Quantitative reverse transcriptase–polymerase chain reaction-based gene expression

Quantitative reverse transcriptase–polymerase chain reaction (RT–PCR) was performed as previously described[13] (see Supplementary material online for details).

Statistical analysis

All data are expressed as means ± SD. All statistical analyses were performed with Ekuseru-Toukei 2006 and 2010 statistical software (Social Survey Research Information Co., Ltd., Tokyo, Japan). Normality was tested with the Kolmogorov–Smirnov test. When data were normally distributed, the statistical significance of differences was assessed with the unpaired *t* test. The Mann–Whitney *U* test was applied when data were not normally distributed. In all analyses, values of $P < 0.05$ were accepted as statistically significant, and all tests were two-sided.

Results

Hepatic peroxisome proliferator-activated receptor-γ overexpression raises blood pressure via the neuronal relay

First, to examine whether hepatic PPARγ2 expression raises BP, we over-expressed PPARγ2 selectively in the livers of C57BL/6 mice fed a normal chow diet (PPARγ2-mice) by systemically infusing a recombinant adenovirus. Recombinant adenovirus infusion through the tail vein resulted in selective transgene expression in the liver (see Supplementary material online, *Figure S1A*) without increased expression in other peripheral tissues, as reported previously.[13,14] Mice given the lacZ adenovirus were used as controls. Hepatic expression of PPARγ2 in normal chow-fed lean mice caused hepatomegaly (*Figure 1A*) due to marked hepatic steatosis (*Figure 1B*), while reducing adipose tissue with suppression of weight gain (see Supplementary material online, *Figure S1B*). In addition, hepatic PPARγ2 expression decreased fasting blood glucose (FBG) (see Supplementary material online, *Figure S1C*), insulin and leptin levels (*Table 1A*). These findings are compatible with the observations in mice with diet-induced obesity.[13] Under these conditions, SBP was significantly elevated in PPARγ2 mice (*Figure 1C*). Thus, PPARγ2 overexpression in the liver raises BP via mechanisms independent of hyperinsulinaemia and hyperleptinaemia. In addition, hepatic PPARγ2 expression increased renal renin and adipose angiotensinogen expressions (see Supplementary material online, *Figure S1D–E*). Since these genes are known to be upregulated by sympathetic stimulation,[3,23,24] these results suggest that hepatic PPARγ2 expression increases sympathetic tonus to the kidney as well as adipose tissue, possibly contributing to BP elevation.

Next, to examine whether vagal afferent signals originating in the liver are the mechanism whereby hepatic PPARγ2 expression raises BP, we surgically dissected the hepatic vagus. Seven days after selective hepatic vagotomy or sham-operation, we administered recombinant adenovirus encoding LacZ or PPARγ2. Liver weights were similarly increased by PPARγ2 overexpression in sham-operated and hepatically vagotomized mice (*Figure 1D*). In contrast, while hepatic PPARγ2 significantly raised SBP in sham-operated mice, selective hepatic vagotomy completely blocked SBP elevation (*Figure 1E*). We next administered PPARγ2 adenovirus to β-less mice.[18] Although adenoviral expression of hepatic PPARγ2 also increased liver weight in β-less mice to an extent similar to that in wild-type controls (*Figure 1F*), SBP was unaffected in β-less mice (*Figure 1G*). Thus, the hepatic PPARγ-modulated neuronal pathway, consisting of afferent vagal and efferent sympathetic nerves, mediates BP elevation.

Knockdown of peroxisome proliferator-activated receptor-γ expression in the liver lowers blood pressure in murine obesity models

Does this system physiologically contribute to obesity-related hypertension? To answer this question, we knocked down hepatic PPARγ expression in murine obesity models. At 8 weeks of age, hepatic PPARγ expression was markedly enhanced in genetically obese KK-Ay mice when compared with lean control KK mice (*Figure 2A*). Administration of recombinant adenovirus expressing shRNA for PPARγ substantially decreased endogenous PPARγ expression in the livers of KK-Ay mice (*Figure 2A*). On Day 3 after adenoviral administration, the knockdown of hepatic PPARγ expression in KK-Ay mice significantly lowered BP, while exerting no effects on BP in KK mice (*Figure 2B*). Note that, at this time point, neither liver weights (*Figure 2C*) nor hepatic triglyceride contents (*Figure 2D*) had yet decreased significantly. Nor were body weights (see Supplementary material online, *Figure S2*), plasma insulin, or leptin levels (*Table 1B*) significantly affected by PPARγ–shRNA administration. Findings were similar in another murine obesity model mice with diet-induced obesity. High fat loading of C57BL/6 mice enhanced endogenous PPARγ expression in the liver (*Figure 2E*) and raised BP (*Figure 2F*). Without significant decreases in liver weights (*Figure 2G*) and hepatic triglyceride contents (*Figure 2H*), hepatic PPARγ knock-down (*Figure 2E*) significantly lowered BP in DIO mice, but not in normal chow-fed lean controls (*Figure 2F*). Thus, upregulation of endogenous PPARγ in the liver during obesity development, rather than hepatic lipid accumulation *per se*, contributes to the development of obesity-related hypertension.

Blockade of hepatic vagal afferents inhibits blood pressure elevation during obesity development

Next, to investigate whether signals mediated by the hepatic vagus are involved in the development of obesity-related hypertension, we performed selective hepatic vagotomy in KK-Ay mice at 8 weeks of age, followed by BP monitoring. Selective hepatic vagotomy did not affect endogenous PPARγ expression in the liver in either KK or KK-Ay mice (*Figure 3A*). Systolic BP rose steadily in sham-operated KK-Ay mice, but hepatic vagotomy blocked this BP elevation (*Figure 3B*), with no changes in plasma insulin or leptin levels (*Table 1C*). In contrast, in KK mice, SBP was unaffected by hepatic vagotomy (*Figure 3B*). Thus, selective hepatic vagotomy

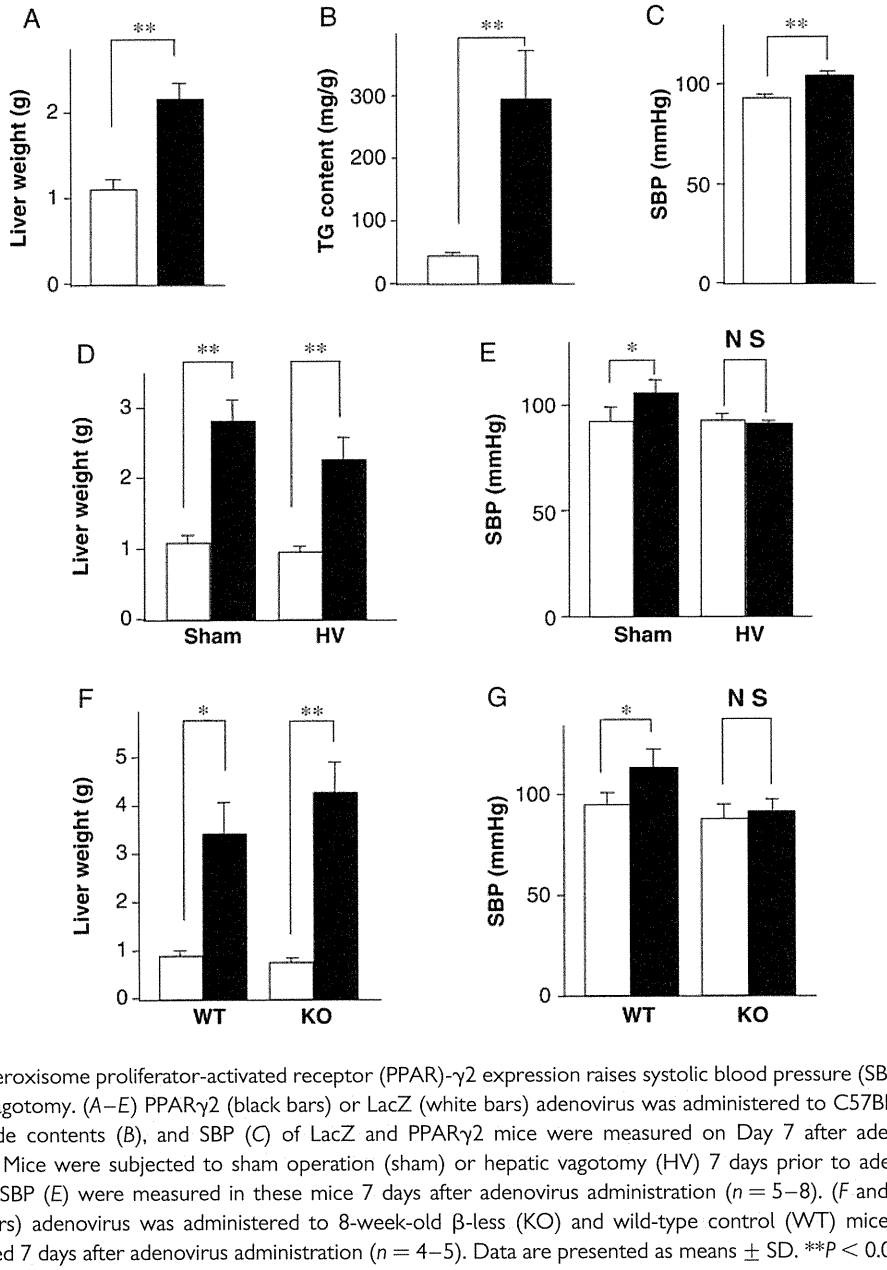

Figure 1 Hepatic peroxisome proliferator-activated receptor (PPAR)-γ2 expression raises systolic blood pressure (SBP), and this elevation is inhibited by hepatic vagotomy. (*A–E*) PPARγ2 (black bars) or LacZ (white bars) adenovirus was administered to C57BL/6 mice. Liver weights (*A*), hepatic triglyceride contents (*B*), and SBP (*C*) of LacZ and PPARγ2 mice were measured on Day 7 after adenovirus administration ($n = 5–7$). (*D* and *E*) Mice were subjected to sham operation (sham) or hepatic vagotomy (HV) 7 days prior to adenoviral administration. Liver weight (*D*) and SBP (*E*) were measured in these mice 7 days after adenovirus administration ($n = 5–8$). (*F* and *G*) LacZ (white bars) or PPARγ2 (black bars) adenovirus was administered to 8-week-old β-less (KO) and wild-type control (WT) mice. Liver weight (*F*) and SBP (*G*) were measured 7 days after adenovirus administration ($n = 4–5$). Data are presented as means ± SD. $^{**}P < 0.01$ and $^{*}P < 0.05$, compared with LacZ mice. NS, not significant.

protected KK-Ay mice from the progression of hypertension associated with obesity.

Hepatic vagotomy involves dissection of both afferent and efferent vagal branches innervating the liver. Therefore, to confirm the involvement of afferent vagal signals originating in the liver, we applied a specific afferent neurotoxin, capsaicin, to the hepatic branch of the vagus in 8-week-old KK and KK-Ay mice. As reported previously,[13] expression of calcitonin gene-related peptide, a sensory neuropeptide, was markedly decreased in the capsaicin-treated vagal nerve, while immunoreactivity for S100 proteins was similar in vehicle- and capsaicin- treated nerves (see Supplementary material online, *Figure S3*), indicating selective deafferentation of the vagal nerve by capsaicin treatment. Perivagal capsaicin treatment did not affect endogenous PPARγ expression in the livers of either KK or KK-Ay mice (*Figure 3C*). Under these conditions, blockade of afferent vagal signals from the liver prevented SBP elevation during obesity development in KK-Ay mice, but had no effect on BP in KK mice (*Figure 3D*). Thus, hepatic PPARγ upregulation associated with obesity is involved in the development of hypertension via the afferent vagal pathway from the liver.

To examine whether this neuronal pathway involves leptin signalling, capsaicin was applied perivagally in a leptin receptor-mutant animal obesity model, ZDF rats, which develop a syndrome with obesity and hypertension.[25] In ZDF rats, endogenous PPARγ expression in the liver is enhanced when compared with that in

Table 1 Fasting plasma insulin and leptin concentrations in the experimental models used in this study

(A)

Adenovirus	LacZ	PPARγ2	*P*-value
Insulin (ng/mL)	0.60 ± 0.20	0.38 ± 0.15	0.049
Leptin (ng/mL)	2.98 ± 0.94	0.61 ± 0.36	<0.001

(B)

	KK			KK-Ay		
Adenovirus	H1-scramble	H1-PPARγ	*P*-value	H1-scramble	H1-PPARγ	*P*-value
Insulin (ng/mL)	0.77 ± 0.24	0.80 ± 0.64	NS	2.90 ± 0.79	1.92 ± 0.79	NS
Leptin (ng/mL)	1.64 ± 0.75	1.64 ± 0.32	NS	23.52 ± 3.08	20.26 ± 6.57	NS

(C)

	KK			KK-Ay		
Operation	Sham	HV	*P*-value	Sham	HV	*P*-value
Insulin (ng/mL)	0.89 ± 0.24	0.79 ± 0.37	NS	5.25 ± 1.58	5.91 ± 2.14	NS
Leptin (ng/mL)	4.99 ± 1.93	3.69 ± 1.23	NS	42.00 ± 5.33	37.41 ± 8.79	NS

(D)

	ZDF		
Treatment	veh	cap	*P*-value
Insulin (ng/mL)	4.31 ± 1.11	3.62 ± 0.29	NS
Leptin (ng/mL)	73.74 ± 8.12	74.76 ± 10.46	NS

control Zucker lean rats (*Figure 3E*). Again, as obesity progressed, BP rose in ZDF rats, but this elevation was blocked (*Figure 3F*) by capsaicin-mediated selective deafferentation of the hepatic vagus (see Supplementary material online, *Figure S4*), with no changes in plasma insulin or leptin levels (*Table 1D*). These findings indicate that afferent signals of the hepatic vagus contribute to the development of obesity-related hypertension in a fashion independent of leptin signalling.

