A new study examined skin biopsy specimens using the immunoperoxidase technique to estimate CML and RAGE content. It was found that the perineurium of peripheral nerves and the *vasa nervorum* in the dermis were more intensely stained for CML, while stronger RAGE immunoreactivity was observed in the bundles of axons and *vasa nervorum* in type 1 diabetic patients receiving kidney transplantation alone, compared to those receiving a successful islet-after-kidney transplantation [87]. Pentosidine levels were also increased in cytoskeletal and myelin protein extracts of human sural nerves [38]. More recently, pronounced AGE immunoreactivity was detected in the axons and myelin sheaths of 90% of type 2 diabetes patients, while no AGE immunoreactivity was detected in control subjects; the intensity of the axonal AGE immunoreactivity was correlated with the severity of the structural changes that are characteristic of diabetic neuropathy, such as perineurial thickening, microvascular luminal narrowing, and axonal loss [88].

SERUM AND SKIN AGES RELATED TO SEVERITY OF HUMAN DIABETIC NEUROPATHY

Accumulation of AGEs in the skin, serum, and other specimens obtained from diabetic patients has been linked to the progression of microvascular complications, including diabetic neuropathy. Earlier studies using gas chromatography-mass spectrometry measured the levels of glycoxidation products such as CML and/or pentosidine in collagen obtained from skin-punch biopsies and demonstrated that the levels of these glycoxidation products were correlated with the severity of nephropathy, retinopathy, and vasculopathy in type 1 diabetic patients [89-91]. A more recent cross-sectional study involving 50 patients with type 2 diabetes measured AGE levels in skin, serum, saliva, and urine using spectrofluometry HPLC; the AGE levels in all specimens except for the urine increased as the patients' neuropathy, retinopathy, and nephropathy progressed [92]. Another recent study [93] measured serum CML levels using enzyme-linked immunosorbent assay with a monoclonal anti-CML antibody (6D12) in 94 type 1 diabetic children and adolescents with or without background retinopathy, microalbuminuria or neuropathy; CML levels were higher in patients with chronic complications than in patients without complications. Taking for granted the fact that CML formation depends on the oxidative condition, the increased CML levels found in the serum and tissue proteins of diabetic patients suggest that CML has a role as an endogenous biomarker of oxidative damage. Meerwaldt el al. [94] developed an AutoFluorescence Reader, a noninvasive method that uses fluorescence properties to specifically measure skin AGE contents. Using this apparatus, they assessed skin AGE accumulation in 24 diabetic patients with a history of foot ulceration, in 23 diabetic patients without clinical neuropathy, and in 21 control subjects; they found that the skin autofluorescence, which reflected tissue AGE accumulation, increased during early stages of diabetic neuropathy and was correlated with the severity of peripheral and autonomic nerve abnormalities, as well as with the presence of foot ulceration [11]. In addition, in a large group of type 2 diabetic patients, the same group confirmed that skin autofluorescence was higher in patients than in age-matched control subjects and was associated with the severity of diabetes-related complications [95]. They proposed that measurement of skin autofluorescence is a rapid and helpful tool that can be used in diabetes outpatient clinics to identify patients who are at risk for developing complications.

EFFFECTS OF ANTI-AGE AGENTS ON DIABETIC NEUROPATHY

Aminoguanidine is a highly reactive nucleophilic reagent that prevents the formation of AGEs by reacting with the carbonyl groups of reducing sugars, as well as alpha- and beta-dicarbonyl compounds such as methylglyoxal, glyoxal, and 3-deoxyglucosone. Aminoguanidine was the most promising agent for preventing AGE-mediated tissue damage caused by diabetes. In fact, initial rat studies confirmed the beneficial effect that aminoguanidine had on the development of retinopathy, nephropathy, and neuropathy. In particular, long-term aminoguanidine treatment improved the nerve conduction deficit and myelinated fiber pathology in diabetic rats [96]. Impaired nerve blood flow in diabetic rats was also normalized after aminoguanidine treatment, but such treatment had no effect on oxygen free radical activity [97]; this suggests that the hemodynamic changes were modulated without affecting oxidative stress. Short-term treatment with aminoguanidine ameliorated nerve conduction slowing and Na^+/K^+-ATPase defects, but not endothelial damage, as reflected by systemic thrombomodulin concentrations [98]. Treating diabetic rats with aminoguanidine improved endoneurial blood flow but not the impairment of endothelium-dependent vasodilation of epineurial vessels of the sciatic nerve [99,100]. The beneficial effects that aminoguanidine had on the structural alterations of endoneurial microvessels were also documented in long-term diabetic rats [101]. In contrast to these rat studies, in type 1 diabetic baboons who had diabetes for less than 5 years, treatment with aminoguanidine for 3 years did not affect impaired nerve conduction velocity, heart rate response, and myelinated fiber pathology. This suggests that, in the type 1 diabetic primate, the accumulation of AGEs is involved only in nerve damage that occurs after a more prolonged observation period [102].

A double-blinded, multiple-dose, placebo-controlled, randomized clinical trial of aminoguanidine in diabetic patients with overt diabetic nephropathy (ACTION) was completed in 1998; ACTION I involved 690 type 1 diabetic patients, and ACTION II involved 599 type 2 diabetic patients. These studies were designed to evaluate the safety and efficacy of aminoguanidine in slowing the rate of progression of renal disease in patients with overt diabetic nephropathy. The primary endpoint was doubling of the baseline serum creatinine. In ACTION I, patients received placebo, high-dose (100-600 mg per day) aminoguanidine, or low-dose (50-300 mg per day) aminoguanidine; the combined aminoguanidine dose group showed decreased progression of diabetic retinopathy and lower triglyceride, LDL cholesterol, and urinary protein levels, as well as a non-significant trend towards a slower doubling of serum creatinine. However, ACTION II was terminated prematurely due to safety concerns and apparent lack of efficacy. Reported side effects of aminoguanidine included gastrointestinal disturbance, liver function test abnormalities, flu-like symptoms, and a rare vasculitis [103]. Based on the outcomes and the side effects noted in these trials, there is no benefit in using aminoguanidine.

Other anti-AGE agents, including the thiazolidine derivative OPB-9195, have been investigated; OPB-9195 has been shown to prevent the progression of diabetic nephropathy in rats [104]. It has also been shown to improve motor nerve conduction slowing without affecting body weight and blood glucose levels in diabetic rats; the improvement was associated with reduced serum AGE levels and peripheral nerve expression of immunoreactive AGE and immunoreactive 8-hydroxy-2'-deoxyguanosine, which is a marker for oxidative stress-related DNA damage, as well as an increase in peripheral nerve (Na^+, K^+)-ATPase activity [105]. An alternative approach is to reduce tissue AGE accumulation by selectively cleaving the resultant AGE crosslinks. Diabetic rats were found to have increased mesenteric vascular AGE accumulation and mesenteric vascular hypertrophy; both of these were prevented by treatment with N-phenacylthiazolium bromide (PTB), which is a prototypic AGE crosslink breaker that attacks covalent carbon-carbon bands of dicarbonyl-derived crosslinks both *in vitro* and *in vivo* [106]. However, a more recent study has demonstrated that although AGE-breakers such as PTB and N-phenacyl-4,5-dimethylthiazolium cleave model crosslinks *in vitro*, they do not significantly cleave AGE crosslinks formed *in vivo* in skin collagen of diabetic rats [107]. Clinical trials of ALT-711, a novel AGE breaker, have shown favorable results with respect to blood pressure and vascular elasticity in aged persons with stiffened vascula-

ture [108], but treatment with ALT-711 for 2 weeks had no effects on motor nerve conduction deficit, C-fiber-mediated nociceptive dysfunction, or impaired pressure-induced vasodilation in diabetic mice after 8 weeks of diabetes [109]. Interestingly, benfotiamine, a lipophilic analogue of thiamine, is a transketolase activator that inhibits three of the four major biochemical pathways implicated in the "unifying hypothesis" of the pathogenesis of hyperglycemia-induced vascular damage: the hexosamine pathway, PKC activation, and AGE formation [110] (Fig. **1**). It has higher bioavailability after oral administration and normalizes cell replication, lactate production, and AGE formation in human umbilical vein cells and bovine retinal endothelial cells cultured in high glucose concentrations [111]. In diabetic rats, the efficacy of benfotiamine with respect to peripheral nerve function and AGE accumulation has been documented, with nearly normalized nerve conduction velocity and inhibition of neural imidazole-type AGE and CML formation after 6 months of benfotiamine treatment [112]. In nondiabetic and diabetic rats, benfotiamine also reduced inflammatory and neuropathic nociception [113]. Therefore, the ability of benfotiamine to inhibit three major pathways simultaneously might be clinically useful in preventing the development and progression of diabetic complications, including neuropathy [114,115]. Finally, the formation of AGEs can also be limited by inhibitors such as pyridoxamine, tenilsetam, and 2,3-diaminophenazone, but their efficacy for treating diabetic neuropathy still remains to be explored [116].

SUMMARY

Taken together, these results indicate that accumulation of AGEs and their binding to RAGE play a key role in the pathogenesis of experimental diabetic neuropathy with increased generation of reactive oxygen species, proinflammatory cytokines, and adhesion molecules, and oxidative stress associated with activation of the NF-kappaB signaling pathway. In addition to polyol pathway hyperactivity, AGE-RAGE-mediated cellular oxidative stress further enhances the accumulation of glycoxidation products such as CML and pentosidine. Although several AGEs have been identified that accumulate in the peripheral nerve of diabetic humans and animals, in diabetic patients the accumulation of AGEs is evident in undamaged axons, Schwann cells, and *vasa nervorum* [36]. Thus, a direct linkage between AGE accumulation and nerve injury that eventually leads to progressive damage and loss of unmyelinated and myelinated nerve fibers is still lacking in the human diabetic nerve. Furthermore, no agents are in current clinical use to block AGE/RAGE signaling in diabetic patients. Therefore, further studies are needed to explore the precise mechanisms that underlie AGE-induced nerve injury and to establish the optimal therapeutic strategy for AGE/RAGE signaling blockade in human diabetic neuropathy.

ACKNOWLEDGMENTS

This review was supported by a grant-in-aid from the Japan Society for the Promotion of Science (to K. Sugimoto; grant no. 18590520). We are grateful to Mrs. Akiko Tamura for her assistance in preparing the manuscript.

