose (mg/dL) × fasting insulin (μU/mL)/405 (Bonora et al., 2000).

2.3. Study protocol

All experiments were carried out in a quiet, electrically shielded room with the subject in the supine position at the same time in the morning (9:00–12:00). After the position of tungsten electrode to obtain MSNA was determined, all subjects were rested for 15 min. The recording of MSNA, HR and continuous noninvasive BP was recorded for 15 min. Then, the patients (not healthy subjects) were changed from alpha-GI to pioglitazone (15 mg/day), which was continued for three months. After three months, all of the parameters were reexamined for the patients treated with pioglitazone. Fig. 1 shows a simultaneous recording of MSNA, arterial pressure and electrocardiogram recording in a DM patient before and after changing to pioglitazone. As the reproducibility of MSNA had been reported (Fagius and Wallin, 1993; Hoffman et al., 1998; Grassi et al., 2009), reexamination was not performed in healthy subjects group. To minimize the confounding effects of hypoglycemia on sympathetic nerve activity, anti-diabetic drugs were withheld on the morning of the study.

2.4. Statistical analysis

Values are expressed as means ± SD. Student's paired *t*-test was used for paired comparisons. Student's unpaired *t*-test was used to compare between healthy subjects and DM patients. Liner regression analysis was used to determine the relationship between the multi-unit MSNA and other variables. All calculations were performed with a personal computer using the StatView-J 5.0 statistical software package (Abacas Concepts, Berkeley, CA). *p* values below 0.05 were considered to indicate statistical significance.

3. Results

3.1. Baseline characteristics

The baseline characteristics are shown in Table 1. No significant differences between the DM patients treated with alpha-GI and the healthy subjects were observed with respect to age, height, body weight, body mass index, systolic arterial pressure, diastolic arterial pressure, and heart rate. The lipid profiles did not differ between the two groups. BNP, a marker of cardiac function, did not differ significantly in the two groups. HbA1c, FBG, and HOMA-IR were significantly higher in DM patients than in the healthy subjects, and IRI tended to increase in DM patients.

3.2. Effect of changing to pioglitazone on hemodynamics and diabetic profiles

Table 2 shows the responses of the hemodynamic and diabetic profiles to changing to pioglitazone. Changing to pioglitazone had no effect on HbA1c and FBG, which remained higher than in healthy subjects. Heart rate, systolic and diastolic arterial pressure, and BNP