Blood pressure is not elevated in murine models of hepatic steatosis without hepatic peroxisome proliferator-activated receptor-γ upregulation

Taking the aforementioned results together, hepatic upregulation of PPARγ, which induces hepatic steatosis, is likely to contribute to the development of obesity-related hypertension via afferent vagal signals. This prompted us to question whether hepatic triglyceride accumulation or hepatic PPARγ upregulation causes hypertension. To answer this question, we examined other models of hepatic steatosis without hepatic upregulation of PPARγ. Acyl-CoA:diacylglycerol acyltransferase (DGAT) is a membrane-bound enzyme that catalyses the last step in the synthesis of triglycerides. DGAT1 and DGAT2 are unrelated proteins that exhibit DGAT activity. Adenoviral expressions of these enzymes, particularly DGAT2, in the liver reportedly result in hepatic steatosis.[19] Indeed, adenoviral DGAT1 or DGAT2 expression in the liver induced hepatomegaly (*Figure 4A*) with hepatic triglyceride accumulation (*Figure 4B*). In particular, hepatic triglyceride contents in DGAT2 mice were similar to those in PPARγ2 mice (*Figure 1B*). However, hepatic PPARγ2 expression was not affected by hepatic expressions of these DGAT enzymes (*Figure 4C*). In contrast to PPARγ2 overexpression, neither DGAT1 nor DGAT2 overexpression in the liver significantly raised BP (*Figure 4D*). White adipose tissue (WAT) weights were not reduced in DGAT1 or DGAT2 mice (see Supplementary material online, *Figure S5*). These findings collectively suggest that DGAT-induced hepatic steatosis has minimal effects on sympathetic tonus.

Fat-specific protein 27 upregulation in the liver contributes to obesity-related hypertension downstream from peroxisome proliferator-activated receptor-γ

What molecules function downstream from PPARγ in the liver? It was recently reported that Fsp27 functions as a direct target gene of PPARγ in the liver and plays a major role in obesity-related hepatic steatosis.[20] In fact, adenovirus-mediated expression of PPARγ2 in the liver markedly enhanced hepatic Fsp27 expression (see Supplementary material online, *Figure S6A*). Therefore, we

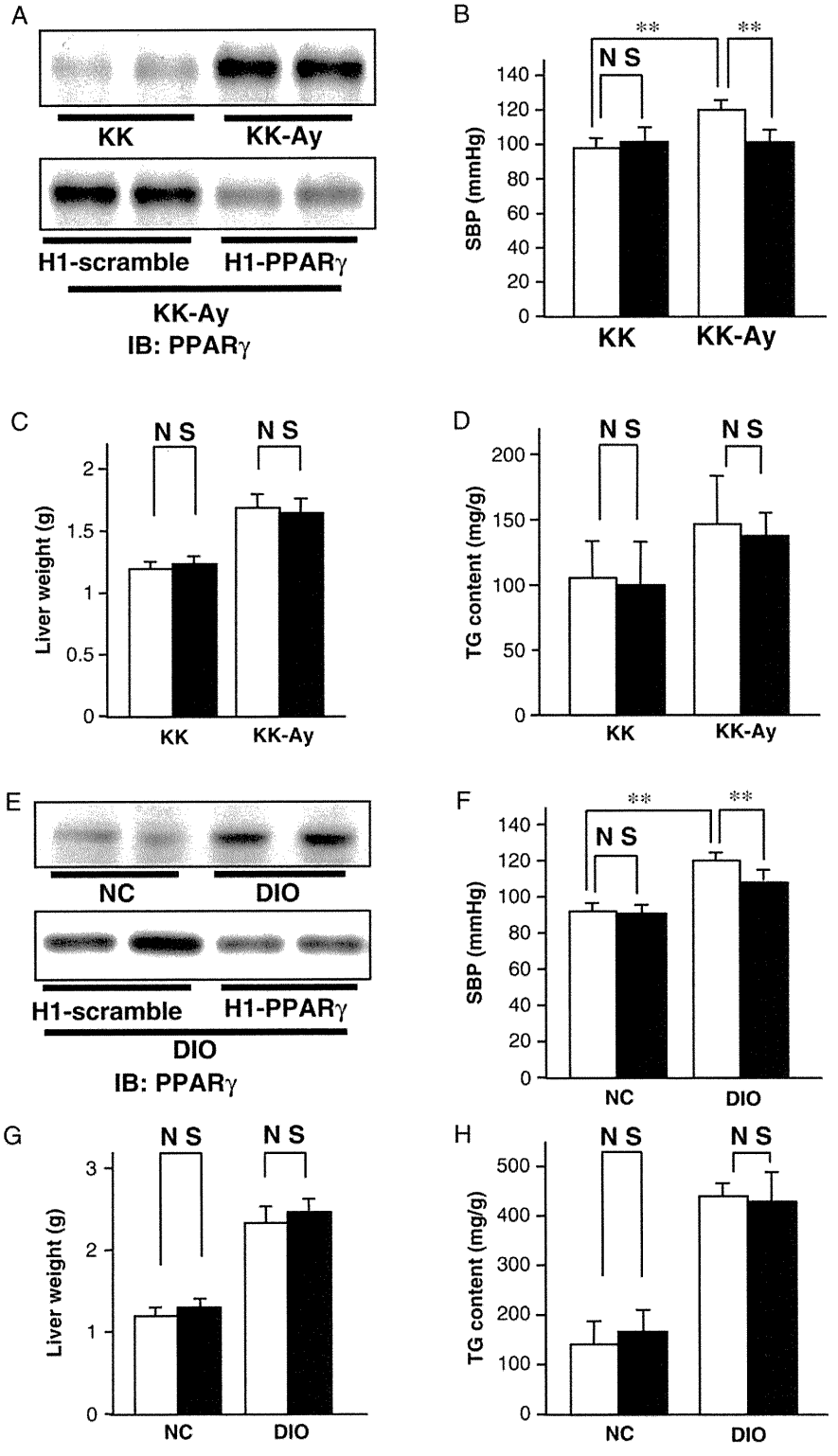

Figure 2 Knockdown of peroxisome proliferator-activated receptor (PPAR)-γ expression in the liver lowers blood pressure (BP) in murine obesity models. Recombinant adenovirus expressing shRNA for PPARγ (H1-PPARγ: black bars) or control scramble (H1-scramble: white bars) was administered to 8-week-old KK or KK-Ay mice ($n = 5–6$) (*A–D*) and C57BL/6 mice fed normal chow (NC) or with high fat diet-induced obesity (DIO) ($n = 4–5$) (*E–H*), followed by measurement of systolic blood pressures (SBPs) (B and F), liver weights (C and G), and hepatic triglyceride contents (D and H) on Day 3 after adenoviral administration. Liver extracts were subjected to immunoblotting with anti-PPARγ antibody (A and E). Data are presented as means ± SD. $^{**}P < 0.01$; NS, not significant.

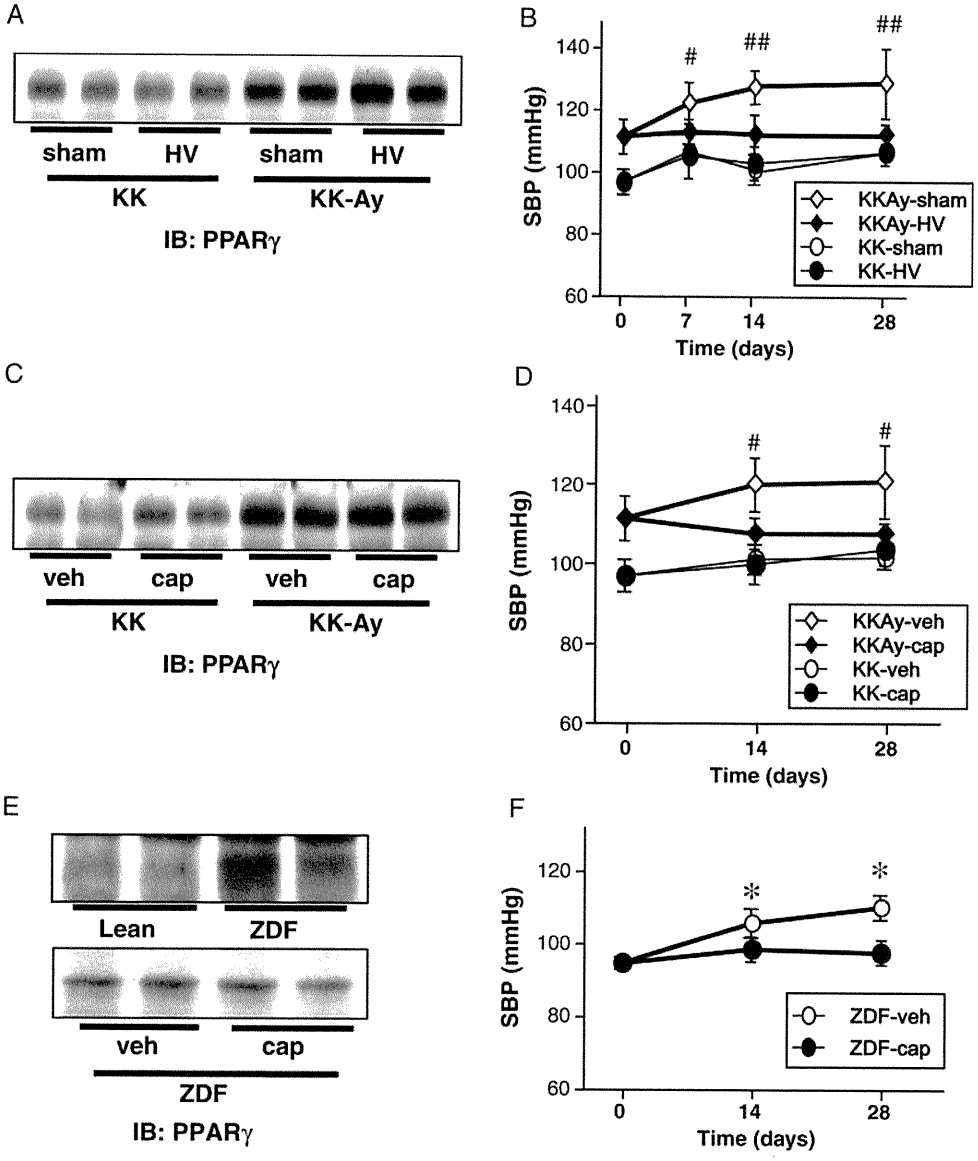

Figure 3 Surgical dissection or pharmacological deafferentation of the hepatic vagus blocks blood pressure (BP) elevation during obesity development. (*A* and *B*) KK and KK-Ay mice at 8 weeks of age were subjected to hepatic vagotomy (HV) or sham operation (sham). (B) Systolic BP (SBP) was monitored for 28 days after these operations. Open circles and squares and closed circles and squares indicate sham operated and KK and KK-Ay mice and hepatically vagotomized-KK and KK-Ay mice ($n = 5–6$), respectively. $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ indicate KK-Ay (sham) vs. KK-Ay (HV) mice. (*C* and *D*) Vehicle (veh) or capsaicin (cap) was applied to the hepatic vagus of 8-week-old KK and KK-Ay mice. (D) SBP was measured on Days 14 and 28 after these operations. Open circles and squares indicate vehicle-treated KK and KK-Ay mice and closed circles and squares indicate capsaicin-treated KK and KK-Ay mice ($n = 4–6$), respectively. $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ indicate vehicle- vs. capsaicin-treated KK-Ay mice. (*E* and *F*) Vehicle (veh) or capsaicin (cap) was applied to the hepatic vagus in 6-week-old Zucker fatty diabetic (ZDF) rats. (*F*) SBP was measured on Days 14 and 28 after these operations. Open and closed circles indicate vehicle- and capsaicin-treated ZDF rats ($n = 4$), respectively. $^{*}P < 0.05$ indicate vehicle- vs. capsaicin-treated ZDF rats. Liver extracts were subjected to immunoblotting with anti-PPARγ antibody (A, C, E). Data are presented as means ± SD.

examined the role of Fsp27 in obesity-related hypertension. First, Fsp27 was overexpressed in the liver by recombinant adenovirus infusion. Similar to the case of hepatic PPARγ2 expression, hepatic Fsp27 expression increased liver weights (*Figure 5A*) with marked steatosis (*Figure 5B*). Consistent with this, hepatic expressions of sterol regulatory element binding protein-1c (see Supplementary material online, *Figure S6B*) and fatty acid synthase (see Supplementary material online, *Figure S6C*) were enhanced in Fsp27-mice. In contrast, WAT weights were reduced (see Supplementary material online, *Figure S6D*) and uncoupling protein-1 expression in brown adipose tissue was significantly upregulated (see Supplementary material online, *Figure S6E*).