ABBREVIATIONS

AGEs	=	Advanced glycation end products
PKC	=	Protein kinase C
CML	=	N-epsilon-(carboxymethyl)lysine
RAGE	=	Receptor for AGEs
GSH	=	Reduced glutathione
MAPK	=	Mitogen-activated protein kinase
TNF	=	Tumor necrosis factor
NF-kappaB	=	Nuclear factor-kappaB
NAD(P)H	=	Reduced nicotinamide adenine dinucleotide (phosphate)
ACTION	=	Aminoguanidine in overt diabetic nephropathy
PTB	=	Phenacylthiazolium bromide

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JOURNAL OF NEUROCHEMISTRY | 2008 | 104 | 491–499 doi: 10.1111/j.1471-4159.2007.05050.x

Correction of protein kinase C activity and macrophage migration in peripheral nerve by pioglitazone, peroxisome proliferator activated-γ-ligand, in insulin-deficient diabetic rats

Shin-Ichiro Yamagishi, Saori Ogasawara, Hiroki Mizukami, Nobuhisa Yajima, Ryu-ichi Wada, Akiko Sugawara and Soroku Yagihashi

Department of Pathology and Molecular Medicine, Hirosaki University School of Medicine, Hirosaki, Japan

Abstract

Pioglitazone, one of thiazolidinediones, a peroxisome proliferator-activated receptor (PPAR)-γ ligand, is known to have beneficial effects on macrovascular complications in diabetes, but the effect on diabetic neuropathy is not well addressed. We demonstrated the expression of PPAR-γ in Schwann cells and vascular walls in peripheral nerve and then evaluated the effect of pioglitazone treatment for 12 weeks (10 mg/kg/day, orally) on neuropathy in streptozotocin-diabetic rats. At end, pioglitazone treatment improved nerve conduction delay in diabetic rats without affecting the expression of PPAR-γ. Diabetic rats showed suppressed protein kinase C (PKC) activity of endoneurial membrane fraction with decreased expression of PKC-α. These alterations were normalized in the treated group. Enhanced expression of phosphorylated extracellular signal-regulated kinase detected in diabetic rats was inhibited by the treatment. Increased numbers of macrophages positive for ED-1 and 8-hydroxydeoxyguanosine-positive Schwann cells in diabetic rats were also corrected by the treatment. Pioglitazone lowered blood lipid levels of diabetic rats, but blood glucose and nerve sorbitol levels were not affected by the treatment. In conclusion, our study showed that pioglitazone was beneficial for experimental diabetic neuropathy via correction of impaired PKC pathway and proinflammatory process, independent of polyol pathway.

Keywords: diabetic neuropathy, peroxisome proliferator activated receptor-γ, proinflammatory process, protein kinase C, thiazolidinedione.

J. Neurochem. (2008) **104**, 491–499.

Neuropathy is a common complication of diabetes that affects nearly 50% of diabetic patients (Shaw and Zimmet 1999; Vinik and Mehrabyan 2004). Most of the patients suffer from intractable signs and symptoms like pain, paresthesia as well as severe autonomic failure, eventually resulting in shortened life expectancy (Vinik and Mehrabyan 2004; Boulton *et al.* 2005). Satisfactory treatments are yet to be available in spite of numerous clinical trials, due in part to the fact that the pathogenesis of diabetic neuropathy is yet to be clear. Polyol pathway hyperactivity, increased non-enzymatic glycation, excessive oxidative stress, and altered protein kinase C (PKC) activity are all invoked in its pathogenesis (Yagihashi 1995; Sima and Sugimoto 1999; Vinik and Mehrabyan 2004). Recent studies disclosed involvement of PKC-mitogen activated protein (MAP)-kinase and nuclear transcription factors such as nuclear factor kappa B (NF-κB) in the injurious process of diabetic neuropathy (Tomlinson 1999; Bierhaus *et al.* 2004; Vincent and Feldman 2004). Impaired cell signaling from these factors related to cell function and structure is now regarded to be a promising target for an effective treatment of diabetic neuropathy (He and King 2004; Obrosova *et al.* 2004).

Peroxisome proliferator-activated receptor is a nuclear receptor that activates cellular metabolism leading to cell growth and differentiation (Murphy and Holder 2000; Hihi *et al.* 2002). Thiazolidine-derivative, thiazolidinedione, is a

Received June 15, 2007; revised manuscript received September 7, 2007; accepted September 11, 2007.

Address correspondence and reprint requests to Dr Soroku Yagihashi, Department of Pathology and Molecular Medicine, Graduate School of Medicine, Hirosaki University, 5 Zaifu-cho, Hirosaki 036-8562, Japan. E-mail: yagihasi@cc.hirosaki-u.ac.jp

Abbreviations used: 8OHdG, 8-hydroxydeoxyguanosine; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; MNCV, motor nerve conduction velocity; NCV, nerve conduction velocity; NF-κB, nuclear factor kappa B; pERK, phosphorylated ERK; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptor; SNCV, sensory nerve conduction velocity; STZ, streptozotocin.

potent ligand to this receptor and is shown to improve insulin sensitivity, thus being used as an anti-diabetic agent for type 2 diabetes. The ligand to PPAR-γ is also known to have anti-inflammatory and antioxidant effects and found to be beneficial for macrovascular complications in diabetes (Hihi *et al.* 2002; Verrier *et al.* 2004). In this setting, it has been proposed that thiazolidinedione modulates cell activity or cell survival through regulation of key molecules of cell signaling like NF-κB (Duez *et al.* 2001; Verrier *et al.* 2004).

In our previous survey, diabetic patients treated with troglitazone, a prototype of PPAR-γ ligand, although now retreated from the market because of its hepatotoxicity, showed less symptoms of neuropathy compared with non-treated group (Satoh *et al.* 2003). It has also been reported that PPAR-γ-ligand ameliorates peripheral nerve function and structure in diabetic animal models but precise mechanisms why they improved neuropathic changes are not well clarified (Qiang *et al.* 1998; Shibata *et al.* 2000). In this study, we therefore explored the effects of pioglitazone, PPAR-γ-ligand, on the development of experimental diabetic neuropathy and attempt to identify factors involved in this process.

Materials and methods

Animals

Male Wistar strain rats 8 weeks of age were made diabetic by intravenous injection of streptozotocin (STZ) (Sigma Co. Ltd., St Louis, MO, USA) (40 mg/kg, i.v.) diluted with 0.1 mol/L citrate buffer. Diabetes was identified by continuous glycosuria and high blood glucose exceeding 14 mmol/L 1 week after STZ injection. Thereafter, a group of diabetic rats was treated orally with pioglitazone (10 mg/kg/day) (kindly donated from Takeda Co., Osaka, Japan) and followed for 12 weeks. All the animals were monitored with body weight and blood glucose during the experimental period. One day before killing, motor and sensory nerve conduction velocities (MNCVs and SNCVs) were examined on the left lower limb. At the time of killing under anesthesia with pentobarbital (Abbot Co., Chicago, IL, USA), blood was withdrawn from the right atrium and centrifuged at 1500 *g* for analysis of blood glucose and lipid levels by autoanalyzer. The sciatic nerve was extirpated for biochemical and immunohistochemical analyses. A portion of the sciatic nerve was frozen for the measurements of sorbitol, fructose, and PKC activity as well as protein expressions of various PKC isoforms, MAP-kinase, and PPAR isoforms. Remaining nerve was fixed in formalin solution for structural examinations.

All animal experimentations followed the Guideline for Animal Experimentation of Hirosaki University (Approval number M0015). The protocol of investigation also conformed to the Guide for the Care and Use of Laboratory Animals as published by NIH (NIH Publication No. 85-23, revised 1996).

Nerve conduction velocity

All animals were anesthetized with isoflurane (Abbot Co.) and placed on a thermostatically controlled heated mat to maintain body temperature at 37°C. The temperature near the sciatic nerve was also kept constant at 37°C by monitoring with an electronic thermometer (PC-9400 Delta; Sato Keiryoki MFG, Tokyo, Japan) with the aid of a warmed blanket.

For MNCV, the left sciatic-tibial nerve was electrically stimulated first at the site of ankle using needle electrodes (MS92 electromyogram device; Medelec, London, UK) and then at the site of sciatic notch and the waves were recorded from the second interosseous muscle of the foot (Yagihashi *et al.* 2001). Supramaximal electrical stimulation of 0.1 ms pulses was identified as the period of latency. The latency differences derived from two stimulating sites were divided by the distance between the stimulating sites, yielding the value of MNCV. For detection of SNCV, the digital nerve was stimulated first at the interdigital metatarsal site and then at the site of the ankle. The initial deflection point of H-reflex was identified as the latency for SNCV. The difference of proximal and distal latency was divided by the distance between the stimulating sites, yielding SNCV. An average of at least five recordings for each was used for measurements.

Tissue carbohydrate levels

Tissue accumulation of sorbitol and fructose was measured in nerve homogenates by HPLC as previously described (Yagihashi *et al.* 2001). Briefly, nerve tissues were homogenized in distilled water. Tricyclic acid and internal standard (D-sorbitol and D-fructose) were added to the homogenate, followed by centrifugation at 10 000 *g* for 5 min at 4°C to obtain the supernatant fraction. To remove tricyclic acid, the supernatant fraction was washed with ethyl ether. Sorbitol and fructose in the supernatant fraction were converted to sorbitol acetate derivative and fructose acetate derivative for the measurement by HPLC (HP1050, Hewlett Packard, Palo Alto, CA, USA) and mass spectrometry (TSQ; Finnigan Mat, San Jose, CA, USA) using a Cadenza CD-C18 COLUMN (75 × 2.0 mm, internal diameter 3 mm; Imtakt, Kyoto, Japan).

Preparation for protein expressions in peripheral nerve

Desheathed nerve tissues were transferred to a tube containing 1.0 mL homogenization buffer [20 mmol Tris–HCl (pH 7.5), 330 mmol sucrose, 0.5 mmol EGTA, 2 mmol EDTA, 2 μg/mL aprotinin, 25 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride] and homogenized with a Polytron. Homogenate was centrifuged at 50 000 *g* for 30 min at 4°C. The pellet was resuspended in 0.6 mL homogenization buffer containing 1% Triton X-100 and stored on ice for 1 h. The protein expression of PPAR, PKC, and MAP-kinase was examined by western blot analysis using each specific antibody. For comparison of PPAR isoform expressions, liver and subcutaneous fatty tissues were also treated in a similar way.