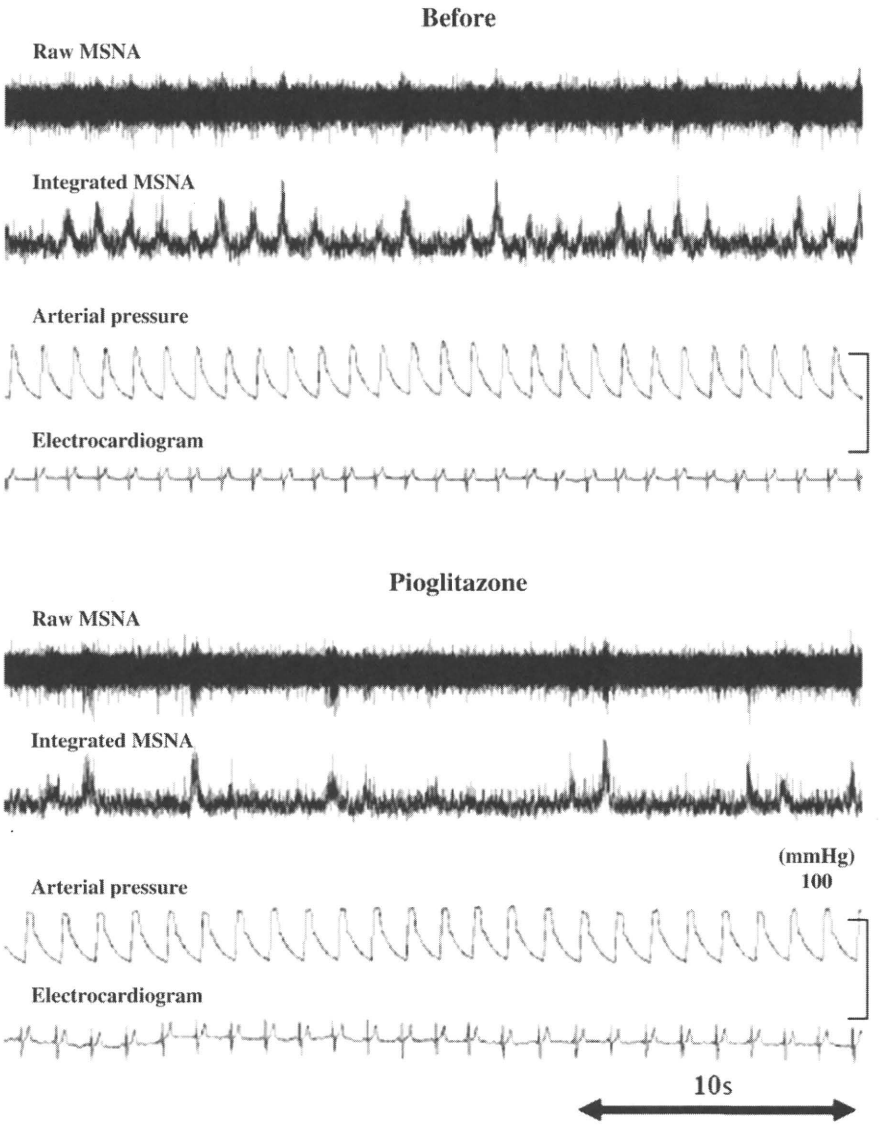

Fig. 1. Typical MSNA recording in a DM patient treated with before and after pioglitazone. In this case, the burst frequency and burst incidence changed from 42 burst/min and 75 burst/100 heartbeats, respectively, to 22 burst/min and 45.8 burst/100 heartbeats. The MSNA decreased after changing to pioglitazone.

did not differ between the two groups. IRI and HOMA-IR decreased after pioglitazone significantly, but HOMA-IR remained higher than in healthy subjects ($p<0.05$).

3.3. Effect of pioglitazone on muscle sympathetic nerve activity

Fig. 2 shows comparison of MSNA between DM patients before and after pioglitazone or healthy subjects. Burst frequency and burst incidence in DM patients treated with alpha-GI were significantly greater than that in healthy subjects (32.0 ± 6.8 vs. 21.5 ± 6.1 burst/min, $p<0.01$ and 52.7 ± 12.8 vs. 37.4 ± 10.4 burst/100 heartbeats, $p<0.01$, respectively). However, both burst frequency and burst incidence were significantly decreased after pioglitazone compared to that in treated with alpha-GI (21.7 ± 5.2 burst/min, $p<0.01$ and 35.8 ± 10.2 burst/100 heartbeats, $p<0.01$, respectively). Additionally, the resting level of MSNA in pioglitazone was similar to that in healthy subjects.

3.4. Relationship between MSNA and the variables in DM

To investigate which parameters were related with the decreased MSNA, regression analysis was performed. Table 3 shows the relationship between the absolute change in burst incidence and the absolute change in the other parameters. No correlations were found between MSNA and the hemodynamic parameters or BNP. However, a significant positive relationship was found between the changes in burst incidence and HOMA-IR ($r=0.65$, $p<0.05$, Fig. 3), and there tended to be a relationship between the change in MSNA and those in the IRI and FBG.

4. Discussion

This is the first study to investigate the effect of improved insulin resistance with pioglitazone on sympathetic nerve activity in controlled type 2 DM patients. We found that changing alpha-GI to pioglitazone reduced MSNA in type 2 DM patients without lowering blood glucose or causing any adverse effects. In addition, the absolute decrease in MSNA was significantly associated with the HOMA-IR. These results suggest that improved insulin resistance with pioglitazone provides an additional effect on sympathetic nerve activity in type 2 DM patients.