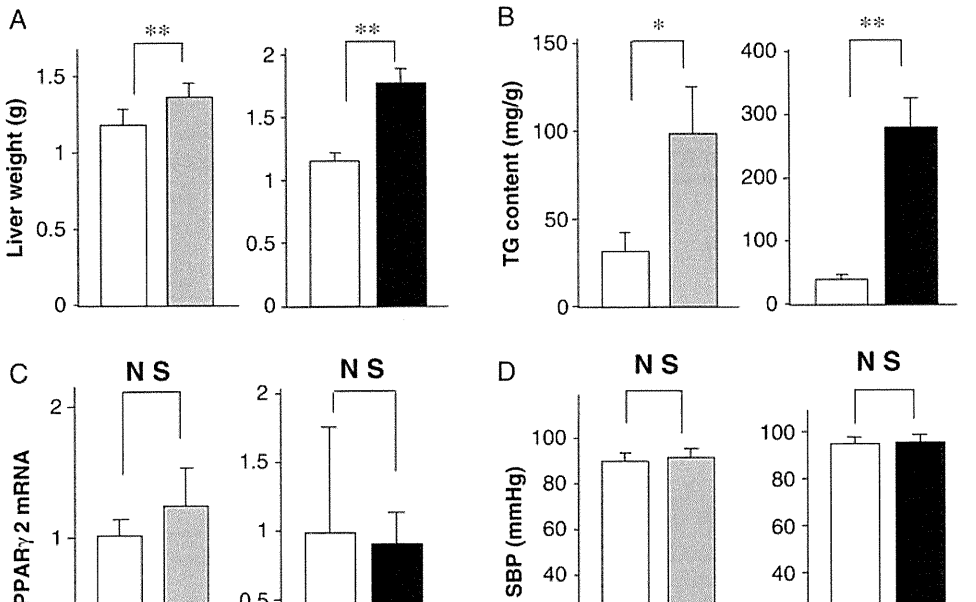

Figure 4 Hepatic steatosis itself does not raise blood pressure (BP) in murine models of hepatic steatosis without hepatic peroxisome proliferator-activated receptor (PPAR)-γ upregulation. DGAT1 (gray bars), DGAT2 (black bars), or LacZ (white bars) adenovirus was administered to C57BL/6 mice. Liver weights (*A*), hepatic triglyceride contents (*B*), hepatic expressions of PPARγ2 mRNA (*C*), and SBPs (*D*) of LacZ-, DGAT1- and DGAT2-mice were measured on Day 7 after adenovirus administration ($n = 4–6$). Data are presented as means ± SD. $^{**}P < 0.01$ and $^{*}P < 0.05$, compared to LacZ mice. NS, not significant.

In addition, hepatic Fsp27 expression increased renal renin expression (see Supplementary material online, *Figure S6F*). These findings, taken together, suggest enhanced sympathetic tonus in Fsp27 mice. Furthermore, hepatic Fsp27 expression significantly lowered FBG (see Supplementary material online, *Figure S6G*). In addition to these metabolic phenotypes, SBP was significantly elevated in Fsp27 mice (*Figure 5C*). Hepatic vagotomy prior to adenoviral administration did not affect liver weights (*Figure 5D*) but did block the BP elevation induced by hepatic Fsp27 expression (*Figure 5E*). These phenotypic features observed in Fsp27 mice, including not only local (in the liver) but also remote effects, are very similar to those induced by hepatic PPARγ2 expression. Therefore, Fsp27 upregulation is likely to mediate the metabolic effects induced by hepatic PPARγ2 expression.

Next, we examined the involvement of hepatic Fsp27 in the development of obesity-related hypertension. In both KK-Ay and DIO mice, hepatic expression of Fsp27 was upregulated when compared with their respective lean controls (*Figure 5F*). Administration of recombinant adenovirus expressing shRNA for Fsp27 decreased endogenous Fsp27 expression in the liver, especially in the murine obesity models (see Supplementary material online, *Figure S7*). Under these conditions, Fsp27 knockdown in the liver significantly lowered SBP in both KK-Ay and DIO mice, but not in their lean counterparts (*Figure 5G*). These findings were similar to the observations in mice with hepatic knockdown of PPARγ (*Figure 2B* and *F*). Thus, hepatic upregulation of Fsp27, a downstream target of PPARγ, is involved in the development of obesity-related hypertension.

Discussion

The first important finding in this study is that hepatic PPARγ upregulation, which is associated with obesity development,[16,17] contributes to obesity-related hypertension via the neuronal relay originating in the liver. This neuronal relay system was shown to enhance systemic energy expenditure and decrease peripheral adiposity and thus appears to function as an anti-obesity mechanism counteracting excess energy accumulation.[13] This, in turn, leads to hypertension development under conditions of chronic excess energy intake. Thus, the endogenous anti-obesity mechanism appears, ironically, to cause pathological states associated with obesity.

This concept might be somewhat contradictory to the effects of thiazolidinediones on obese subjects. Thiazolidindione treatment of mice as well as human subjects with obesity reportedly increases adiposity as well as lowering BP,[8,9] while hepatic PPARγ2 expression in the present study decreased adiposity and raised BP. These inconsistencies raise the possibility that unbalanced overexpression of PPARγ2 is characterized by a relative lack of its coactivators, leading to non-physiological effects. However, this possibility is quite unlikely for the following reasons. First, hepatic PPARγ2 expression increased hepatic lipid

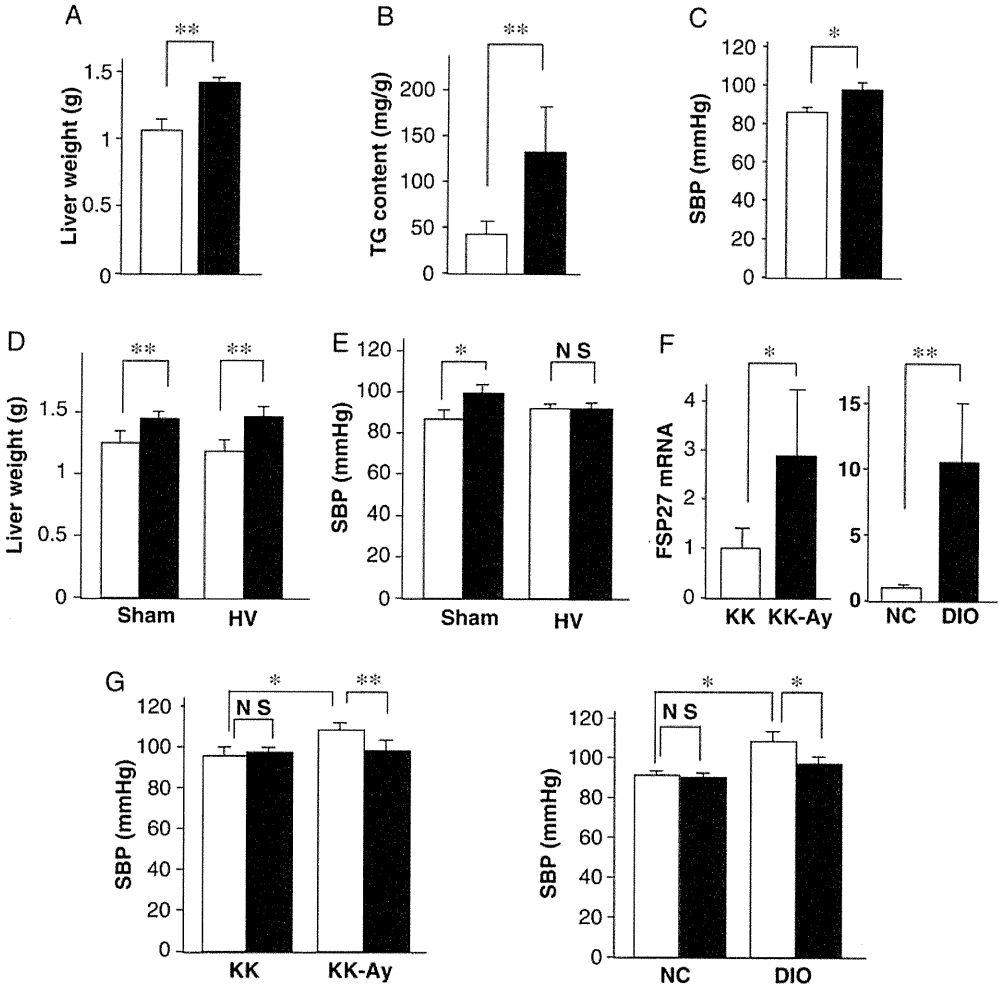

Figure 5 Hepatic fat-specific protein 27 (Fsp27) expression is involved in obesity-related hypertension. (*A–C*) Fsp27 (black bars) or LacZ (white bars) adenovirus was administered to C57BL/6 mice. Liver weights (A), hepatic triglyceride contents (B), and systolic blood pressures (SBPs) (C) of LacZ and Fsp27 mice were measured on Day 7 after adenovirus administration ($n = 5–7$). (*D* and *E*) Mice were subjected to sham operation (sham) or hepatic vagotomy (HV) 7 days prior to adenoviral administration. Liver weights (D) and SBPs (E) of LacZ and Fsp27 mice were measured on Day 7 after adenovirus administration ($n = 4–6$). (*F*) Expression of Fsp27 mRNA was measured by quantitative reverse transcriptase–polymerase chain reaction (RT–PCR) in 8-week-old KK or KK-Ay mice and C57BL/6 mice fed normal chow (NC) or with high fat diet-induced obesity (DIO) ($n = 5–6$). (*G*) Recombinant adenovirus expressing shRNA for Fsp27 (black bars) or control scramble (white bars) was administered to KK or KK-Ay mice ($n = 5$) and NC or DIO mice ($n = 4$). SBP was measured on Day 3 after adenoviral administration. Data are presented as means ± SD. $^{**}P < 0.01$ and $^{*}P < 0.05$, compared with LacZ mice. NS, not significant.

accumulation, suggesting activation of physiological pathways downstream from PPARγ2. In addition, loss of function of PPARγ reversed these phenotypes: i.e. liver-specific knockout of PPARγ reportedly increased peripheral adiposity in murine models of obesity[26] and liver-selective knockdown reversed obesity-related hypertension (*Figure 2B* and *F*). Furthermore, and most importantly, hepatic expression and knockdown of Fsp27, a physiological target of PPARγ,[20] exerted effects similar to those of PPARγ2. Therefore, activation of the PPARγ–Fsp27 pathway in the liver is likely to induce sympathetic activation, leading to reduced peripheral adiposity and elevated BP. Systemic administration of thiazolidinediones activates PPARγ not only in the liver but also other organs/tissues throughout the body, especially adipose tissue, where PPARγ expression is markedly higher than that in the liver. In contrast, liver-selective expression and knockdown, as performed in this study, enabled us to examine the specific role of hepatic PPARγ and revealed its importance in the development of obesity-related hypertension.

In mammals, visceral innervation is generally recognized as being based on a dual nerve branch structure, involving both parasympathetic (vagal) and sympathetic nerves. Furthermore, each nerve branch consists of both afferent and efferent neurons. The sympathetic afferents from the aorta[27] or abdominal viscera[28] reportedly play important roles in BP regulation. Splanchnic (sympathetic) afferents from the liver are also involved in insulin hypersecretion during obesity development.[14] In this study, selective

pharmacological deafferentation of the hepatic vagus blocked BP elevation during obesity development in both KK-Ay mice and ZDF rats. In addition to the sympathetic afferent signals, therefore, vagal afferents from the liver modulate BP, contributing to the development of obesity-related hypertension. Infusion of fatty acids into the portal vein reportedly activates hepatic vagal function[29] and the efferent sympathetic nervous system,[30] as well as raising BP.[31] Intraperitoneal dexamethasone administration has also been shown to raise BP via a mechanism involving PPARα and vagal afferents.[21] Thus, neuronal signals, mediated by the afferent vagal nerve from the liver, are involved in determining BP in various settings.

There are numerous reports on the relationship between obesity and sympathetic activation. Several pathological states combining to produce the metabolic syndrome have been shown to be associated with increased adrenergic drive.[32] Humoral factors, including insulin and leptin, have been proposed to be involved in the development of obesity-related disorders. Insulin resistance and the resultant hyperinsulinaemia reportedly contribute to pathological phenotypes of the metabolic syndrome.[4] Hyperleptinaemia is also associated with hypertension[6,7] and renal sympathetic activation[33] in human subjects. Despite resistance to leptin's anorexigenic effect in obese states, the sympathoexcitatory effect of leptin is reportedly preserved,[34] a state termed selective leptin resistance. Thus, sympathetic activation in response to hyperleptinaemia may contribute to hypertension development in patients afflicted with the metabolic syndrome. However, in the present study, adenoviral overexpression of PPARγ in the liver raised BP despite marked decrements in both plasma insulin and leptin levels. Furthermore, dissection or deafferentation of the hepatic vagus did not decrease fasting plasma insulin or leptin levels but did block hypertension development in obesity models. In particular, in leptin receptor mutant ZDF rats, deafferentation of the hepatic vagus blocked BP elevation during obesity development, confirming that this effect is independent of leptin signalling. Thus, in addition to these humoral factors, the present study revealed the importance of the neuronal mechanism underlying obesity-related hypertension; an afferent nerve pathway from the liver which mediates pressor signals from the periphery to the brain.