Protein kinase C activity

Protein kinase C activities were assayed by the method described previously (Yagihashi *et al.* 2001). Excised nerve was homogenized in 1.0 mL of the same buffer as used for other protein expressions and centrifuged at 50 000 *g* for 30 min at 4°C. Supernatant was collected and used as cytosolic fraction. The pellet was resuspended in 0.6 mL homogenization buffer containing 1% Triton X-100 and stored on ice for 1 h. Resuspended solution was centrifuged at 50 000 *g* for 30 min at 4°C, after which supernatant was used as membrane fraction. Phosphorylation assay was carried out in a

reaction mixture [20 mmol Tris pH 7.5, 1 mmol $CaCl_2$, 10 mmol $MgCl_2$, 33 μmol octapeptide (RKRTLRRL), 5 mmol EGTA, and 10 μmol γ-^{32}P-ATP (5–10 × 105 cpm) (Perkin Elmer Life Sciences, Boston, MA, USA) in the presence or absence of 6.4 μg/mL diorein and 96 μg/mL phosphatidylserine. The reaction was started by the addition of 30 μL cytosol or membrane fraction, incubated at 30°C for 10 min and terminated by the spotting the reaction mixture onto P-81 paper (Whatman; Maidstone, Kent, UK). P-81 paper was washed by 75 mmol phosphate buffer four times for 15 min. Radioactivity was counted by liquid scintillation spectrometer (Aloka, Tokyo, Japan).

Western blot analysis

Western blot analysis was performed using supernatant proteins of nerve homogenates for PPAR isoforms and MAP-kinase and those that were extracted as cytosol and membrane fraction for PKC isoforms. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed using the Xcell SureLock system (Invitrogen, San Diego, CA, USA) in the reducing condition. Aliquots of 100-μg samples of protein were dissolved in the sample buffer [2.5% 2-mercaptoethanol, 62.5 mmol Tris–HCl, 10% glycerol, 2% sodium dodecyl sulfate, 0.0025% bromophenol blue, 50 mmol reducing agent (dithiothreitol), pH 6.8] and loaded onto the Novex Tris–glycine PreCast Gel (Invitrogen). After completion of the migration, the proteins were transferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore, Bedford, MA, USA) in a transfer buffer (25 mmol Tris, 0.2 mol/L glycine, and 20% methanol) using a wet transfer unit of Xcell SureLock system. For blocking, blot membranes were incubated with 5% skimmed milk in phosphate buffered saline–Triton X-100 (137 mmol NaCl, 2.7 mmol KCl, 1.5 mmol KH_2PO_4, 8.0 mmol Na_2HPO_4, pH 7.4, and 1% Triton-X 100) for overnight at 4°C. After washing with phosphate buffered saline–Triton X-100 membrane was incubated with antibodies to PPAR-α (Santa Cruz BioTech Inc., Santa Cruz, CA, USA), PPAR-γ (RDI, Flanders, NJ, USA), extracellular signal-regulated kinase (ERK)-I and II (Santa Cruz BioTech Inc.), phosphorylated ERK (pERK)-I and -II (Santa Cruz BioTech Inc.), JNK-I (Santa Cruz BioTech Inc.), PKC-α, -βI, and -βII specific antibodies (Santa Cruz BioTech Inc.), and β-actin (Santa Cruz BioTech Inc.) for 1 h at 25°C. The dilution of all antibodies was 1 : 1000. A final incubation was carried out with peroxidase-conjugated anti-rabbit or anti-goat IgG (Santa Cruz BioTech Inc.) for 45 min at 25°C. Immunodetection was performed by ECL system (Amersham-Pharmacia, Buckinghamshire, UK). Quantitative analysis of exposed films was performed using NIH image software (version 1.61; Bethesda, MD, USA).

Immunohistochemical evaluation

For immunohistochemical analysis, 4-μm thick transverse sections of formalin-fixed nerve tissues were deparaffinized and pre-treated with methanol containing 0.3% H_2O_2 to eliminate endogenous peroxidase activity. Antibodies to PPAR-γ (RDI) and PPAR-α (Santa Cruz BioTech Inc.), as well as PKC-α, -βI, -βII antibodies (Santa Cruz BioTech Inc.) were applied to the sections overnight at 4°C. To demonstrate the migration of macrophage, antibody to specific rat macrophage (CD68, clone ED1) (Dako Cytomation, Tokyo, Japan) was used. The effect of oxidative stress-induced DNA damage was examined by detection of 8-hydroxydeoxyguanosine (8OHdG) using antibody to 8OHdG (Nihon Yushi, Jika, Shizuoka, Japan) (*Wada et al. 2001*). After the application of first antibodies, the sections were incubated with secondary and tertiary agents using a streptavidin-biotin-peroxidase detection kit (Histofine SAB-PO Kit; Nichirei, Tokyo, Japan). *N,N'*-diaminobenzidine was used to visualize peroxidase deposition at the antigenic sites, and these sections were counterstained with hematoxylin. Specificity of the staining was confirmed by (i) omission of the first antibody during the process of the immunostaining, (ii) replacement of the first antibody by excessive antigens, (iii) absorption of the antibody by non-immune rabbit sera, and (iv) absorption of the antibody by excessive antigens of PKC-α, -βI, and -βII. Some nerve samples from groups of rats were distorted during tissue sampling and therefore discarded for the structural investigations.

Population of macrophage and 8OHdG-positive cells

For the evaluation of 8OHdG-positive and ED-1 positive cells, transverse sections of the sciatic nerve stained immunohistochemically were incorporated into NIH image analysis. Positive cells for 8OHdG and ED-1 were identified as strongly positive reaction which intensity was more than five times compared with background intensity evaluated by NIH image analysis (Wada *et al.* 2001; Wang *et al.* 2005) . The number of macrophages were counted on the sections of individual animals and expressed as cell number per unit area. Only the cells with nuclei were selected.

Statistical analysis

Data were expressed as mean ± SE. Statistical analysis was carried out on a Macintosh computer (Apple Inc., Cupertino, CA, USA) using a commercially available statistical program (StatView, version 4.11J; Huilinks, Tokyo, Japan). Comparison of the values among the groups was carried out using one-way ANOVA, followed by Bonferroni's correction for multiple comparisons. p-values < 0.05 were considered to be significant.

Results

Laboratory data

The laboratory data are summarized in Table 1. At the end of the experiment, average body weight in diabetic group was smaller compared with that in non-diabetic group. Pioglitazone-treatment did not influence these values. Blood glucose concentrations were significantly greater in diabetic group and pioglitazone treatment did not influence the levels. Serum lipid levels of total cholesterol and triglyceride were both elevated in diabetic group compared with those in controls and pioglitazone treatment reduced the levels of triglyceride but not of cholesterol (Table 1).

Nerve conduction velocity

At 12 weeks of diabetes, diabetic group showed 30% reduction of MNCV and 35% reduction of SNCV compared with those in normal control rats, respectively ($p < 0.01$ for both) (Fig. 1). Pioglitazone treatment improved both MNCV

Table 1 Body weight, concentrations of blood glucose, lipids, and nerve carbohydrates in experimental animals

Group	Number of animals (*n*)	Body weight (g)		Blood glucose (nmol/L)		Blood lipids		Nerve sorbitol (nmol/mg protein)	Nerve fructose (nmol/mg protein)
		Initial	End	Initial	End	T-cholesterol (mmol/L)	Triglyceride (mmol/L)		
Control	9	270 ± 9	491 ± 16	6.8 ± 0.4	6.9 ± 0.4	2.26 ± 0.11	2.60 ± 0.31	3.5 ± 0.24	17.9 ± 1.79
Diabetic	10	247 ± 3*	352 ± 13*	30.4 ± 1.2*	27.5 ± 0.1*	2.88 ± 0.11*	3.80 ± 0.63*†	44.0 ± 2.15*	170.2 ± 5.19*
Diabetic + Pioglitazone	7	249 ± 4*	345 ± 18*	30.2 ± 1.3*	27.7 ± 1.2*	2.34 ± 0.08†	1.72 ± 0.24†	48.7 ± 4.21*	162.8 ± 5.12*

Values are mean ± SE. *$p < 0.01$ versus control, **$p < 0.05$ versus control, †$p < 0.05$ versus diabetic, ‡$p < 0.01$ versus diabetic rats. Initial is the point of starting the treatment.

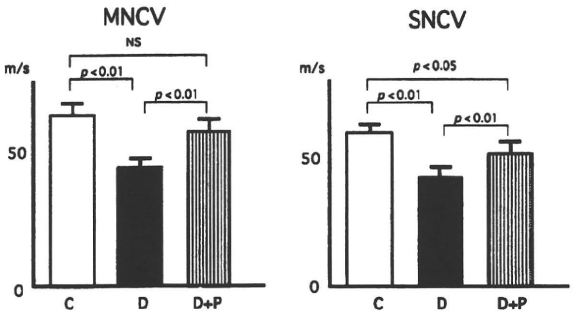

Fig. 1 Nerve conduction studies on experimental animals. There was a significant delay of motor and sensory nerve conduction velocity (MNCV and SNCV) in untreated diabetic rats (D) compared with normal control rats (C). The delay of both MNCV and SNCV was significantly improved by pioglitazone treatment (D + P). The number of animals was nine in control, ten in diabetic, and seven in pioglitazone-treated group.

by 15% ($p < 0.01$ vs. untreated diabetic rats) and SNCV by 25% ($p < 0.01$ vs. untreated diabetic rats).

Nerve carbohydrate metabolites

Nerve sorbitol was increased more than 12-fold in diabetic group compared with normal controls and pioglitazone treatment did not influence the levels (Table 1). Similarly, nerve fructose was increased ninefold in diabetic group compared with controls and again pioglitazone treatment did not influence the level.

Peroxisome proliferator-activated receptor expressions

Immunohistochemistry revealed the localization of PPAR-γ in Schwann cells of myelinated fibers and endothelial cells of endoneurial vessels in non-diabetic control nerves (Fig. 2). There was no positive reaction for PPAR-α. By western blot analyses, while the liver contained both PPAR-α and PPAR-γ, only PPAR-γ was detected in peripheral nerve and subcutaneous fatty tissues (Fig. 2). Expression levels of PPAR were not affected by pioglitazone-treatment.

Protein kinase C alterations

We separated homogenates of the endoneurial tissues into membrane and cytosolic fractions. PKC activity of the membrane fraction was significantly reduced by 35% level in diabetic rats compared with the level of control rats, while pioglitazone-treated group showed a significant recovery of the activity to near normal levels (Fig. 3a). On the other hand, PKC activity of the cytosolic fraction was not significantly altered in diabetic rats but increased in pioglitazone-treated diabetic rats compared with the levels of control and untreated diabetic rats.