Several studies have shown that insulin augments sympathetic outflow to skeletal muscle in humans (Anderson et al., 1991; Berne et al., 1992; Vollenweider et al., 1993). The sympathoexcitatory effects of insulin are thought to be mediated by two mechanisms: 1) a direct

Table 1
Baseline characteristics.

	Healthy subjects	DM patients
		Alpha-GI
Age (years)	61.8 ± 4.6	64.0 ± 6.5
Height (m)	1.60 ± 0.7	1.56 ± 0.1
Weight (kg)	57.2 ± 11.2	56.5 ± 8.1
BMI (kg/m^2)	22 ± 2.8	23.2 ± 2.5
Heart rate (bpm)	58.0 ± 8.5	62.4 ± 14.7
SAP (mmHg)	119.2 ± 14.9	129.3 ± 14.4
DAP (mmHg)	62.5 ± 6.6	68.4 ± 8.3
Cholesterol (mg/dL)	202 ± 10.8	200 ± 30.3
Triglycerides (mg/dL)	85.6 ± 33.9	89.5 ± 30.2
HDL-c (mg/dL)	60.4 ± 3.3	60.9 ± 10.9
LDL-c (mg/dL)	124.9 ± 9.7	120.9 ± 27.8
BNP (pg/mL)	15.2 ± 5.3	16.0 ± 11.6
Hemoglobin A1c (%)	5.2 ± 0.2	6.5 ± 0.6[a]
FBG (mg/dL)	92.0 ± 11.1	147.5 ± 21.0[a]
IRI (μU/mL)	4.8 ± 3.1	5.7 ± 3.0
HOMA-IR	1.1 ± 0.7	2.09 ± 1.2[b]

Results are expressed as the means ± SD. SAP = systolic arterial pressure, DAP = diastolic arterial pressure, FBG = fasting blood glucose, IRI = immunoreactive insulin, HOMA-IR = homeostasis model assessment of insulin resistance, BNP = brain natriuretic peptide.
[a] $p<0.01$ compared to healthy controls.
[b] $p<0.05$ compared to healthy controls.

increase in central sympathetic outflow, and 2) baroreceptor reflex-mediated sympathetic activation in response to the peripheral vasodilatory action of insulin. Insulin enters the central nervous system through the blood–brain barrier via receptor-mediated transport, and insulin receptors exist in several distinct regions, such as the median hypothalamus (Muntzel et al., 1995). The thiazolidinedione, pioglitazone, acts as a ligand of peroxisome proliferator-activated receptor (PPAR) γ and induces the differentiation of adipose tissue (Forman et al., 1995). PPAR γ reduces insulin resistance and then incorporates glucose into the cell. Nagai et al. reported an obese DM patient with obstructive sleep apnea syndrome in whom pioglitazone improved the sympathetic nerve activity (Nagai et al., 2003). This report was consistent with our results that pioglitazone reduced the sympathetic outflow to the peripheral nervous system. Furthermore, our results clarified that the suppression of MSNA with pioglitazone was significantly correlated with the HOMA-IR in DM patients.

Interestingly, the decreased level of MSNA after pioglitazone was similar to that in the age-matched healthy subjects. Regarding insulin resistance, the HOMA-IR in DM treated with pioglitazone was still higher than in the healthy subjects. This inhibition of MSNA cannot be explained by a reduction in insulin resistance. Previous reports have shown that pioglitazone also reduces reactive oxygen species

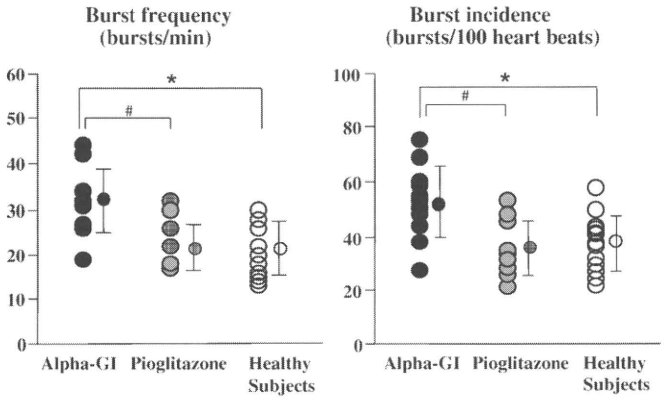

Fig. 2. Comparison of the burst frequency and burst incidence between DM treated with before and after pioglitazone, and healthy subjects. Both the burst frequency and burst incidence in DM patients treated with pioglitazone were significantly lower than those in alpha-GI. In addition, both MSNA in pioglitazone were similar to those in healthy subjects. The results are expressed as means ± SD. #$p<0.01$ compared between alpha-GI and pioglitazone. *$p<0.01$ compared between alpha-GI and healthy subjects.