As we reported previously,[13] hepatic PPARγ2 expression enhances systemic metabolic rates and lipolysis in adipose tissue. These effects were blocked by administration of a pan-β adrenergic blocker, bupranorol, suggesting involvement of sympathetic activation. The results obtained using β-less mice in this study further support the hypothesis that hepatic PPARγ2 expression enhances sympathetic tonus, leading to BP elevation. Since sympathetic activities were not measured directly, however, we cannot rule out the possibility that other, including renal and vascular, potential mechanisms are also involved in BP elevation induced by hepatic PPARγ2 expression. As described above, various mechanisms have been proposed to underlie obesity-induced hypertension, including, hyperinsulinaemia, hyperleptinaemia, and activation of the renin–angiotensin system. Oxidative and inflammatory stress, endothelial dysfunction, and decreased sensitivity to natriuretic peptides are also reportedly involved in hypertension development associated with obesity.[2,3] Therefore, it was quite unexpected that BP elevation was almost completely blocked by either hepatic PPARγ2 knockdown or hepatic vagotomy. These findings suggest the contributions of various mechanisms to differ among stages of obesity. We used young rodents and measured BP during obesity development rather than after the establishment of obesity, indicating that the neuronal mechanism originating in hepatic PPARγ2 expression is likely to play important roles in BP elevation, at least, during the course of obesity development. All results presented herein were obtained from animal obesity models, such as KK-Ay and DIO mice as well as ZDF rats. Animal studies have limitations and further examinations are, of course, required to conclude whether this neuronal mechanism is also relevant to human obese subjects.

Hepatic PPARγ expression induced both hepatic lipid accumulation and BP elevation. In search of the target downstream from PPARγ in the liver, therefore, we first examined the possibility that hepatic lipid accumulation *per se* raises BP. However, BP was not elevated in other models of hepatic steatosis, DGAT1 and DGAT2 overexpressions, in which PPARγ is not upregulated in the liver. In addition, hepatic knockdown of PPARγ in KK-Ay and DIO mice lowered BP prior to hepatic triglyceride reduction. These findings together suggest that hepatic PPARγ upregulation, rather than hepatic lipid accumulation, contributes to the development of obesity-related hypertension. Then we examined the role of Fsp27, a direct target of PPARγ.[20] Fsp27 is a member of the Cide family of proteins, also known as Cidec in humans.[35] Fat-specific protein 27 is expressed mainly in WAT but high fat feeding increases hepatic Fsp27 expression.[36] Fat-specific protein 27 deficiency inhibited lipid accumulation in the liver during high fat feeding,[37] indicating that Fsp27 mediates hepatic lipid accumulation downstream from PPARγ.[20] In the present study, overexpression of Fsp27 in the liver also resulted in remote metabolic phenotypes, including adipose tissue reduction, blood glucose lowering, and BP elevation, all of which were blocked by hepatic vagotomy. In addition, hepatic Fsp27 is upregulated in murine obesity models and its knockdown reversed the BP elevation associated with obesity development. These findings indicate that the hepatic PPARγ–Fsp27 pathway contributes to the development of obesity-related hypertension.

In conclusion, afferent vagal signals triggered by the hepatic PPARγ–Fsp27 pathway are likely to play an important role in the development of obesity-related hypertension. Thus, the liver may sense metabolic states and transmit neuronal signals modulating energy and glucose metabolism to maintain metabolic homeostasis. However, these signals may also have adverse impacts due to sympathetic overflow[13] and hyperinsulinaemia[14] during obesity development. Therefore, neuronal signals from the liver may contribute to the development of various pathological phenotypes characteristic of the metabolic syndrome.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

Acknowledgements

We thank Drs B. B. Lowell, F. J. Gonzalez, and H. Mizuguchi for generous gifts of β-less mice, Fsp27 adenoviruses, and PPARγ

shRNA adenoviruses, respectively, and Drs M. Nakazato and Y. Minokoshi for technical advice and helpful discussions.

Funding

This work was supported by Grants-in-Aid for Scientific Research, to H.K. (B2, 20390252) and Y.O. (A2, 19209034), a Research Fellowship to K.U. (19007896) from the Ministry of Education, Science, Sports and Culture of Japan, and a Grant-in-Aid for Scientific Research (H19-genome-005) to Y.O. from the Ministry of Health, Labor and Welfare of Japan. This work was also supported by the Global-COE Program for 'Network Medicine' to Y.O. and H.K. from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflict of interest: none declared.

References

1. Katagiri H, Yamada T, Oka Y. Adiposity and cardiovascular disorders: disturbance of the regulatory system consisting of humoral and neuronal signals. *Circ Res* 2007;**101**:27–39.
2. Haffner S, Taegtmeyer H. Epidemic obesity and the metabolic syndrome. *Circulation* 2003;**108**:1541–1545.
3. Sarzani R, Salvi F, Dessi-Fulgheri P, Rappelli A. Renin-angiotensin system, natriuretic peptides, obesity, metabolic syndrome, and hypertension: an integrated view in humans. *J Hypertens* 2008;**26**:831–843.
4. Reaven GM, Lithell H, Landsberg L. Hypertension and associated metabolic abnormalities—the role of insulin resistance and the sympathoadrenal system. *N Engl J Med* 1996;**334**:374–381.
5. Bays HE, Gonzalez-Campoy JM, Bray GA, Kitabchi AE, Bergman DA, Schorr AB, Rodbard HW, Henry RR. Pathogenic potential of adipose tissue and metabolic consequences of adipocyte hypertrophy and increased visceral adiposity. *Expert Rev Cardiovasc Ther* 2008;**6**:343–368.
6. Rosmond R, Chagnon YC, Holm G, Chagnon M, Perusse L, Lindell K, Carlsson B, Bouchard C, Bjorntorp P. Hypertension in obesity and the leptin receptor gene locus. *J Clin Endocrinol Metab* 2000;**85**:3126–3131.
7. Shankar A, Xiao J. Positive relationship between plasma leptin level and hypertension. *Hypertension* 2010;**56**:623–628.
8. Walker AB, Chattington PD, Buckingham RE, Williams G. The thiazolidinedione rosiglitazone (BRL-49653) lowers blood pressure and protects against impairment of endothelial function in Zucker fatty rats. *Diabetes* 1999;**48**:1448–1453.
9. Raji A, Seely EW, Bekins SA, Williams GH, Simonson DC. Rosiglitazone improves insulin sensitivity and lowers blood pressure in hypertensive patients. *Diabetes Care* 2003;**26**:172–178.
10. Morse SA, Zhang R, Thakur V, Reisin E. Hypertension and the metabolic syndrome. *Am J Med Sci* 2005;**330**:303–310.
11. Grassi G, Dell'Oro R, Facchini A, Quarti Trevano F, Bolla GB, Mancia G. Effect of central and peripheral body fat distribution on sympathetic and baroreflex function in obese normotensives. *J Hypertens* 2004;**22**:2363–2369.
12. Guyenet PG. The sympathetic control of blood pressure. *Nat Rev Neurosci* 2006;**7**:335–346.
13. Uno K, Katagiri H, Yamada T, Ishigaki Y, Ogihara T, Imai J, Hasegawa Y, Gao J, Kaneko K, Iwasaki H, Ishihara H, Sasano H, Inukai K, Mizuguchi H, Asano T, Shiota M, Nakazato M, Oka Y. Neuronal pathway from the liver modulates energy expenditure and systemic insulin sensitivity. *Science* 2006;**312**:1656–1659.
14. Imai J, Katagiri H, Yamada T, Ishigaki Y, Suzuki T, Kudo H, Uno K, Hasegawa Y, Gao J, Kaneko K, Ishihara H, Niijima A, Nakazato M, Asano T, Minokoshi Y, Oka Y. Regulation of pancreatic beta cell mass by neuronal signals from the liver. *Science* 2008;**322**:1250–1254.
15. Fajas L, Auboeuf D, Raspe E, Schoonjans K, Lefebvre AM, Saladin R, Najib J, Laville M, Fruchart JC, Deeb S, Vidal-Puig A, Flier J, Briggs MR, Staels B, Vidal H, Auwerx J. The organization, promoter analysis, and expression of the human PPARgamma gene. *J Biol Chem* 1997;**272**:18779–18789.
16. Rahimian R, Masih-Khan E, Lo M, van Breemen C, McManus BM, Dube GP. Hepatic over-expression of peroxisome proliferator activated receptor gamma2 in the ob/ob mouse model of non-insulin dependent diabetes mellitus. *Mol Cell Biochem* 2001;**224**:29–37.
17. Westerbacka J, Kolak M, Kiviluoto T, Arkkila P, Siren J, Hamsten A, Fisher RM, Yki-Jarvinen H. Genes involved in fatty acid partitioning and binding, lipolysis, monocyte/macrophage recruitment, and inflammation are overexpressed in the human fatty liver of insulin-resistant subjects. *Diabetes* 2007;**56**:2759–2765.
18. Bachman ES, Dhillon H, Zhang CY, Cinti S, Bianco AC, Kobilka BK, Lowell BB. betaAR signaling required for diet-induced thermogenesis and obesity resistance. *Science* 2002;**297**:843–845.
19. Yamazaki T, Sasaki E, Kakinuma C, Yano T, Miura S, Ezaki O. Increased very low density lipoprotein secretion and gonadal fat mass in mice overexpressing liver DGAT1. *J Biol Chem* 2005;**280**:21506–21514.
20. Matsusue K, Kusakabe T, Noguchi T, Takiguchi S, Suzuki T, Yamano S, Gonzalez FJ. Hepatic steatosis in leptin-deficient mice is promoted by the PPARgamma target gene Fsp27. *Cell Metab* 2008;**7**:302–311.
21. Bernal-Mizrachi C, Xiaozhong L, Yin L, Knutsen RH, Howard MJ, Arends JJ, Desantis P, Coleman T, Semenkovich CF. An afferent vagal nerve pathway links hepatic PPARalpha activation to glucocorticoid-induced insulin resistance and hypertension. *Cell Metab* 2007;**5**:91–102.
22. Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, Meng Q, Mustafa AK, Mu W, Zhang S, Snyder SH, Wang R. H2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. *Science* 2008;**322**:587–590.
23. Kim SM, Chen L, Faulhaber-Walter R, Oppermann M, Huang Y, Mizel D, Briggs JP, Schnermann J. Regulation of renin secretion and expression in mice deficient in beta1- and beta2-adrenergic receptors. *Hypertension* 2007;**50**:103–109.
24. Serazin V, Dieudonne MN, Morot M, de Mazancourt P, Giudicelli Y. cAMP-positive regulation of angiotensinogen gene expression and protein secretion in rat adipose tissue. *Am J Physiol Endocrinol Metab* 2004;**286**:E434–E438.
25. Fujiwara K, Hayashi K, Matsuda H, Kubota E, Honda M, Ozawa Y, Saruta T. Altered pressure-natriuresis in obese Zucker rats. *Hypertension* 1999;**33**:1470–1475.
26. Matsusue K, Haluzik M, Lambert G, Yim SH, Gavrilova O, Ward JM, Brewer B Jr, Reitman ML, Gonzalez FJ. Liver-specific disruption of PPARgamma in leptin-deficient mice improves fatty liver but aggravates diabetic phenotypes. *J Clin Invest* 2003;**111**:737–747.
27. Pagani M, Pizzinelli P, Bergamaschi M, Malliani A. A positive feedback sympathetic pressor reflex during stretch of the thoracic aorta in conscious dogs. *Circ Res* 1982;**50**:125–132.
28. Fu LW, Longhurst JC. Reflex pressor response to arterial phenylbiguanide: role of abdominal sympathetic visceral afferents. *Am J Physiol* 1998;**275**(Pt 2):H2025–H2035.
29. Randich A, Spraggins DS, Cox JE, Meller ST, Kelm GR. Jejunal or portal vein infusions of lipids increase hepatic vagal afferent activity. *Neuroreport* 2001;**12**:3101–3105.
30. Benthem L, Keizer K, Wiegman CH, de Boer SF, Strubbe JH, Steffens AB, Kuipers F, Scheurink AJ. Excess portal venous long-chain fatty acids induce syndrome X via HPA axis and sympathetic activation. *Am J Physiol Endocrinol Metab* 2000;**279**:E1286–E1293.
31. Grekin RJ, Vollmer AP, Sider RS. Pressor effects of portal venous oleate infusion. A proposed mechanism for obesity hypertension. *Hypertension* 1995;**26**:193–198.
32. Grassi G. Sympathetic overdrive and cardiovascular risk in the metabolic syndrome. *Hypertens Res* 2006;**29**:839–847.
33. Eikelis N, Schlaich M, Aggarwal A, Kaye D, Esler M. Interactions between leptin and the human sympathetic nervous system. *Hypertension* 2003;**41**:1072–1079.
34. Correia ML, Haynes WG, Rahmouni K, Morgan DA, Sivitz WI, Mark AL. The concept of selective leptin resistance: evidence from agouti yellow obese mice. *Diabetes* 2002;**51**:439–442.
35. Gong J, Sun Z, Li P. CIDE proteins and metabolic disorders. *Curr Opin Lipidol* 2009;**20**:121–126.
36. Guillen N, Navarro MA, Arnal C, Noone E, Arbones-Mainar JM, Acin S, Surra JC, Muniesa P, Roche HM, Osada J. Microarray analysis of hepatic gene expression identifies new genes involved in steatotic liver. *Physiol Genomics* 2009;**37**:187–198.
37. Nishino N, Tamori Y, Tateya S, Kawaguchi T, Shibakusa T, Mizunoya W, Inoue K, Kitazawa R, Kitazawa S, Matsuki Y, Hiramatsu R, Masubuchi S, Omachi A, Kimura K, Saito M, Amo T, Ohta S, Yamaguchi T, Osumi T, Cheng J, Fujimoto T, Nakao H, Nakao K, Aiba A, Okamura H, Fushiki T, Kasuga M. FSP27 contributes to efficient energy storage in murine white adipocytes by promoting the formation of unilocular lipid droplets. *J Clin Invest* 2008;**118**:2808–2821.