Western blot analysis disclosed that PKC-α was reduced in the membrane fraction from diabetic rats, while PKC-α in the cytosolic fraction was increased, although the protein content

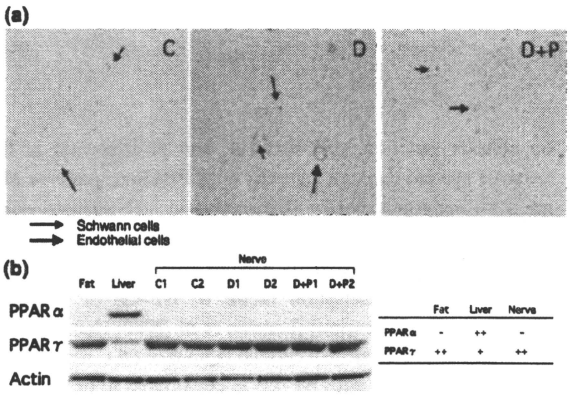

Fig. 2 (a) Expression of PPAR in peripheral nerve tissues and the effect of diabetes. Immunohistochemically, PPAR-γ was specifically expressed on the Schwann cell cytoplasm (arrow) and endothelial cells of endoneurial microvessels (thick arrow) in non-diabetic control nerves. There was no positive reaction of PPAR-α in peripheral nerve tissues (not shown). (b) By western blot analysis, PPAR-γ was expressed in peripheral nerve, liver, and fatty tissue, while PPAR-α was contained only in liver. Diabetic condition did not alter the PPAR-γ expression in the nerve. C, control rats; D, diabetic rats; D + P, pioglitazone-treated diabetic rats (C1, C2, D1, D2, D + P1, and D + P2 represent the individual animal number and P for D + P group).

in the cytosolic fraction was much less than in the membrane fraction (Fig. 3b). Pioglitazone treatment corrected these changes in both membrane and cytosolic fractions. There was an elevated expression of PKC-βII in the membrane fraction in diabetic rats and pioglitazone-treatment improved this change. The membrane and cytosolic fractions of PKC-βI did not alter in diabetic rats and pioglitazone treatment did not affect the expressions. Comparsion of the average values obtained from densitometric analysis among the groups confirmed these changes (Fig. 3c).

Mitogen-activated protein kinase

The expression levels of ERK-I and -II in diabetic rats were comparable with those in normal control rats, whereas the levels of pERK-I and -II were significantly elevated in diabetic rats and this increase was corrected by pioglitazone treatment (Fig. 4). There was no significant alteration of the expression level in JNK-I (data not shown).

Macrophage and 8OHdG-positive cells in peripheral nerve

Immunohistochemistry exhibited only a few endoneurial ED-1-positive macrophages in normal control rats (Fig. 5a). In contrast, diabetic rats showed an abundance of ED-1-positive cells often with vacuolar appearance. Such cells appeared to be less marked in pioglitazone-treated diabetic rats. Similarly, 8OHdG-positive cells were marked in diabetic rats compared with normal control rats and this expression was suppressed in pioglitazone-treated diabetic rats (Fig. 5b). Quantitative analysis confirmed the above findings (Fig. 5c).

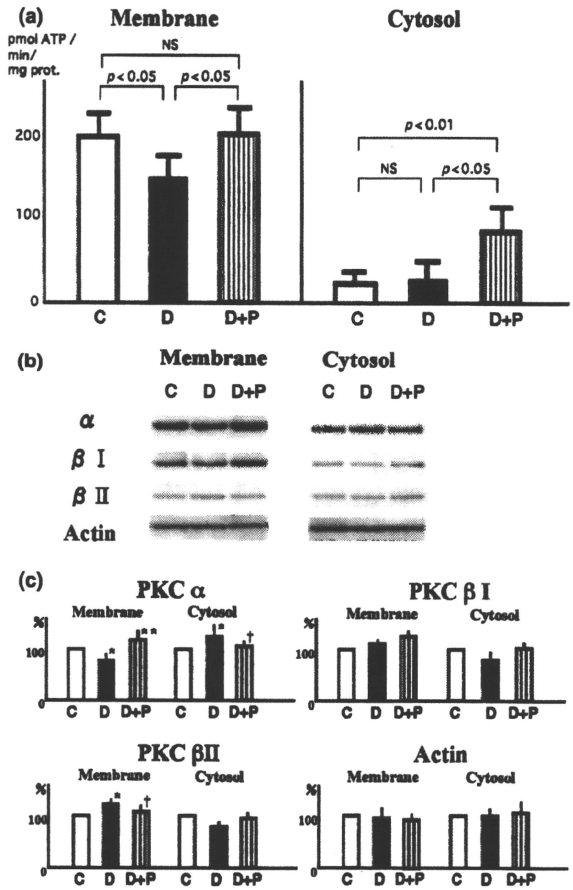

Fig. 3 (a) PKC activity of endoneurial tissues of peripheral nerve in experimental rats. There was significant reduction of membrane PKC activity in diabetic rats (D) compared with normal controls (C). Pioglitazone-treated group (D + P) showed normalization of PKC activity. Cytosolic PKC activity was not significantly altered in diabetic rats but there was elevation of cytosolic PKC activity in pioglitazone-treated diabetic rats. The number of animals was seven in each group. (b) PKC isoform expressions of endoneurial tissues of peripheral nerve in experimental rats. There was a decrease in PKC-α expression in membrane fraction in diabetic rats and pioglitazone treatment corrected this decrease. PKC-α in cytosolic fraction was increased in diabetic rats and pioglitazone treatment corrected this change. There was no significant change in PKC-βI, but an increase in membranous PKC-βII expression in diabetic rats and pioglitazone inhibited this change, whereas there was no change in cytosolic PKC-βII expression. (c) Densitometric analysis shows the average value of each group and bar stands for SE. The number of animals was seven in each group. C, control rats; D, diabetic rats; D + P, pioglitazone-treated diabetic rats. $^{*}p < 0.01$ versus C, $^{**}p < 0.01$ versus D, and $\dagger p < 0.05$ versus D.

Discussion

We have shown that PPAR-γ-ligand, pioglitazone, significantly improved nerve conduction velocity (NCV) in

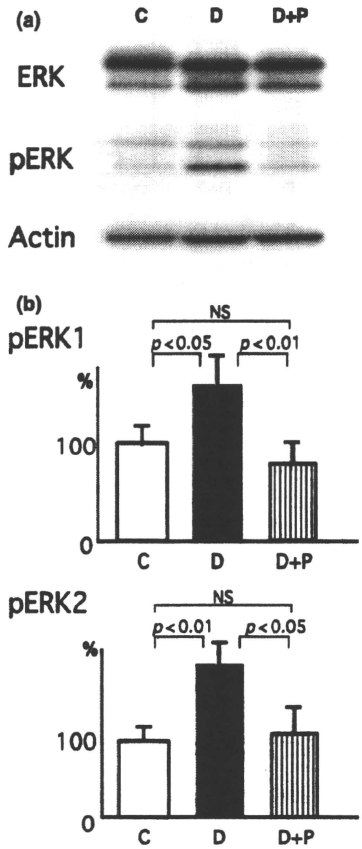

Fig. 4 MAP-kinase expression in experimental rats. There was no significant change in the expression of non-phosphorylated ERK among groups. In contrast, expression of phosphorylated ERK (pERK)-I and -II was enhanced in diabetic rats. Pioglitazone treatment inhibited the elevation of pERK-I and -II. The number of animals was seven in each group. C, control rats; D, diabetic rats; D + P, pioglitazone-treated diabetic rats.

insulin-deficient STZ-diabetic rats, indicating beneficial effects of pioglitazone on experimental diabetic neuropathy. It is of note that the effects were independent of the levels of hyperglycemia or accumulated polyols in nerve tissues but associated with correction of PKC activity and pERK. The recovery of PKC activity was associated with restoration of membrane PKC-α expression. In addition, pioglitazone treatment suppressed the proinflammatory condition possibly elicited by oxidative stress-induced injury and migration of macrophages in diabetic nerve. These results provide a new facet of the multifactorial cause of diabetic neuropathy and a novel direction of the treatment for neuropathic complications of human diabetes.

Thiazolidinedione was originally developed as an anti-diabetic agent acting as an insulin sensitizer to promote adipocyte differentiation and glucose utilization (Olefsky 2000; Evans *et al.* 2004). This agent has pleiotropic effects on cellular function, cell survival, and proliferation in the nervous system through binding with PPAR-responsive element via retinoid receptor (transactivation) (Nishijima *et al.* 2001; Wada *et al.* 2006). It also exerts neuro-glial cells to protect neuronal cell death by suppressing the gene activation to repress the transcription factor of NF-κB or activated protein-1 (transrepression) (Lennon *et al.* 2002; Heneka *et al.* 2005). In fact, correction of cellular signals of PKC activity and MAP-kinase in line with NF-κB is considered to be the main action of thiazolidinedione (Straus *et al.* 2000; Hung *et al.* 2001), serving as a potent anti-inflammatory agent (Duez *et al.* 2001; Verrier *et al.* 2004). The experimental approach has been conducted to circumvent a variety of degenerative neuronal disorders including Alzheimer's disease (Coombs *et al.* 2000; Landreth and Heneka 2001), experimental allergic encephalomyelitis (Feinstein *et al.* 2002), amyotrophic lateral sclerosis (Kiaei *et al.* 2005), and ischemic damage of the brain (Zhao *et al.* 2005; Ou *et al.* 2006).

There are only two reports in the literature that showed beneficial effects of thiazolidinedione on neuropathic changes in diabetic animals models (Qiang *et al.* 1998; Shibata *et al.* 2000). Qiang *et al.* (1998) reported that troglitazone improved NCV and fiber atrophy in STZ rats in which suppression of blood tumor necrosis factor-α and lipid peroxides was thought to be a key process for the thiazolidinedione effects. Our results may well be in keeping with the above data showing less migration of macrophages and less numbers of cells positive with 8OHdG *in situ* in pioglitazone-treated diabetic nerve compared with those in untreated rats. Shibata *et al.* (2000) reported an improvement of NCV in diabetic ZDF rats treated with a new thiazolidine-derivative. It is not clear, however, whether the NCV change was caused by this compound or blood glucose control itself as the treated animals also showed better metabolic controls. The current study is the first to demonstrate the expression of PPAR-γ, but not PPAR-α in the Schwann cells. The presence of PPAR-γ suggests that improvement of neuropathy may be ascribed to a direct action of pioglitazone on the nerve as well as endoneurial vessels, although other factors may also be involved.