(Fujisawa et al., 2009), stimulating central sympathetic nerve activity accompanied by angiotensin II (Gao et al., 2004). We did not examine the relationship between reactive oxygen species and sympathetic excitation. However, the sympathetic excitation might be restrained with the reduction of reactive oxygen species together with improving insulin resistance with pioglitazone.

In this study, the DM patients had relatively higher heart rates than did the healthy subjects, although apparent neuropathy was excluded. An increased heart rate and reduced heart rate variability are signs of early cardiovascular autonomic neuropathy. The mechanism underlying these abnormalities has been reported to be a disorder of the autonomic nervous system especially impaired parasympathetic nerve activity (Stein et al., 2007). Although pioglitazone reduced the sympathetic nerve activity in our study, the resting heart rate still remained higher in DM patients than in healthy controls. Restoring parasympathetic nerve activity would be needed to decrease heart rate to normal levels.

One of the notable adverse effects of pioglitazone treatment is fluid retention that results from the stimulation of ENaC-mediated renal salt absorption (Guan et al., 2005). In the PROactive study, edema was a reported complication of pioglitazone therapy (Dormandy et al., 2005); consequently, pioglitazone is not recommended for treating DM patients with heart failure. Lago et al. reported that congestive heart failure occurred in 1.8% of patients taking thiazolidinediones (Lago et al., 2007). In our study, body weight and BNP did not increase after changing from alpha-GI to pioglitazone (56.5 vs. 56.7 kg). All our DM patients had no complications or nephropathy (albumin excretion ratio <20 μg/min and normal renal function). Our strict screening

Table 2
Effect of changing to pioglitazone on hemodynamic and diabetic profiles.

	DM patients	
	Alpha-GI	Pioglitazone
Heart rate (bpm)	62.4 ± 14.7	62.2 ± 10.4
SAP (mmHg)	129.3 ± 14.4	126.8 ± 15.7
DAP (mmHg)	68.4 ± 8.3	65.4 ± 10.6
BNP (pg/mL)	16.0 ± 11.6	19.0 ± 9.5
Hemoglobin A1c (%)	6.5 ± 0.6	6.7 ± 0.6
FBG (mg/dL)	147.5 ± 21.0	144.4 ± 23.7
IRI (μU/mL)	5.7 ± 3.0	4.5 ± 2.7[a]
HOMA-IR	2.09 ± 1.2	1.50 ± 0.89[a]

Results are expressed as means ± SD. SAP = systolic arterial pressure, DAP = diastolic arterial pressure, FBG = fasting blood glucose, IRI = immunoreactive insulin, HOMA-IR = homeostasis model assessment of insulin resistance, BNP = brain natriuretic peptide.
[a] $p<0.05$ compared to alpha-GI.

Table 3
Regression analysis between MSNA and variables.

	ΔBurst incidence	
	r	p
ΔHeart rate (bpm)	−0.15	0.65
ΔSAP (mmHg)	0.03	0.94
ΔDAP (mmHg)	−0.11	0.97
ΔBNP (pg/mL)	−0.33	0.31
ΔFBG (mg/dL)	0.51	0.09
ΔIRI (μU/mL)	0.51	0.09
ΔHOMA-IR	0.65	<0.05

SAP = systolic arterial pressure, DAP = diastolic arterial pressure, FBG = fasting blood glucose, IRI = immunoreactive insulin, HOMA-IR = homeostasis model assessment of insulin resistance, BNP = brain natriuretic peptide.