ORIGINAL

Advance Publication
doi: 10.1507/endocrj. EJ11-0145

Recurrent hypoglycemia during pregnancies in a woman with multiple autoantibodies including anti-insulin receptor antibody and anti-platelet antibody, whose serum lowered murine blood glucose levels and phosphorylated insulin receptor of CHO-IR cells

Makiko Toshihiro[1)], Hideki Katagiri[2)], Koujiro Kataoka[3)], Akimune Fukushima[4)], Toshie Segawa[5)], Takuya Fujiwara[5)], Isao Hikichi[6)], Noriko Takebe[1)] and Jo Satoh[1)]

[1)]*Division of Diabetes and Metabolism, Iwate Medical University School of Medicine, Japan*
[2)]*Department of Metabolic Diseases, Center for Metabolic Diseases, Tohoku University Graduate School of Medicine, Japan*
[3)]*Division of Gastroenterology and Hepatology, Iwate Medical University School of Medicine, Japan*
[4)]*Department of Obstetrics and Gynecology, Iwate Medical University School of Medicine, Japan*
[5)]*Division of Endocrinology, Department of Internal Medicine, Iwate Medical University School of Medicine, Japan*
[6)]*Department of Diabetes, Morioka City Hospital, Japan*

Abstract. We report a rare case of recurrent hypoglycemia in a pregnant woman during the period of pregnancies. She suffered from severe hypoglycemia and intrauterine fetal death during the first pregnancy. Thereafter, there was no hypoglycemia, and no obvious cause of hypoglycemia was found by close examinations. Two years later, at eight weeks into the second pregnancy, hypoglycemia recurred. The patient had multiple auto-antibodies including anti-insulin receptor antibody and anti-platelet antibody associated with decreased platelet count. She completed the pregnancy with continuous intravenous administration of glucose that prevented hypoglycemia and finally delivered a healthy baby by Caesarian section. Both the hypoglycemia and thrombocytopenia, and the auto-antibodies disappeared after the delivery. We analyzed the patient's serum as a possible cause of hypoglycemia. Administration of the serum lowered blood glucose levels of mice more strongly than control serum. In addition, the serum phosphorylated tyrosine of insulin receptor of *Chinese hamster ovary cells* overexpressing human insulin receptors (CHO-IR cells) in vitro. These results suggest that multiple auto-antibodies might have been induced by a trigger of pregnancy, although the precise mechanism was unclear, and the anti-insulin receptor antibody and anti-platelet antibody might have induced hypoglycemia and thrombocytopenia, respectively, during the pregnancy.

Key words: Hypoglycemia, Pregnancy, Anti-insulin receptor antibody, Anti-platelet antibody, Phosphorylation of insulin receptor

HYPOGLYCEMIA is induced by various causes [1]. We experienced a case of recurrent hypoglycemia in a pregnant woman during the period of pregnancy on two separate occasions. The patient had multiple autoantibodies including anti insulin receptor (IR) antibody and anti-platelet antibody associated with thrombocytopenia. After the delivery, the titers of autoantibodies were decreased, the hypoglycemia disappeared, and thrombocytopenia improved. We analyzed the patient's serum as a possible cause of hypoglycemia. Here we report the results that the serum lowered murine blood glucose levels and phosphorylated tyrosine of insulin receptor of CHO-IR cells.

Submitted Jul. 19, 2011; Accepted Aug. 24, 2011 as EJ11-0145
Released online in J-STAGE as advance publication Sep. 17, 2011

Correspondence to: Jo Satoh, Division of Diabetes and Metabolism, Department of Internal Medicine, Iwate Medical University School of Medicine, 19-1 Uchimaru, Morioka, Iwate 020-8505, Japan. E-mail: jsatoh@iwate-med.ac.jp

Case Report

The patient (a 32-year-old woman) has no particular family history and past history except acute pneumonia

Table 1 Laboratory data on admission

TSH (0.5-5.0 μIU/mL)	1.72	FPG (70-109 mg/dL)	50
FT3 (2.3-4.3 pg/mL)	2.58	F-IRI (3.1-16.9 μU/mL)	0.5
FT4 (0.9-1.7 pg/mL)	0.98	Proinsulin (5-10 pmol/mL)	6.7
		Serum-CPR (0.6-2.1 ng/mL)	0.11
ACTH (7.2-63.3 pg/mL)	42.6	Urine-CPR (29.2-167 μg/day)	51.5
Cortisol (4.0-18.3 μg/dL)	19.7	Insulin antibody (<0.4%)	(-)
		Insulin receptor antibody (<24.2%)	39.6%
Glucagon (50-150 pg/mL)	120	PA IgG (9-25 ng/10^7 platelets)	149.6
LH (1.1-14.2 mIU/mL)	0.20	GAD antibody (<1.5 U/mL)	(-)
FSH (1.5-8.5 mIU/mL)	<0.05	ICA antibody (<1.25 JDF U)	(-)
PRL (4.9-29.3 ng/mL)	157.2	Pituitary antibody (-)	(-)
ADH (0.3-3.5 pg/mL)	0.7	Anti-thyroglobulin antibody (-)	(+)
GH (0.66-3.68 ng/mL)	0.46	Ant-microsome antibody (-)	(+)
IGF-1 (73-311 ng/mL)	180	Anti-DNA antibody (-)	(-)
IGF-2 (-)	(-)	Anti-nuclear antibody (-)	(-)
		CEA (<5.0 ng/mL)	0.6
Urine-cortisol (11.2-80.3 μg/day)	117	CA19-9 (<37.0 U/mL)	6.5
Urine-17-OHCS (2.6-7.8 mg/day)	3.1	CA125 (<35.0 U/mL)	25.6
Urine-17-KS (1.0-10.9 mg/day)	7.7		

(Normal range)

Abbreviations: S, serum; U, urine; TSH, thyroid stimulating hormone; FT3, free triiodothyronine; FT4, free thyroxine; ACTH, adrenocorticotropic hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; PRL, prolactin; ADH, antidiuretic hormone; GH, growth hormeone; IGF, insulin-like growth factor; OHCS, hydroxycorticosteroid; KS, ketosteroid; FPG, fasting plasma glucose; IRI, immunoreactive insulin; CPR, connecting peptide immunoreactivity; PA IgG, platelet-associated IgG; GAD, glutamic acid decarboxylase; ICA, islet cell antibody; CEA, carcinoma embryonic antigen

at 15 years of age. She was brought into the advanced emergency center of Iwate Medical University Hospital due to a sudden loss of consciousness at 9 weeks of the first pregnancy in December 2001. Her blood glucose level was as low as 26 mg/dl and she was immediately treated for hypoglycemia, and then hospitalized in Morioka City Hospital on the same day for close examination. An intrauterine fetal death was found during the examination. The hypoglycemia did not occur thereafter. The glucose tolerance test and various hormone measurements conducted thereafter showed that all results were within the normal ranges. Also, no abnormal findings suggesting insulinoma were observed in the imaging tests, including abdominal ultrasonography, computed tomography (CT) and angiography.

Two years later, her hypoglycemia had recurred at 8 weeks into the second pregnancy in September 2003, and she was re-hospitalized in the Morioka City Hospital for treatment of hypoglycemia. In order to prevent night-time hypoglycemia, glucose solution was intravenously infused every day. However, due to the persistence of the low blood glucose level, at 15 weeks of pregnancy (October 2003) the patient was transferred to the Department of Obstetrics and Gynecology of the Iwate Medical University Hospital for whole-body care and close examination, and was referred to the Department of Diabetes and Metabolism on the same day.

On admission, physical examination revealed that she had a low grade fever (37.2°C), but no particular abnormalities relating to acanthosis nigricans, hirsutism, and Sjogren syndrome. Laboratory data showed that there were no abnormalities in peripheral blood and biochemical examinations except a mild anemia (RBC: 367 x 10^4/μl; Hb: 11.3 g/dl), thrombocytopenia (11.3 × 10^4/μl) and an increase in serum γ-globulin fraction (27%). Fasting plasma glucose (FPG) level was 50 mg/dl, and fasting serum insulin (IRI) and serum C-peptide levels were lower than normal, whereas the pro-insulin level was within the normal range (Table 1). The growth hormone (GH) level was lower than normal, whereas the insulin-like growth factor-1 (IGF-1)

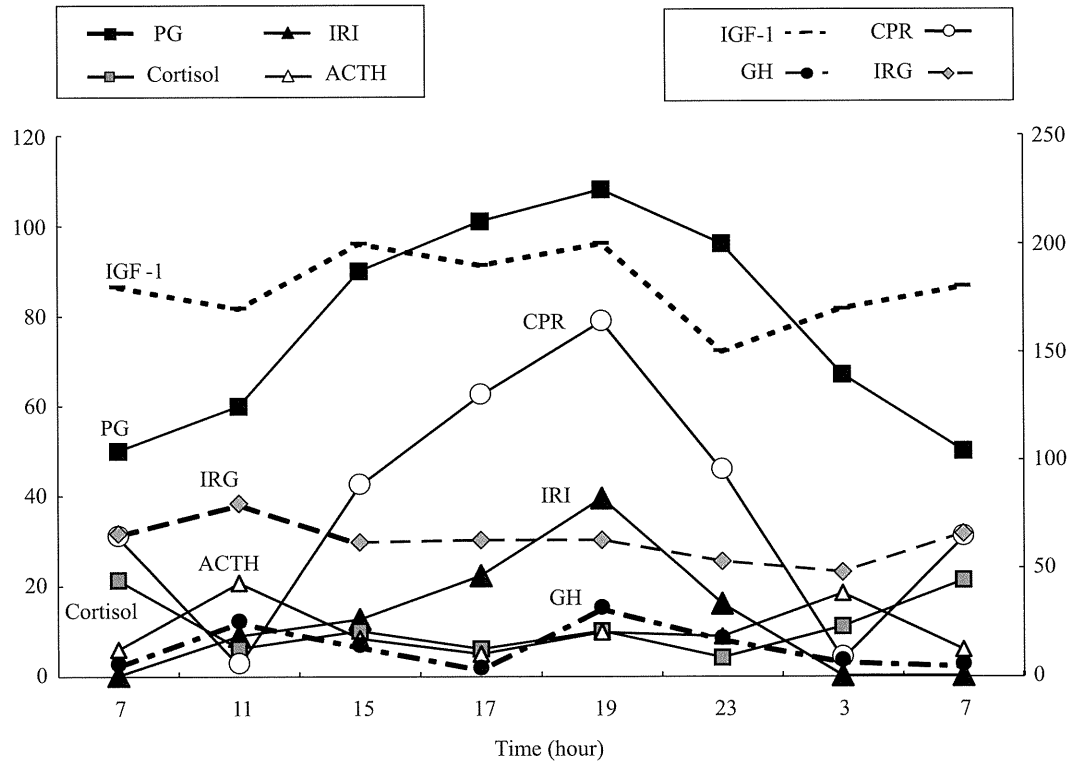

Fig. 1 Circadian variations of plasma hormone levels in the patient.
Abbreviations: PG, plasma glucose (mg/dL); IRI, immunoreactive insulin (μU/mL); GH, growth hormone ($\times 10^{-1}$ ng/mL); IRG, immunoreactive glucagon (pg/mL); ACTH, adrenocorticotropic hormone (pg/mL); CPR, connecting peptide immunoreactivity ($\times 10^{-2}$ ng/mL); IGF, insulin-like growth factor (ng/mL).

level was within the normal range and IGF-2 was not detected (measured by Prof. Naomi Hizuka, Tokyo Women's Hospital). The thyroid hormone levels (free T_3 [FT_3] and free T_4 [FT_4]) were normal. The luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels were lower and the prolactin level was higher than normal. As for autoantibodies, insulin antibody was negative, whereas anti-insulin receptor antibody was positive; the inhibition rate of insulin binding was 39.6% (measured by BML, Inc., Tokyo, Japan). Anti-thyroglobulin antibody and anti-microsome antibody were positive, whereas anti-DNA and anti-nuclear antibodies were negative. No abnormality was observed in the abdominal ultrasonography and magnetic resonance imaging of the brain. CT was not performed because of the pregnancy.

After admission, since hypoglycemia (50-60 mg/dl at 4:00 a.m.) persisted even with supplementary meals at 3 p.m. and 9 p.m. and a 5% glucose drip-infusion from midnight to 6 a.m., 5% glucose was changed to 10% glucose. But the mean glucose level at 4 a.m. remained as low as 50-60 mg/dl. At one time, she lost consciousness because of hypoglycemia when she was out of the hospital in the daytime without the glucose infusion, and was brought back to the hospital by an ambulance.

In January 2004, since the platelet count fell to as low as 7.0×10^4/μl and anti-platelet antibody (PA IgG; 149.6 fg/platelet) was positive, a bone marrow puncture was performed at the Hematology Department of our hospital and she was diagnosed as having idiopathic thrombocytopenic purpura (ITP).