The correction of altered MAP-kinase or PKC activity is an important mechanism that occurs in improvement of neuropathic changes in diabetic animals when treated with aldose reductase inhibitor or anti-oxidants (Tomlinson 1999; Purves *et al.* 2001; Ho *et al.* 2006). The metabolic changes correlate with amelioration of nerve Na,K-ATPase activity and NCV (Kim *et al.* 1991; Yagihashi *et al.* 2001) and lead to inhibition of nerve fiber atrophy with improved cytoskeletal protein synthesis (Scott *et al.* 1999). Our findings suggest that transactivating effects of pioglitazone mainly contribute to the activation of PKC activity with increased expression of PKC-α isoform that finally resulted in the alleviated nerve function. Indeed, activation of PKC activity is crucial for neuronal differentiation of PC12 cells when the

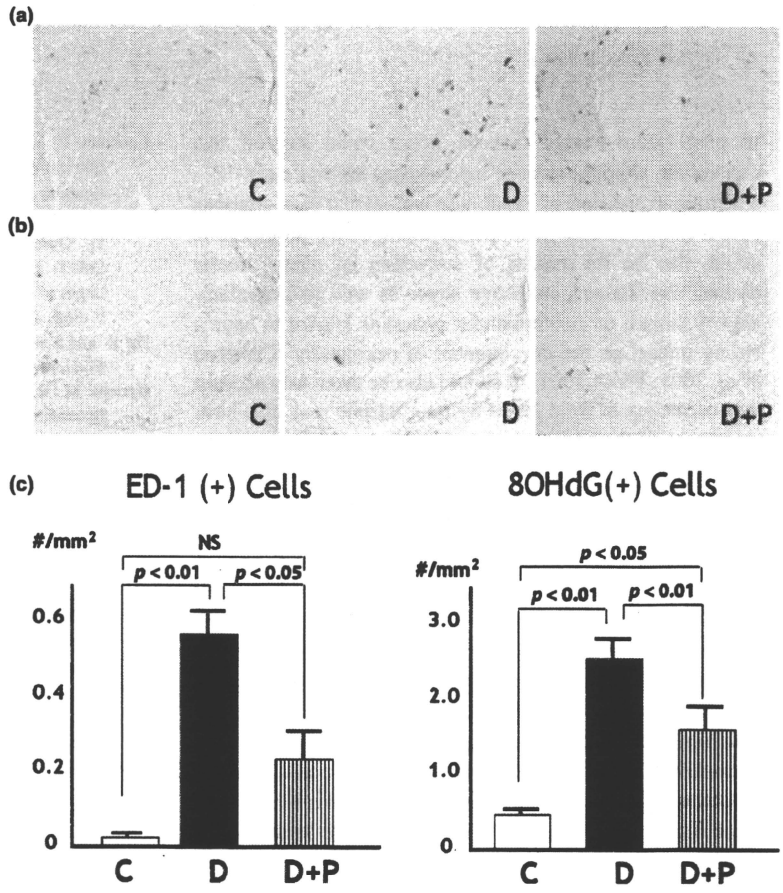

Fig. 5 (a) Immunohistochemical detection of ED-1-positive macrophages in sciatic nerve of experimental rats. In normal controls (C), only a few positive cells were noted. There was a considerable increase in ED-1-positive cells in diabetic rats (D). Pioglitazone-treated rats (D + P) showed only a few positive cells. (b) Immunohistochemical detection of 8OHdG in sciatic nerve of experimental rats. There was an only weak reaction of Schwann cells to 8OHdG in normal control rats (C), whereas diabetic rats (D) contained many Schwann cells positive to 8OHdG and this reaction was suppressed in pioglitazone-treated rats (D + P). (c) Quantitative analysis confirmed the increases in ED-1- and 8OHdG-positive cells in diabetic rats (D) compared with normal control rats (C). These changes were suppressed in pioglitazone-treated group (D + P). The number of animals was five in each group.

cells are stimulated by nerve growth factor which action is mediated by PPAR-γ after its binding with nerve growth factor receptor (Fuenzalida *et al.* 2005).

Protein kinase C activity in diabetic nerves is differently reported in previous studies as elevated (Roberts and McLean 1997; Kim *et al.* 2003), reduced (Kim *et al.* 1991) or not altered (Nakamura *et al.* 1999). We consider that different tissue sampling or different methods for the measurement may have yielded the discrepancy. In our studies on diabetic transgenic mice over-expressing human aldose reductase, we showed clearly different PKC alterations between endoneurial and vessel-rich epineurial tissues and between membrane and cytosolic fractions. Similar to the current results, endoneurial membrane fraction displayed reduced PKC activity with reduced expression of PKC-α isoform (Yamagishi *et al.* 2003; Uehara *et al.* 2004). Recent studies on the effects of rosiglitazone in diabetic Zucker rats also demonstrated tissue-specific alterations of PKC activity, in which PKC-β was activated in microvessels but not consistently so in the retina (Sotiropoulos *et al.* 2006). They further showed that rosiglitazone enhanced PKC activity in microvessels and the retina, but not in mononuclear cells (Sotiropoulos *et al.* 2006).

Increased migration of macrophages or oxidative-stress induced DNA damage appeared to be characteristic in peripheral nerve of STZ-diabetic rats. These changes were clearly inhibited by pioglitazone treatment. As the elevated sorbitol and fructose levels were not affected, the effects could be independent of polyol pathway. AGE-RAGE system or oxidative stress is suggested to elicit an inflammatory process in diabetic nerve (Bierhaus *et al.* 2001; Chawla *et al.* 2001; Wada and Yagihashi 2005), in which activation of MAP-kinase and NF-κB is central for neural tissue damage (Bierhaus *et al.* 2001; Wada and Yagihashi 2005). Our results indicated that MAP-kinase may not be directly connected with the alteration of total PKC activity in diabetic nerve. It can be speculated that proinflammatory processes may activate MAP-kinase related to PKC-βII activation (He and King 2004; Sotiropoulos *et al.* 2006), while impaired cellular signaling of phopshoinositide metabolism may lead to suppressed PKC activity with reduced membrane PKC-α expression (Kim *et al.* 1991; Yamagishi *et al.* 2003; Uehara *et al.* 2004). Under such circumstances, pioglitazone raised PKC activity to promote cell activity and inhibited phosphorylation of MAP-kinase by transrepression. Supranormal levels of PKC activity in the cytosolic fraction

in pioglitazone-treated diabetic nerve could support this contention, although precise mechanisms are not clear.

Strong expression of PPAR-γ in endothelial cells suggests that improvement of neuropathic changes by pioglitazone could also be the results of correction of neurovascular dysfunction. Indeed, oxidative stress as well as proinflammatory stimuli on neurovascular system is known to have a strong impact on the development of neuropathy (Cameron *et al.* 2001; Yorek 2003). It should also be taken into account that correction of lipid levels by pioglitazone may also have an impact on the nerve function. High lipid levels may exert enhancement of oxidative stress, resulting in neurovascular deficits in diabetes. In this study, we did not specifically examine the parameters of vascular system like nerve blood flow or vascular structure. Therefore, this possibility as well as an impact of hyperlipidemia needs to be evaluated by future investigations.

In conclusion, we explored the effects of pioglitazone on experimental diabetic neuropathy. PPAR-γ was expressed in the peripheral nerve and pioglitazone improved the peripheral nerve function. The changes were associated with correction of PKC activity and inhibition of proinflammatory processes independent of polyol pathway. We expect that these findings provide a new direction for the treatment of diabetic neuropathy in humans.

Acknowledgements

This study is supported by a Grant-in-Aid from the Ministry of Science, Education, Culture and Sport, Japan (#14370073) and by the Ministry of Health and Welfare, Japan.

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nature medicine

Serotonin regulates pancreatic beta cell mass during pregnancy

Hail Kim[1], Yukiko Toyofuku[2], Francis C Lynn[1,9], Eric Chak[1], Toyoyoshi Uchida[2], Hiroki Mizukami[3], Yoshio Fujitani[2,4], Ryuzo Kawamori[2,4–6], Takeshi Miyatsuka[1], Yasuhiro Kosaka[1], Katherine Yang[1], Gerard Honig[7], Marieke van der Hart[7], Nina Kishimoto[1], Juehu Wang[1], Soroku Yagihashi[4], Laurence H Tecott[7], Hirotaka Watada[2,6] & Michael S German[1,8]

During pregnancy, the energy requirements of the fetus impose changes in maternal metabolism. Increasing insulin resistance in the mother maintains nutrient flow to the growing fetus, whereas prolactin and placental lactogen counterbalance this resistance and prevent maternal hyperglycemia by driving expansion of the maternal population of insulin-producing beta cells[1–3]. However, the exact mechanisms by which the lactogenic hormones drive beta cell expansion remain uncertain. Here we show that serotonin acts downstream of lactogen signaling to stimulate beta cell proliferation. Expression of serotonin synthetic enzyme tryptophan hydroxylase-1 (Tph1) and serotonin production rose sharply in beta cells during pregnancy or after treatment with lactogens *in vitro*. Inhibition of serotonin synthesis by dietary tryptophan restriction or Tph inhibition blocked beta cell expansion and induced glucose intolerance in pregnant mice without affecting insulin sensitivity. Expression of the $G\alpha_q$-linked serotonin receptor 5-hydroxytryptamine receptor-2b (Htr2b) in maternal islets increased during pregnancy and normalized just before parturition, whereas expression of the $G\alpha_i$-linked receptor Htr1d increased at the end of pregnancy and postpartum. Blocking Htr2b signaling in pregnant mice also blocked beta cell expansion and caused glucose intolerance. These studies reveal an integrated signaling pathway linking beta cell mass to anticipated insulin need during pregnancy. Modulators of this pathway, including medications and diet, may affect the risk of gestational diabetes[4].

Although lactogenic hormones regulate beta cell mass during pregnancy, they alone cannot explain all of the maternal changes in this process[2,5–8]. Despite persistent elevations of placental lactogen and prolactin through the end of pregnancy and the end of lactation, respectively, beta cell proliferation peaks at midgestation in rodents and drops to the normal nonpregnant rate or below by parturition, prompting rapid normalization of beta cell mass postpartum[2,9,10] (**Supplementary Fig. 1**). Therefore, to identify other genes potentially involved in regulating maternal beta cell mass, we compared the global gene expression patterns in islets from nonpregnant and pregnant (gestational days 13–15 (G13–G15)) female mice by high-throughput sequencing of cDNA (**Supplementary Table 1** and **Supplementary Fig. 2**) and by hybridization to oligonucleotide microarrays (**Supplementary Table 2**).

Among the genes most markedly induced during pregnancy were *Tph1* and *Tph2*, which encode the two isoforms of tryptophan hydroxylase, the rate-limiting enzyme in the synthesis of serotonin (5-hydroxytryptamine, 5-HT). We have observed that beta cells share with serotonergic neurons a common gene expression program and the ability to synthesize, store and secrete serotonin (Y. Ohta and M.S.G., unpublished observations). Indeed, expression of several other serotonergic transcripts was substantially increased in islets from pregnant mice, including those encoding aromatic L-amino acid decarboxylase, the enzyme that catalyzes the second and final step in serotonin synthesis, and vesicular monoamine transporter VMAT1 (encoded by *Slc18a1*) (**Supplementary Table 1**).

Real-time RT-PCR for *Tph1* and *Tph2* confirmed the genomic analyses (**Fig. 1a**). Expression of *Tph1* and *Tph2* increased from the nonpregnant baseline by G6 and peaked at G12 with 527-fold and sevenfold increases, respectively, whereas levels in gut, heart and brain did not change (**Supplementary Fig. 3a–c**). *Tph1* expression remained high postpartum until the end of lactation, followed by a return to prepregnancy levels (**Fig. 1a**). Western blotting with specific antiserum against Tph1 detected the induction of Tph1 protein expression in pregnant mouse islets[11] (**Fig. 1b**). HPLC detected small tissue amounts of 5-HT in islets of nonpregnant mice, which increased 420-fold in islets from pregnant mice but did not change in gut (**Fig. 1c,d**).

[1]Diabetes Center, University of California–San Francisco (UCSF), San Francisco, California, USA. [2]Department of Medicine, Metabolism and Endocrinology, Juntendo University Graduate School of Medicine, Tokyo, Japan. [3]Department of Pathology and Molecular Medicine, Hirosaki University Graduate School of Medicine, Hirosaki, Japan. [4]Center for Therapeutic Innovations in Diabetes, Juntendo University Graduate School of Medicine, Tokyo, Japan. [5]Center for Beta Cell Biology and Regeneration, Juntendo University Graduate School of Medicine, Tokyo, Japan. [6]Sportology Center, Juntendo University Graduate School of Medicine, Tokyo, Japan. [7]Department of Psychiatry, UCSF, San Francisco, California, USA. [8]Department of Medicine, UCSF, San Francisco, California, USA. [9]Current address: Departments of Surgery and Cellular and Physiological Sciences, Faculty of Medicine, University of British Columbia, Vancouver, British Columbia, Canada. Correspondence should be addressed to M.S.G. (mgerman@diabetes.ucsf.edu).

Received 25 February; accepted 28 May; published online 27 June 2010; doi:10.1038/nm.2173

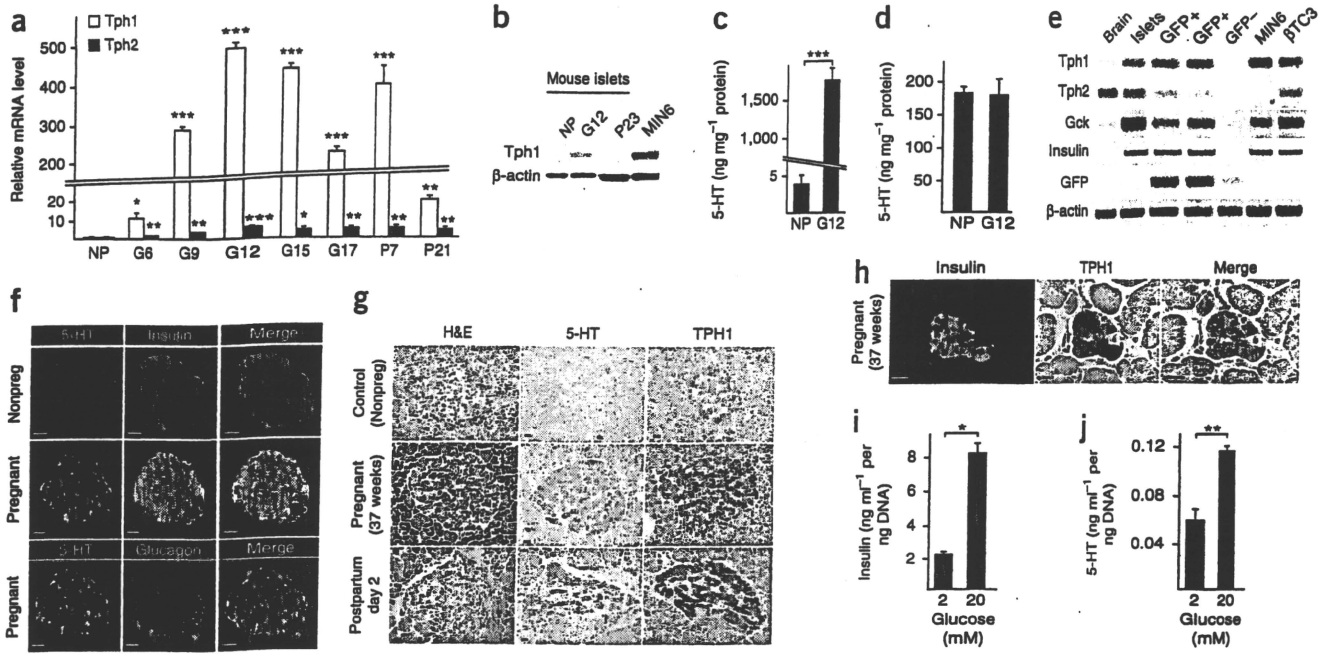

Figure 1 5-HT production in islets during pregnancy. (**a**) mRNA levels for *Tph1* and *Tph2* measured by real time RT-PCR in islet RNA at the dates of gestation (G) or postpartum (P), as indicated, relative to the RNA levels in islets from nonpregnant female mice. $n = 3$–6 mice per data point. (**b**) Western blotting with Tph1-specific and β-actin–specific antisera on protein extracted from islets of nonpregnant female (NP), pregnant (G12) and postpartum day 23 (P23) mice and MIN6 insulinoma cells. (**c**,**d**) Tissue 5-HT concentrations, as assayed by HPLC in islets (**c**) and duodenum (**d**) isolated from three nonpregnant (NP) and 3 pregnant (G12) female mice. (**e**) mRNA detection by RT-PCR from mouse islets, beta cells (GFP$^+$) nonbeta islet cells (GFP$^-$), brain tissue and the beta cell lines MIN6 and βTC3. (**f**) Immunofluorescent staining of pancreata from nonpregnant female (Nonpreg) and G12 pregnant mice for insulin or glucagon (both green) and 5-HT (red). (**g**) Immunoperoxidase staining for 5-HT and TPH1 in pancreata from nonpregnant, pregnant (37 weeks gestation) and postpartum day 2 human autopsies. (**h**) Immunohistochemical staining for insulin (red, fluorescent) and TPH1 (black, peroxidase) in pregnant human autopsy pancreas. (**i**,**j**) Secreted insulin (**i**) and 5-HT (**j**), as assayed by ELISA (**i**) and HPLC (**j**), after a 1-h incubation of islets isolated from pregnant (G13–G15) mice in 2 mM or 20 mM glucose. $n = 5$ groups of 40 islets. All data are presented as means ± s.e.m. Statistical significance versus islets cultured in 2mM glucose (**i**,**j**) or versus nonpregnant control (**a**,**c**) was analyzed by Student's t test: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Scale bars, 20 μm.

RT-PCR amplified both *Tph1* and *Tph2* mRNA from beta cells purified from mice expressing EGFP in beta cells (MIP-GFP mice[12]) (**Fig. 1e**). Immunohistochemical staining detected 5-HT and TPH1 co-localized with insulin in islets from pregnant mice (**Fig. 1f**) and pregnant and postpartum humans (**Fig. 1g,h** and **Supplementary Fig. 3d**). In a test of the ability of beta cells to secrete this stored 5-HT, glucose stimulation induced secretion of insulin and 5-HT from islets isolated from pregnant mice (**Fig. 1i,j**). Taken together, these data indicate that beta cells markedly increase synthesis, storage and secretion of 5-HT during pregnancy. This increase may modestly boost circulating 5-HT concentrations (**Supplementary Fig. 4**) but probably increases local islet amounts of 5-HT much more. Therefore, we tested whether 5-HT affects beta cell function and glucose metabolism during pregnancy.

Because tryptophan is an essential amino acid and the K_m of Tph for tryptophan is higher than tissue tryptophan concentrations, dietary restriction of tryptophan can sharply drop 5-HT levels[13]. We found that mice fed a tryptophan-free diet from G6 to G12 and tested at G13 (**Fig. 2a**) developed severe glucose intolerance compared to mice fed either a normal or histidine-free diet (**Fig. 2b**). Furthermore, a less restricted diet, with 20% of normal tryptophan content, or treatment with the Tph inhibitor 4-chloro-DL-phenylalanine methyl ester hydrochloride (PCPA) also induced glucose intolerance in pregnant mice (**Fig. 2c**) but not in nonpregnant female mice (**Fig. 2d**). These results support the conclusion that 5-HT production is necessary for the maintenance of glucose homeostasis during pregnancy.

To determine how 5-HT might regulate islet function, we assayed the expression in islets of the 14 mouse 5-HT receptors by RT-PCR[14]. Several were expressed in relatively high amounts in mouse islets (**Fig. 2e**), and transcripts encoding the $G\alpha_i$-linked receptor Htr1d and the $G\alpha_q$-linked receptor Htr2b were detected in purified beta cells (**Fig. 2f**). In pregnant mice, *Htr2b* expression increased significantly from G6 through G15 (**Fig. 2g**) and normalized at the end of gestation, whereas *Htr1d* expression increased at the end of gestation (G17) and postpartum (**Fig. 2h**). Notably, increased *Htr2b* expression closely correlated with the period of increased beta cell proliferation, and increased *Htr1d* expression correlated with the cessation of beta cell proliferation and regression of beta cell mass[2,9].