Since the blood and urinary cortisol levels were lower than those during usual pregnancy, although the serum aldosterone level was as high as that during usual pregnancy, secondary adrenocortical insufficiency was suspected. Although the baseline levels of serum ACTH and cortisol were within the normal range, the circadian rhythm of these hormones showed low levels as a whole (Fig. 1). Therefore, we considered hypocortisolemia as a part of the possible causes of her hypoglycemia, and administered orally a small

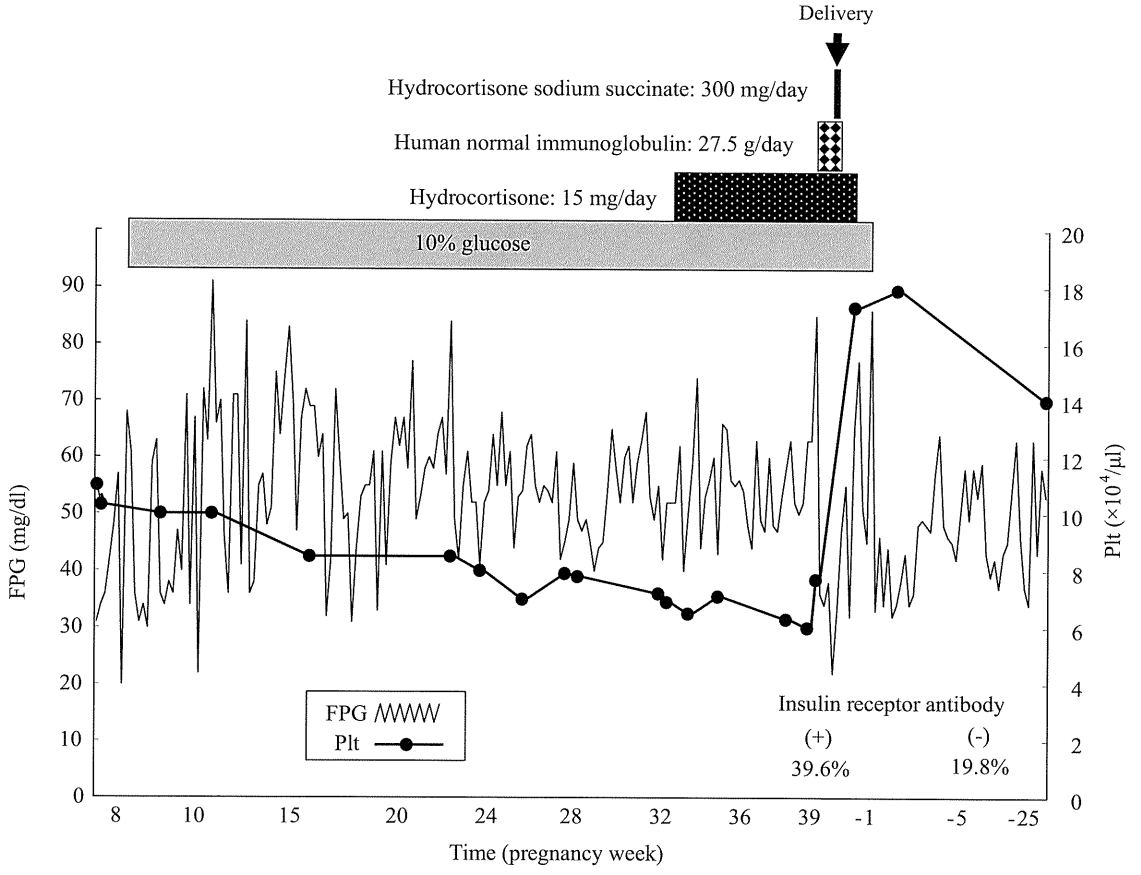

Fig. 2 Time course of FPG and platelet counts according to pregnancy week of the patient.
Abbreviations: FPG, fasting plasma glucose; Plt, platelet

dose of hydrocortisone (15 mg/day) to the patient from March 2004 (at 33 weeks of pregnancy) (Fig. 2).

Because the platelet count further decreased to as low as $6.0 \times 10^4/\mu l$, we decided to carry out a planned delivery by Caesarian section. To increase the platelet count, human immunoglobulin was administered to the patient at a dose of 27.5 g/day for three days from April 3, 2004 (39 weeks of pregnancy), and the platelet count increased to $15.2 \times 10^4/\mu l$. Also, on April 6, 2004, to avoid any adrenal crisis which could be induced by stress at delivery, hydrocortisone sodium succinate was administered to the patient at a dose of 300 mg/day, and on the same day a healthy baby was delivered by Caesarian section (Fig. 2).

After the delivery, her blood glucose level rose gradually to the point at which the patient did not develop hypoglycemia even with regular meals and without glucose infusion. Since a starvation test conducted one month after the delivery showed that the patient did not develop hypoglycemia during 7 hours of fasting, she was discharged from the hospital.

An examination conducted in July 2004 (3 months after the delivery) showed that anti-platelet antibody was negative (<25 ng/10^7 platelets) and the platelet count had returned to normal. Furthermore, an examination in October 2004 (6 months after the delivery) showed that anti-insulin receptor antibody was also negative (19.8%). Thereafter, we did not follow her laboratory data because of her moving to a city far from our hospital. However, according to the recent telephone information from her on June, 2011, the patient had not become pregnant and not developed hypoglycemia and her boy grew satisfactorily since the delivery on 2004.

Materials and Methods

Measurement of anti-insulin receptor antibody

Anti-insulin receptor antibody in serum was mea-

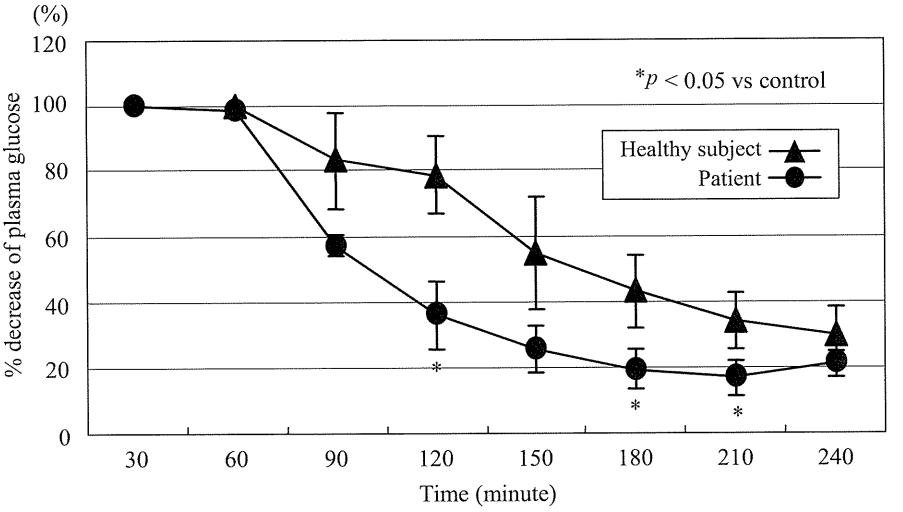

Fig. 3 Percent decrease of plasma glucose levels after injection with serum from the patient or a healthy subject.

sured in the BML, Inc. (Tokyo, Japan) by a radioreceptor assay as previously reported [2]. Binding activity of insulin receptor antibody with insulin receptor from human B lymphoblastic IM-9 cells [3] was expressed as % inhibition by insulin receptor antibody of ^{125}I-insulin binding with human insulin receptor. Cutoff value of this assay was 24.2% (mean + 3SD).

Treatment of mice with human serum

Sera were taken from the patient during the pregnancy and a person with normal glucose tolerance (control), and one ml of each serum was intraperitoneally administered to 5 mice in each group, and the blood glucose level of the mice was measured by a glucometer. The mice were fasted after the administration of the serum (water was fed freely), and the changes in their blood glucose level were shown as % decrease in comparison with the level before the serum administration. The protocol of the animal experiment was approved by the Animal Care and Use Committee of the Iwate Medical University.

Phosphorylation of IR of CHO-IR cells

The patient's serum and the healthy subject's (control) serum were dialyzed in with Ham' sF12 buffer. After incubation of CHO-IR cells for 5 hours in a serum-free culture medium (serum starvation), the medium was replaced with the sera from the patient or control subjects after dialysis and incubated for 5-30 minutes. After incubation, the cells were solubilized, subjected to immunoprecipitation using anti-IR ß chain antibody, subjected to electrophoresis in 7.5% acrylamide gel and blotted using an anti-phosphotyrosine antibody (4G10) as reported previously [4].

Results

The patient's serum lowered mouse blood glucose levels

To determine whether the serum of the patient had any hypoglycemic effect. One ml of each serum was intraperitoneally administered to 5 mice in each group. As shown in Fig. 3, the % decrease in the blood glucose level of the mice administered with the patient's serum was significantly greater than that of the control. This result suggested the presence of a factor lowering the blood glucose level in the serum of the patient.

The effect of the patient's serum on phosphorylation of insulin receptors of CHO-IR cells.

To determine whether the patient's serum, which was positive for the IR antibody and had the hypoglycemic effect in mice (Fig. 3), phosphorylates IR, CHO-IR cells were incubated with the serum. As shown in Fig. 4, the patient's serum (lane 3 and 5) showed a positive blot band in the 90-kDa region just like insulin as the positive control (lane 1), and the serum with longer incubation (30 min. with lane 5 vs. 5 min. with lane 3) showed a more distinct positive band. On the other hand, for negative control (lane 4 for 5 min. and lane 6 for 30 min.), the blot band was negative. This suggested that the patient's serum stimulated the IR and induced the tyrosine phosphorylation of IR ß chain.

Discussion

Hypoglycemia is induced by a variety of endogenous and exogenous causes [5]. Endogenous causes are classified as insulin-mediated (insulinoma, nesidoblastosis, non-insulinoma pancreatogenous hypoglycemia syndrome [NIPHS], insulin antibody and reactive hypoglycemia) and insulin-independent causes (critical organ failure, sepsis, hormone deficiency such as cortisol, growth hormone and hypopituitarism, insulin receptor antibodies, and non-islet cell tumor). Exogenous causes include therapeutic drugs such as oral hypoglycemic agents and others, factitious cause, and alcohol or toxins.

In this case, exogenous causes could be neglected, because she was not administered any medicines. Among endogenous causes, insulin-mediated causes such as insulinoma, nesidoblastosis and NIPHS are unlikely, because serum insulin and c-peptide levels were not high at the time of hypoglycemia and anti-insulin antibody was negative. As insulin-independent causes, she had no critical organ failure, sepsis and hormone deficiencies. Although serum cortisol levels were relatively low as a pregnant woman, serum ACTH levels were within normal limits and she had no signs and symptoms of Addison's disease, nor autoimmune polyglandular syndrome type 1 and type 2. A relationship between hypocortisolemia and autoantibodies such as anti-insulin receptor antibody and anti-platelet antibody is unknown. The relative hypocorticosolemia may not be a major cause of hypoglycemia, rather it might be a factor exacerbating hypoglycemia.

It is most likely in this case that anti-IR antibody may have induced hypoglycemia, because of several lines of evidence. First, the patient's serum positive for anti-IR antibody lowered plasma glucose levels of mice as compared with control serum from a healthy subject (Fig. 3). Second, the patient's serum phosphorylated tyrosineof IR of CHO-IR cells as did insulin (Fig. 4). Finally, the improvement of hypoglycemia was associated with decreased titer of anti-IR antibody. Eventually she can be diagnosed as having type B insulin resistance syndrome associated with hypoglycemia [6].

There remain some questions, e.g., why hypoglycemia was induced and why multiple autoantibodies such as anti-IR antibody and PA IgG were produced only during the period of pregnancy, although anti-IR antibody and PA-IgG was not measured at time of the first pregnancy, when she had no thrombocytopenia. There

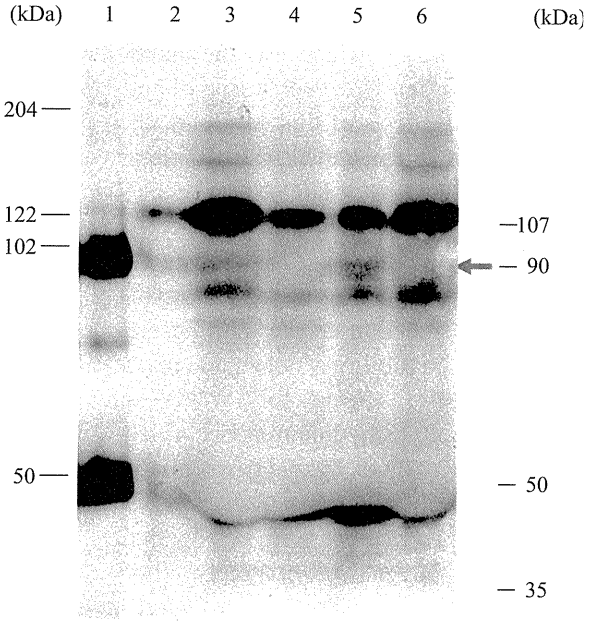

Fig. 4 Tyrosine phosphorylation of the insulin receptor of CHO cells by the treatment with the patient's serum.
M: Markers Lane 1: Insulin (positive control) Lane 2: Blank Lane 3 and 5: Patients serum incubated 5 min. (lane 3) and 30 min. (lane 5). Lane 4 and 6: Control serum incubated 5 min. (lane 4) and 30 min. (lane 6).

has been implications that the immune system becomes aberrant and some autoimmune diseases such as systemic lupus erythematosus (SLE) are exacerbated during pregnancy [7]. SLE is associated with various multiple autoantibodies and there are some reports on cases with SLE associated anti-insulin receptor antibody [8-10]. Our case did not show symptoms of SLE and did not fulfill the criteria of SLE by the American Rheumatoid Association [9].

It has been reported that approximately 7-8% of pregnant woman have thrombocytopenia, the cause of which includes gestational thrombocytopenia and ITP [12]. Pregnancy does not increase the risk of ITP, but it exacerbates preexisting ITP [12]. Therefore, this case is not rare in terms of pregnancy associated with ITP. However, there has not been reported a pregnant woman associated with both anti-IR antibody and anti-platelet antibody. Recently, a very rare case of type B insulin resistance syndrome and ITP has been reported [13]. In this case, helicobacter pylori (HP) infection was indicated as a cause of ITP, and eradication therapy of HP resulted in an increase of platelet number and decrease of anti-IR antibody. In our case, low titer of anti-HP antibody (14 U/mL) (normal < 10 U/ml, SRL

Co. Ltd, Tokyo, Japan) was detected in the serum during the pregnancy, which was measured years later in the stored serum. However, a role of anti-HP antibody in the pathogenesis of this case was unclear, because platelet number and anti-IR antibody improved without eradication therapy of HP.

After the successful delivery, hypoglycemia was improved and the anti-IR antibody anti-platelet antibody became negative. The completion of the pregnancy may have resulted in decrease of these autoantibodies. However, a possibility is not denied that administration of a low dose (15 mg/day for 6 weeks) and a high dose (300 mg once) of hydrocortisone, which was given to compensate relative hypocortisolemia and to prevent adrenal crisis during the Caesarian section, respectively, might have reduced autoanitbodies including anti-IR antibody and anti-platelet antibody.

In summary, we report here an interesting rare case of a pregnant woman who suffered from severe hypoglycemia only during two occasions of her pregnancies. She had anti-IR antibody and anti-platelet antibody. Administration of the serum lowered blood glucose levels in mice, and the serum phosphorylated tyrosine of insulin receptor of CHO-IR cells. These autoantibodies and both the hypoglycemia and thrombocytopenia disappeared after the delivery. From these findings, we concluded that anti-insulin receptor antibody and anti-platelet antibody during pregnancies might have lead to hypoglycemia and thrombocytopenia, respectively.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

We thank Prof. N. Hizuka, Tokyo Women's Hospital, for the measurement of IGF-II, Prof. T. Suda, Department of Endocrinology, Hirosaki University, for the helpful discussion and Dr. Paul Langman, Ph.D. for his kind assistance with English usage.

References

1. Service FJ (1995) Hypoglycemia disorders. Hypoglycemia disorders. *N Engl J Med* 332: 1144-1152.
2. Satoh K, Kawaguchi R, Yonezawa M, Kubono K, Hikiji K, Ishigami T, Tsukada Y, Takahashi M (1990) A determination of anti-insulin receptor antibody in serum--a radioreceptor assay excluded the influence of insulin and anti-insulin antibody. *Rinsho Byori* 38: 311-316.
3. Van Obberghen E, De Meyts P, Roth J (1976) Cell surface receptors for insulin and human growth hormone. Effect of microtubule and microfilament modifiers. *J Biol Chem* 251: 6844-6851.
4. Yamada T, Katagiri H, Asano T, Inukai K, Tsuru M, Kodama T, Kikuchi M , Oka Y (2001) 3-phosphoinositide-dependent protein kinase 1, an Akt1 kinase, is involved in dephosphorylation of Thr-308 of Akt1 in Chinese hamster ovary cells. *J Biol Chem* 276: 5339-5345.
5. Glaser B, Leibowitz G (2005) Hypoglycemia. In: Kahn CR, Weir GC, King GL, Jacobson AM, Moses AC, Smith RJ (eds) Joslin's Diabetes Mellitus 14th edn, Lippincott Williams & Wilkins, New York: 1147-1175.
6. Lupsa BC, Chong AY, Cochran EK, Soos MA, Semple RK, Gorden P (2009) Autoimmune forms of hypoglycemia. *Medicine* 88: 141-153.
7. Carvalheiras G, Vita P, Marta S, Trovão R, Farinha F, Braga J, Rocha G, Almeida I, Marinho A, Mendonça T, Barbosa P, Correia J, Vasconcelos C (2010) Pregnancy and systemic lupus erythematosus: Review of clinical features and outcome of 51 pregnancies at a single institution. *Clin Rev Allergy Immunol* 38: 302-306.
8. Moller DE, Ratner RE, Borenstein DG, Taylor, SI (1988) Autoantibodies to the insulin receptor as a cause of autoimmune hypoglycemia in systemic lupus erythematosus. *Am J Med* 84: 334-338.
9. Varga J, Lopatin M, Boden G (1990) Hypoglycemia due to antiinsulin receptor antibodies in systemic lupus erythematosus. *J Rheumatol* 17: 1226-1229.
10. Kato Y, Ichiki, Kitajima Y (2008) A case of systemic lupus erythematosus presenting as hypoglycemia due to anti-insulin receptor antibodies. *Rheumatol Int* 29: 103-105.
11. Parodi A, Rebora A (1997) ARA and EADV criteria for classification of systemic lupus erythematosus in patients with cutaneous lupus erythematosus. *Dermatology* 194: 217-220.
12. Sukenik-Halevy R, Ellis MH, Fejgin MD (2008) Management of immune thrombocytopenic purpura in pregnancy. *Obstet Gynecol Surv* 63: 182-188.
13. Imai J, Yamada T, Saito T, Ishigaki Y, Hinokio Y, Kotake H, Oka Y, Katagiri H (2009) Eradication of insulin resistance. *Lancet* 374: 264.

Hypertension Research (2011) 34, 1302–1308

www.nature.com/hr

Open

ORIGINAL ARTICLE

Reduction of circulating superoxide dismutase activity in type 2 diabetic patients with microalbuminuria and its modulation by telmisartan therapy

Hiroki Fujita[1], Takuya Sakamoto[2], Koga Komatsu[2], Hiromi Fujishima[1], Tsukasa Morii[1], Takuma Narita[1], Takamune Takahashi[3] and Yuichiro Yamada[1]

Growing evidence indicates that oxidative stress induced by excessive superoxide has a central role in the pathogenesis of diabetic nephropathy (DN). Telmisartan, one of the currently available angiotensin II type 1 receptor blockers (ARBs), has been shown to exert a more powerful proteinuria (albuminuria) reduction in patients with DN, but whether the prominent renoprotective effect of telmisartan is mediated through enhancing antioxidant defense capacity and reducing oxidative stress has not been fully elucidated. The present study first revealed that the serum activity of superoxide dismutase (SOD) responsible for superoxide removal is reduced in the DN stage of microalbuminuria, but not in normoalbuminuria in type 2 diabetic patients. We next examined the alteration of SOD and oxidative stress following an 8-week treatment with telmisartan (40 mg per day) in 12 type 2 diabetic patients with microalbuminuria. Interestingly, the telmisartan treatment not only reduced the circulating levels of two oxidative stress markers, 8-hydroxy-2′-deoxyguanosine (8-OHdG) and nitrotyrosine (NT), but also enhanced serum SOD activity. Notably, a significant correlation was observed between the increase in serum SOD activity and the reduction in albuminuria. We further compared the anti-oxidative effect of telmisartan with that of losartan, another member of the ARB class, by implementing an 8-week interval crossover treatment with these ARBs in another 12 microalbuminuric type 2 diabetic patients. The patients showed higher serum SOD activity, and lower circulating levels of 8-OHdG and NT, during treatment with telmisartan than with losartan. These results suggest that telmisartan has a more potent antioxidative effect through its ability to enhance SOD activity in type 2 diabetic patients with microalbuminuria.
Hypertension Research (2011) **34**, 1302–1308; doi:10.1038/hr.2011.127; published online 4 August 2011

Keywords: angiotensin II type 1 receptor blocker; diabetic nephropathy; oxidative stress; superoxide dismutase; telmisartan

INTRODUCTION

Oxidative stress induced by superoxide anion ($O_2^{\bullet -}$) overproduction is considered a major cause of diabetic vascular injury, including diabetic nephropathy (DN). An excess of the superoxide anion causes vascular cell injury through the formation of cytotoxic secondary reactive oxygen species, such as peroxynitrite ($ONOO^-$) and hydroxyl radicals ($^{\bullet}OH$).[1] The superoxide is produced by multiple pathogenic pathways, including increased nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase activity, uncoupled endothelial nitric oxide (NO) synthase and enhanced angiotensin II signaling.[2] In contrast to the superoxide-producing enzymes such as NAD(P)H oxidase and endothelial NO synthase, superoxide dismutase (SOD) serves as an antioxidant enzyme responsible for superoxide removal. The SOD converts superoxide anion into hydrogen peroxide (H_2O_2) and molecular oxygen.[3,4] The hydrogen peroxide is further detoxified into water (H_2O) by catalase in peroxisomes or glutathione peroxidase in mitochondria.[1,5] Growing evidence indicates that chronic hyperglycemia causes superoxide overproduction by activating NAD(P)H oxidase[6–9] and uncoupling endothelial NO synthase.[9] Therefore, the SOD antioxidant defense system has a key role in protecting vascular cells from increased oxidative stress in the diabetic state.

Telmisartan is a unique angiotensin II type 1 (AT1) receptor blocker (ARB) that functions as a partial agonist of the peroxisome proliferator-activated receptor-γ.[10–12] A recent clinical study showed that telmisartan is superior to another ARB, losartan, in reducing proteinuria effect in patients with DN, despite a comparable blood pressure reduction.[13] Given this compelling evidence, it was expected that telmisartan, among various ARBs, may exert a more powerful protective effect against oxidative stress in the diabetic state. In the present study, we first determined the stage of DN that alters the SOD antioxidant defense capacity and enhances oxidative stress. Our data demonstrate that reduced SOD antioxidant defense capacity and markedly increased oxidative stress are observed in the DN stage of

[1]Division of Endocrinology, Metabolism and Geriatric Medicine, Akita University Graduate School of Medicine, Akita, Japan; [2]Division of Gastroenterology, Honjo Daiichi Hospital, Akita, Japan and [3]Division of Nephrology, Vanderbilt University Medical Center, Nashville, TN, USA
Correspondence: Dr H Fujita, Division of Endocrinology, Metabolism and Geriatric Medicine, Akita University Graduate School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan.
E-mail: hirofuji@gipc.akita-u.ac.jp
Received 29 January 2011; revised 13 May 2011; accepted 6 June 2011; published online 4 August 2011

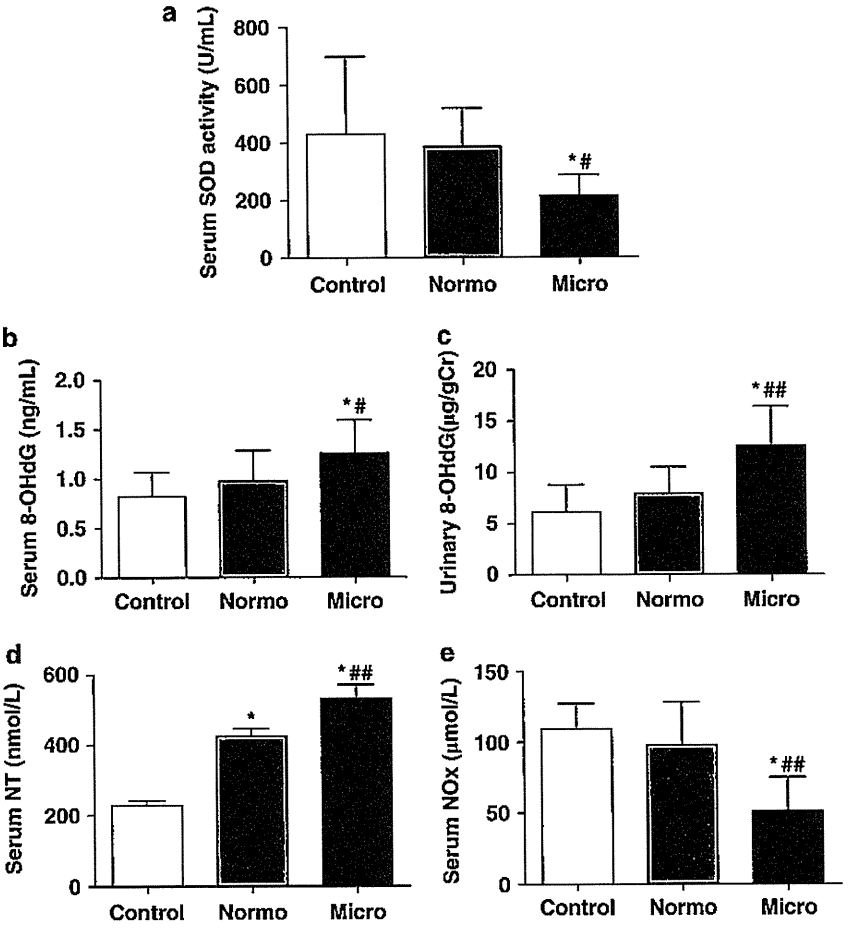

Figure 1 Oxidative stress markers in non-diabetic healthy subjects (control, n=18) and type 2 diabetic patients with normoalbuminuria (normo, n=19) and microalbuminura (micro, n=16). (a) serum SOD activity; (b) serum 8-OHdG; (c) urinary 8-OHdG; (d) serum NT; (e) serum NOx. Data are presented as the means ± s.d. $^{*}P<0.001$ *vs.* control; $^{\#}P<0.05$ *vs.* normo; $^{\#\#}P<0.001$ *vs.* normo. NT, nitrotyrosine.