To assess the contribution of 5-HT signaling, we treated mice with Htr antagonists (**Fig. 2a**). The nonspecific Htr antagonist methysergide and the selective Htr2b antagonist SB204741 induced glucose intolerance in pregnant mice (**Fig. 2i**) but not in nonpregnant female mice (SB204741, **Fig. 2d**) or when given acutely (**Supplementary Fig. 5a**), whereas the selective Htr2a antagonist ketanserin had no effect on glucose tolerance (**Fig. 2i**). Treatment with PCPA or SB204741 or a tryptophan-free diet all lowered insulin levels in pregnant mice (**Fig. 2j**), but insulin sensitivity was not reduced with PCPA or SB204741 treatment (**Supplementary Fig. 5b**). To test the requirement for Htr2b

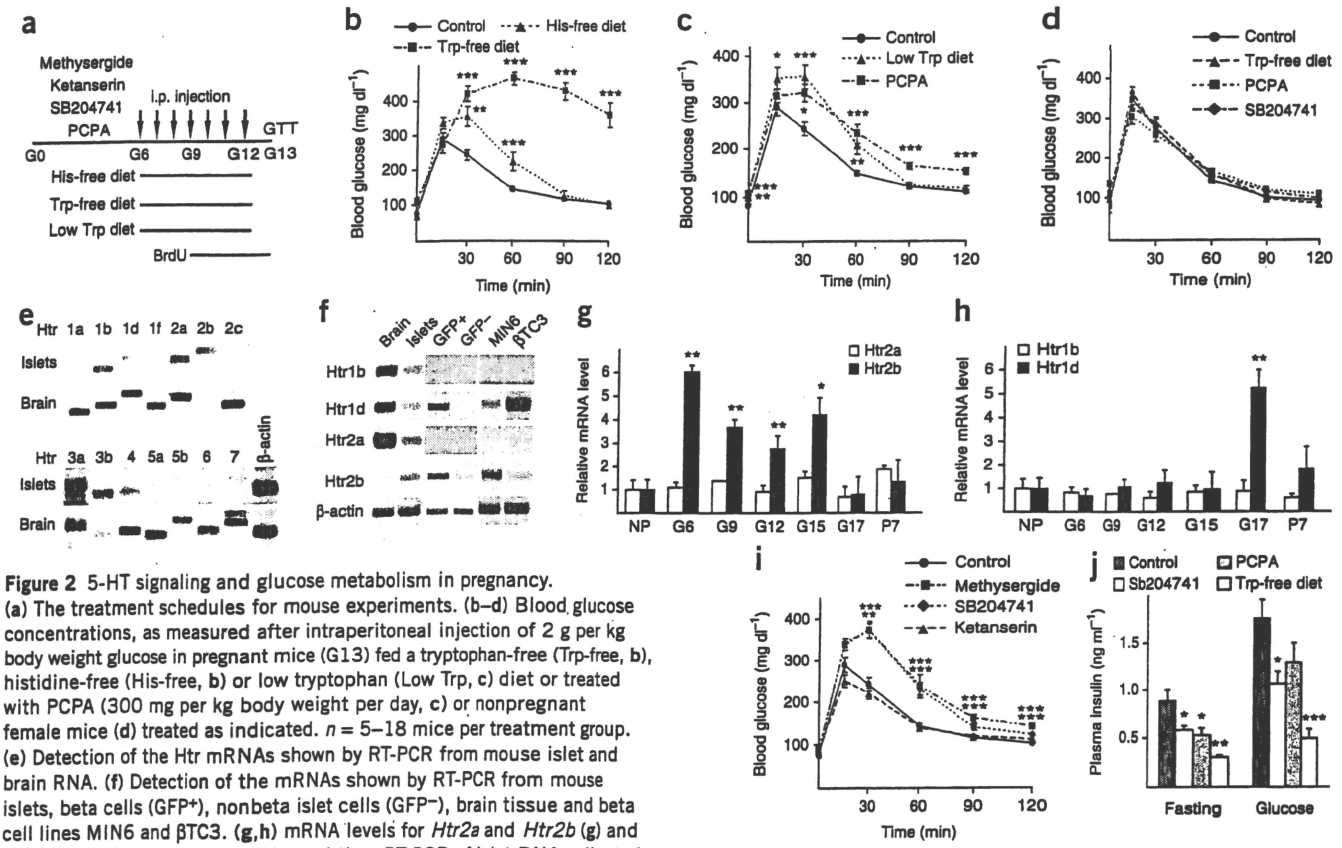

Figure 2 5-HT signaling and glucose metabolism in pregnancy. (**a**) The treatment schedules for mouse experiments. (**b–d**) Blood glucose concentrations, as measured after intraperitoneal injection of 2 g per kg body weight glucose in pregnant mice (G13) fed a tryptophan-free (Trp-free, **b**), histidine-free (His-free, **b**) or low tryptophan (Low Trp, **c**) diet or treated with PCPA (300 mg per kg body weight per day, **c**) or nonpregnant female mice (**d**) treated as indicated. $n = 5–18$ mice per treatment group. (**e**) Detection of the Htr mRNAs shown by RT-PCR from mouse islet and brain RNA. (**f**) Detection of the mRNAs shown by RT-PCR from mouse islets, beta cells (GFP$^+$), nonbeta islet cells (GFP$^-$), brain tissue and beta cell lines MIN6 and βTC3. (**g,h**) mRNA levels for *Htr2a* and *Htr2b* (**g**) and *Htr1b* and *Htr1d* (**h**) measured by real-time RT-PCR of islet RNA collected at the gestational dates indicated, shown relative to the levels in islets from nonpregnant female mice. $n = 3–6$ mice per data point. (**i**) Blood glucose concentrations, as measured after intraperitoneal injection of glucose (2 g per kg body weight) in pregnant mice treated with SB204741 (1 mg per kg body weight per day), methysergide (3 mg per kg body weight per day) or ketanserin (1 mg per kg body weight per day). $n = 5–18$ mice per treatment group. (**j**) Insulin concentrations in pregnant mice treated as in **a** with tryptophan-free diet, PCPA or SB204741 and fasted for 6 h at G13 before measuring insulin in plasma collected before and 30 min after intraperitoneal injection of 2 g per kg body weight glucose. $n = 4–7$ mice per group. All data are presented as means ± s.e.m. Statistical significance versus untreated control (**b,c,i,j**) or versus nonpregnant control (**g,h**) was analyzed by Student's *t* test: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

signaling during pregnancy genetically, we assessed *Htr2b*-null mice and observed glucose intolerance during pregnancy (**Fig. 3a,b**).

Given the known role of Htr2b signaling in driving cell proliferation in other tissues[15–21], we tested whether 5-HT signaling contributes to increased beta cell proliferation during pregnancy. Treatment with PCPA or SB204741 as well as *Htr2b* loss reduced the normal increase in beta cell proliferation in pregnant mice at G13, as assayed by BrdU incorporation (**Figs. 2a** and **3c**) and prevented the pregnancy-induced expansion of beta cell mass (**Fig. 3d**) but did not reduce insulin secretion acutely from islets *in vitro* (**Supplementary Fig. 5c**). In addition, both PCPA and SB204741 attenuated the growth of the MIN6 mouse beta cell tumor line but not of NIH-3T3 mouse fibroblasts (**Supplementary Fig. 6a–c**). Therefore, expansion of beta cell mass during pregnancy requires both Tph1-driven 5-HT production and signaling through Htr2b.

To determine whether the requirement for Htr2b signaling resides specifically in the islets, we transplanted islets isolated from *Htr2b*$^{+/+}$ or *Htr2b*$^{-/-}$ mice under the kidney capsule of *Htr2b*$^{+/+}$ mice (**Fig. 3e**) and assessed beta cell proliferation in the transplanted islets during pregnancy. Although this approach removes the islets from their normal anatomical context, it avoids the use of Cre recombinase, which, when expressed under the control of pancreatic promoters, often recombines targeted genes in other tissues, as well[22]. In transplanted beta cells, pregnancy induced 5-HT production regardless of the presence of *Htr2b* (**Fig. 3f**). However, whereas both the host and the transplanted *Htr2b*$^{+/+}$ beta cells similarly increased BrdU incorporation during pregnancy, fewer transplanted *Htr2b*$^{-/-}$ beta cells incorporated BrdU, indicating a local requirement for Htr2b signaling in islets during pregnancy (**Fig. 3g,h**).

Because the pregnancy hormones prolactin and placental lactogen stimulate beta cell proliferation[2,3,5,6] and prolactin induces *Tph1* expression in the mammary gland[23], we tested the ability of lactogenic hormones to induce *Tph1* expression in islets isolated from pregnant mice at G3. Moderate concentrations of prolactin (200 ng ml^{-1}) increased *Tph1* expression 28-fold, whereas high concentrations of prolactin (1,000 ng ml^{-1}) corresponding to midgestation lactogen levels increased *Tph1* expression by 147-fold (**Fig. 4a**) without affecting expression of *Tph2*, *Htr1d* or *Htr2b* (**Fig. 4b–d**), which therefore may be regulated by some other pregnancy-related signals. Human placental lactogen induced Tph1 expression to similar levels in G3 mouse islets, but nonpregnant islets responded less robustly (**Supplementary Fig. 6d**), consistent with evidence that prolactin receptor expression and signaling increase in islets early during pregnancy[24] (**Supplementary Table 1**).

Next we tested whether 5-HT can independently induce islet cells to proliferate. Treatment of mouse islets with prolactin (1,000 ng ml^{-1}) or 5-HT (10 μM) induced similar increases in proliferation, as