Not surprisingly, the telmisartan treatment lowered systolic and diastolic blood pressure and reduced albuminuria. Figure 2 shows the changes in the oxidative stress markers after 8 weeks of telmisartan treatment in the diabetic patients with microalbuminuria. Notably, an increase in serum SOD activity was observed after 8 weeks of telmisartan treatment (Figure 2a). Interestingly, there was a significant correlation between the increase in serum SOD activity and the reduction in albuminuria (Figure 2b). In agreement with the improvement in antioxidant defense capacity, serum and urinary 8-OHdG levels and serum NT levels were significantly reduced after 8 weeks of telmisartan treatment (Figures 2c–e). The serum NOx levels were significantly increased after 8 weeks of telmisartan treatment (Figure 2f). Thus, we found that the telmisartan treatment can improve systemically increased oxidative stress and reduce SOD antioxidant defense capacity in microalbuminuric type 2 diabetic patients.

Changes in oxidative stress markers by crossover treatment with telmisartan and losartan in type 2 diabetic patients with microalbuminuria

To compare the antioxidative effects of telmisartan with those of other ARBs, we performed a crossover treatment with telmisartan and losartan in type 2 diabetic patients with microalbuminuria, and investigated changes in oxidative stress markers. Table 3 shows clinical and biochemical parameters at the end of each 8-week treatment period: telmisartan 40 mg per day (first period), losartan 50 mg per day (second period) and telmisartan 40 mg per day (third period). There were no significant differences in body mass index, blood pressure, plasma glucose or serum lipid levels between the three

Table 2 Changes in clinical parameters after 8 weeks of telmisartan treatment in type 2 diabetic patients with microalbuminuria

	Baseline	*Telmisartan 8W*
n	12	
Age (years)	64±7	
Gender (male/female)	6/6	
Body mass index ($kg\,m^{-2}$)	24.8±2.6	24.8±2.6
Systolic blood pressure (mm Hg)	138±5	127±10*
Diastolic blood pressure (mm Hg)	73±7	69±7†
Fasting plasma glucose ($mg\,dl^{-1}$)	116±12	115±13
HbA1c (%)	7.2±0.7	7.2±0.7
LDL-cholesterol ($mg\,dl^{-1}$)	97.1±24.2	92.7±26.3
HDL-cholesterol ($mg\,dl^{-1}$)	54.7±8.8	56.5±7.9
Triglyceride ($mg\,dl^{-1}$)	130.8±66.7	121.4±61.5
Serum creatinine ($mg\,dl^{-1}$)	0.71±0.16	0.70±0.14
Urinary albumin ($mg\,g^{-1}$ creatinine)	110.5±72.5	66.8±59.7*

Abbreviations: HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
Data were presented as means ± s.d. $^{*}P<0.001$, $^{\dagger}P<0.01$ *vs.* baseline.

Table 3 Changes in clinical parameters by crossover treatment with telmisartan and losartan in type 2 diabetic patients with microalbuminuria

	First period telmisartan 8W	*Second period losartan 8W*	*Third period telmisartan 8W*
n	12		
Age (years)	68±6		
Gender (male/female)	5/7		
Body mass index ($kg\,m^{-2}$)	23.9±3.7	23.7±3.8	23.8±3.8
Systolic blood pressure (mm Hg)	126±12	132±13	128±11
Diastolic blood pressure (mm Hg)	69±8	75±8	71±7
Fasting plasma glucose ($mg\,dl^{-1}$)	113±13	119±17	115±17
HbA1c (%)	6.5±0.8	6.6±0.9	6.6±0.9
LDL-cholesterol ($mg\,dl^{-1}$)	107.9±24.7	107.9±24.5	110.0±24.5
HDL-cholesterol ($mg\,dl^{-1}$)	59.8±14.7	58.6±14.6	58.2±13.5
Triglyceride ($mg\,dl^{-1}$)	86.3±38.4	72.4±25.2	79.7±22.7
Serum creatinine ($mg\,dl^{-1}$)	0.65±0.19	0.61±0.15	0.62±0.15
Urinary albumin ($mg\,g^{-1}$ creatinine)	68.4±38.5	86.0±53.1*	73.5±49.3†

Abbreviations: HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
Data were presented as means±s.d. *$P<0.05$ *vs.* first period; †$P<0.05$ *vs.* second period.

stood. Recent experimental studies have shown that telmisartan attenuates oxidative stress by downregulating NAD(P)H oxidase, a major superoxide-producing enzyme.[22,23] By contrast, the effect of telmisartan on a superoxide-scavenging enzyme, SOD, has not been clarified. Therefore, we next tested whether telmisartan treatment ameliorates the reduced SOD antioxidant defense capacity in the DN stage of microalbuminuria. Our data clearly demonstrate that telmisartan treatment enhances serum SOD activity and systemically reduces oxidative and nitrosative stress in patients with microalbuminuric DN. Importantly, the increase in serum SOD activity by telmisartan treatment showed a correlation with the reduction of albuminuria in these patients with microalbuminuric DN. This finding indicates that the mechanism by which telmisartan provides renoprotective effects may involve improvement of the SOD antioxidant defense capacity. Considering the present results along with recent compelling evidence, it is likely that telmisartan exerts antioxidative effects by modulating both superoxide-producing and superoxide-scavenging enzymes.

Angiotensin II has been shown to promote superoxide generation through NAD(P)H oxidase activation, independently of its systemic vasoconstriction ability.[24,25] Therefore, blockade of the angiotensin II signaling pathway via AT1 receptors is expected to reduce the superoxide-induced oxidative stress. Although all ARBs may share the antioxidative effects to some extent, there seems to be a difference

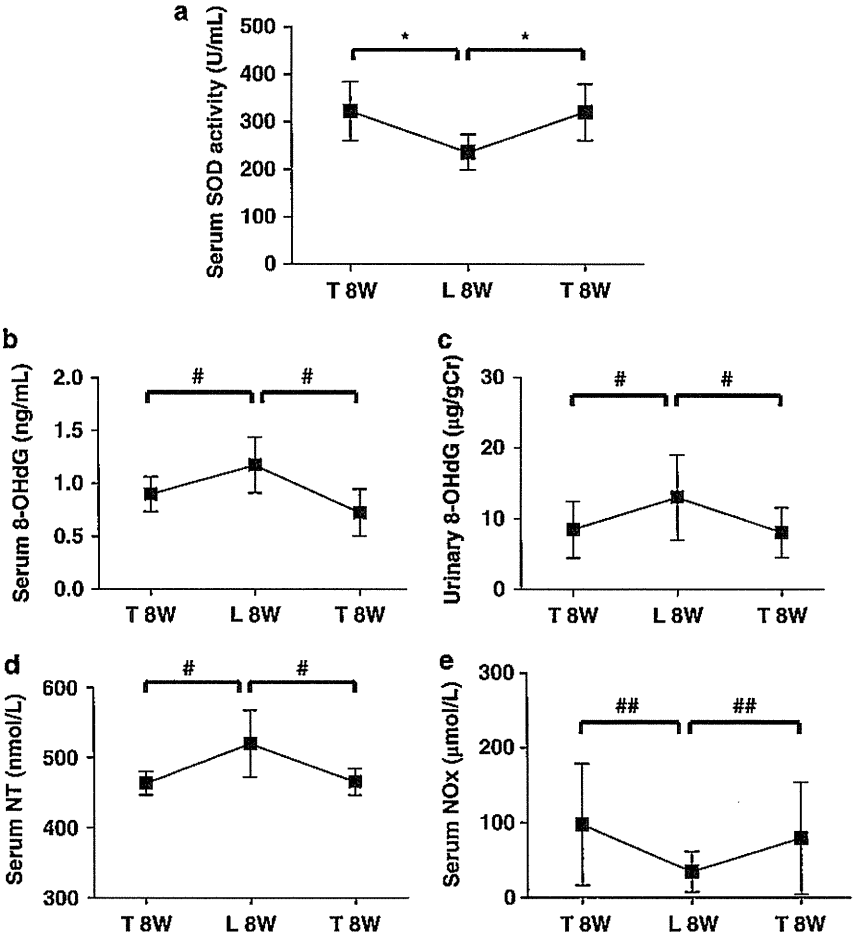

Figure 3 Changes in oxidative stress markers by crossover treatment with telmisartan and losartan in type 2 diabetic patients with microalbuminuria. (a) serum SOD activity; (b) serum 8-OHdG; (c) urinary 8-OHdG; (d) serum NT; (e) serum NOx. Data are presented as the means±s.d. ($n=12$). *$P<0.01$, #$P<0.001$, ##$P<0.05$. T 8W, telmisartan treatment for 8 weeks; L 8W, losartan treatment for 8 weeks; NT, nitrotyrosine.

levels with cardiovascular disease and modulation by statin therapy. *JAMA* 2003; **289**: 1675–1680.
18 Cosentino F, Hishikawa K, Katusic ZS, Luscher TF. High glucose increases nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. *Circulation* 1997; **96**: 25–28.
19 Fujita H, Fujishima H, Chida S, Takahashi K, Qi Z, Kanetsuna Y, Breyer MD, Harris RC, Yamada Y, Takahashi T. Reduction of renal superoxide dismutase in progressive diabetic nephropathy. *J Am Soc Nephrol* 2009; **20**: 1303–1313.
20 Galle J, Schwedhelm E, Pinnetti S, Boger RH, Wanner C. Antiproteinuric effects of angiotensin receptor blockers: telmisartan versus valsartan in hypertensive patients with type 2 diabetes mellitus and overt nephropathy. *Nephrol Dial Transplant* 2008; **23**: 3174–3183.
21 Makino H, Haneda M, Babazono T, Moriya T, Ito S, Iwamoto Y, Kawamori R, Takeuchi M, Katayama S. Prevention of transition from incipient to overt nephropathy with telmisartan in patients with type 2 diabetes. *Diabetes Care* 2007; **30**: 1577–1578.
22 Sugiyama H, Kobayashi M, Wang DH, Sunami R, Maeshima Y, Yamasaki Y, Masuoka N, Kira S, Makino H. Telmisartan inhibits both oxidative stress and renal fibrosis after unilateral ureteral obstruction in acatalasemic mice. *Nephrol Dial Transplant* 2005; **20**: 2670–2680.
23 Takaya T, Kawashima S, Shinohara M, Yamashita T, Toh R, Sasaki N, Inoue N, Hirata K, Yokoyama M. Angiotensin II type 1 receptor blocker telmisartan suppresses superoxide production and reduces atherosclerotic lesion formation in apolipoprotein E-deficient mice. *Atherosclerosis* 2006; **186**: 402–410.
24 Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res* 1994; **74**: 1141–1148.
25 Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA, Griendling KK, Harrison DG. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J Clin Invest* 1996; **97**: 1916–1923.
26 Kakuta H, Sudoh K, Sasamata M, Yamagishi S. Telmisartan has the strongest binding affinity to angiotensin II type 1 receptor: comparison with other angiotensin II type 1 receptor blockers. *Int J Clin Pharmacol Res* 2005; **25**: 41–46.
27 Burnier M. Telmisartan: a different angiotensin II receptor blocker protecting a different population? *J Int Med Res* 2009; **37**: 1662–1679.
28 Yano Y, Hoshide S, Ishikawa J, Noguchi C, Tukui D, Takanori H, Tada M, Kanemaru Y, Yano A, Ishikawa S, Shimada K, Kario K. The differential effects of angiotensin II type 1 receptor blockers on microalbuminuria in relation to low-grade inflammation in metabolic hypertensive patients. *Am J Hypertens* 2007; **20**: 565–572.
29 Dobrian AD, Schriver SD, Khraibi AA, Prewitt RL. Pioglitazone prevents hypertension and reduces oxidative stress in diet-induced obesity. *Hypertension* 2004; **43**: 48–56.
30 Tessari P, Cecchet D, Cosma A, Vettore M, Coracina A, Millioni R, Iori E, Puricelli L, Avogaro A, Vedovato M. Nitric oxide synthesis is reduced in subjects with type 2 diabetes and nephropathy. *Diabetes* 2010; **59**: 2152–2159.
31 Prabhakar S, Starnes J, Shi S, Lonis B, Tran R. Diabetic nephropathy is associated with oxidative stress and decreased renal nitric oxide production. *J Am Soc Nephrol* 2007; **18**: 2945–2952.
32 Kanetsuna Y, Takahashi K, Nagata M, Gannon MA, Breyer MD, Harris RC, Takahashi T. Deficiency of endothelial nitric-oxide synthase confers susceptibility to diabetic nephropathy in nephropathy-resistant inbred mice. *Am J Pathol* 2007; **170**: 1473–1484.
33 Wago T, Yoshimoto T, Akaza I, Tsuchiya K, Izumiyama H, Doi M, Hirata Y. Improvement of endothelial function in patients with hypertension and type 2 diabetes after treatment with telmisartan. *Hypertens Res* 2010; **33**: 796–801.