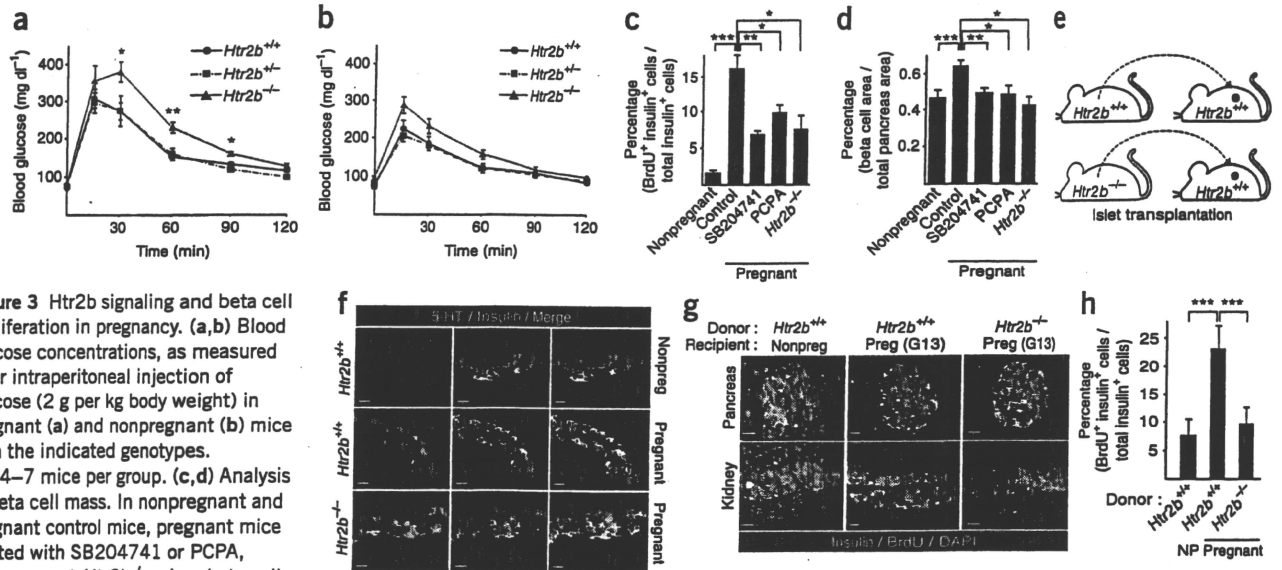

Figure 3 Htr2b signaling and beta cell proliferation in pregnancy. (**a**,**b**) Blood glucose concentrations, as measured after intraperitoneal injection of glucose (2 g per kg body weight) in pregnant (**a**) and nonpregnant (**b**) mice with the indicated genotypes. $n = 4–7$ mice per group. (**c**,**d**) Analysis of beta cell mass. In nonpregnant and pregnant control mice, pregnant mice treated with SB204741 or PCPA, and pregnant *Htr2b*$^{-/-}$ mice, beta cell proliferation (**c**) was quantified by counting the percent of insulin-positive cells labeled with BrdU, and relative beta cell mass (**d**) was calculated as the area of insulin-positive cells/total pancreatic area. $n = 4–6$ mice per group. (**e**) A schematic showing the islet transplantation model used in **f**–**h**. Islets isolated from *Htr2b*$^{+/+}$ (black) or *Htr2b*$^{-/-}$ (red) mice were transplanted into the kidney capsule of *Htr2b*$^{+/+}$ mice. (**f**) Immunofluorescence staining for 5-HT (red) and insulin (green) in donor islets with the indicated genotypes, transplanted under the kidney capsule in nonpregnant or G13 pregnant hosts. (**g**) Immunofluorescence staining for insulin (red) and BrdU (green) together with DNA staining with DAPI (blue) in host pancreatic islets and donor islets with the indicated genotypes, transplanted under the kidney capsule in nonpregnant and G13 pregnant mice. (**h**) Beta cell proliferation in transplanted islets with the indicated genotypes, as quantified by determining the percentage of insulin-positive cells labeled with BrdU in nonpregnant (NP) and pregnant mice. $n = 3$ or 4 mice per group. All data are presented as means ± s.e.m. Statistical significance versus *Htr2b*$^{+/+}$ control (**a**), versus pregnant untreated *Htr2b*$^{+/+}$ control (**c**,**d**) or versus pregnant *Htr2b*$^{+/+}$ control (**h**) was analyzed by Student's *t* test: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Scale bars, 20 μm.

measured by BrdU incorporation (**Fig. 4e**) and Ki67 staining (**Supplementary Fig. 6e**). Finally, treatment of islets with 5-HT (10 μM) *in vitro* altered cyclin gene expression in a pattern similar to that seen in islets from pregnant mice (**Fig. 4f,g**).

Taken together, these studies suggest a simple model for the regulation of beta cell mass during pregnancy (**Fig. 4h**). During pregnancy, lactogenic signaling induces *Tph1* expression and 5-HT synthesis in islets. 5-HT, in turn, functions in a paracrine-autocrine fashion

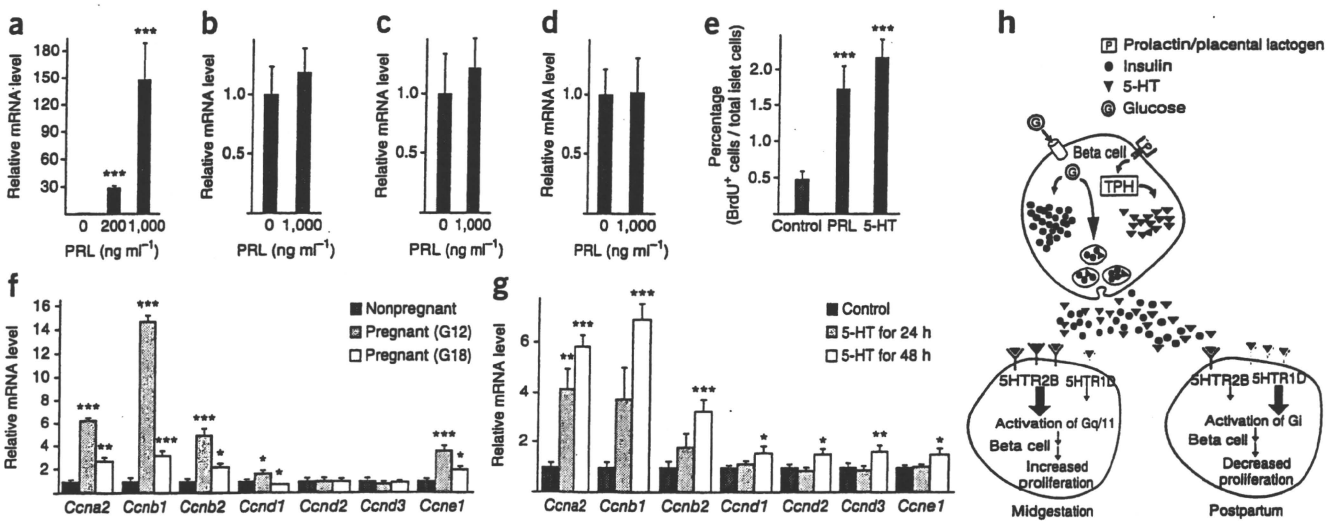

Figure 4 5-HT– and lactogen-induced beta cell proliferation. (**a**–**d**) mRNA levels for *Tph1* (**a**), *Tph2* (**b**), *Htr1d* (**c**) and *Htr2b* (**d**), as measured by real-time RT-PCR of RNA from islets isolated from G3 pregnant mice and cultured with the indicated concentration of mouse prolactin (PRL) for 72 h. $n = 3–6$ groups of islets per data point. (**e**) Beta cell proliferation, as quantified by determining the percentage of insulin-positive cells labeled with BrdU in mouse islets cultured with prolactin (1,000 ng ml^{-1}, PRL), 5-HT (10 μM) or no added hormone (control) for 4 d. $n = 5–10$ groups of islets per data point. (**f**,**g**) Levels of cyclin mRNAs, as measured by real-time RT-PCR in RNA from islets collected at the indicated dates and shown relative to the levels in control islets from nonpregnant female mice (**f**) or from islets cultured with 5-HT (10 μM) for the indicated times and shown relative to the levels in control islets cultured without 5-HT for 24 h (**g**). $n = 4–10$ groups of islets per data point. (**h**) A proposed model for 5-HT regulation of beta cell proliferation during pregnancy. All data are presented as means ± s.e.m. Statistical significance versus untreated control (**a**,**e**,**g**) or versus nonpregnant control (**f**) was analyzed by Student's *t* test: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

through $G\alpha_q$-coupled Htr2b to stimulate beta cell proliferation. Shortly before parturition, expression of Htr2b decreases and expression of $G\alpha_i$-coupled Htr1d increases, generating an inhibitory signal capable of reducing beta cell proliferation and beta cell mass (M. Berger and M.S.G., unpublished data), so that beta cell mass returns rapidly to prepregnancy levels. Although this model focuses on the regulation of beta cell mass, increasing evidence suggests that beta cell mass influences insulin production capacity and thus the risk of diabetes[25–27]. In addition, serotonin signaling may also directly affect insulin secretion independently of its effect on beta cell mass. Finally, similar shifts in receptor expression might contribute to other peripartum changes, such as the mobilization of calcium from bone[18] that occurs with lactation.

Modulators of this pathway, including drugs, diet and genetic inheritance, may affect the risk of gestational diabetes and, possibly, the long-term risk of developing type 2 diabetes. The dual roles of serotonin in regulating mood and beta cell mass provide a possible link that could explain the association of depression with both type 2 diabetes[28] and gestational diabetes[29], as well as the diabetogenic effects of some classes of psychiatric medications[30]. A more complete understanding of the function of this pathway may suggest improved methods for both preventing and treating diabetes.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Accession codes. All high-throughput sequencing and microarray data have been deposited in the Gene Expression Omnibus with Series accession numbers GSE21860 and GSE22125, respectively.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

We thank G. Grodsky, W. Rutter and members of the German laboratory for helpful discussions; F. Schaufle and the UCSF Diabetes and Endocrinology Research Center Microscopy Core Laboratory; G. Szot and the UCSF Diabetes and Endocrinology Research Center Islet Core; Y. Zhang, Z. Li and S. Zhao for technical assistance with mouse husbandry and genotyping; N. Daimaru, E. Magoshi and K. Nakamura for technical assistance; K. Takahashi, W. Inaba, M. Tsujii and S. Nakayama for immunohistochemical studies; Y. Katayama (Hirosaki University) and P. Ursell (UCSF) for providing human pancreatic samples; and D. Kuhn (Wayne State University), L. Maroteaux (Institut National de la Santé et de la Recherche Médicale) and M. Hara (University of Chicago) for generously providing the Tph1-specific antisera, *Htr2b*-targeted mice and MIP-GFP mice, respectively. This work was supported by grants from the Larry L. Hillblom Foundation, the Juvenile Diabetes Research Foundation, the American Diabetes Association, the US National Institutes of Health–National Institute of Diabetes and Digestive and Kidney Diseases and the Ministry of Education, Sports and Culture of Japan.

AUTHOR CONTRIBUTIONS

H.K., H.W. and M.S.G. designed the research; H.K., Y.T., F.C.L., E.C., T.U., H.M., Y.F., T.M., Y.K., G.H., M.v.d.H., K.Y., N.K. and J.W. performed the research; H.K., F.C.L., R.K., S.Y., H.W., L.H.T. and M.S.G. analyzed the data; and H.K., F.C.L., H.W. and M.S.G. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mouse studies. C57BL/6J mice were housed on a 12-h light-dark cycle in climate-controlled, pathogen-free barrier facilities. The Institutional Animal Care and Use Committees at UCSF and Juntendo University approved all studies involving mice. *Htr2b* targeted mice were provided by L. Maroteaux[16] and backcrossed with C57BL/6J mice for more than seven generations. C57BL/6J mice were purchased from Jackson Laboratories. Mating was confirmed by the presence of a vaginal plug the next morning, designated day 0 of gestation (G0). 4-Chloro-DL-phenylalanine methyl ester hydrochloride (PCPA, Sigma-Aldrich, 300 mg per kg body weight per da^1), SB204741 (Sigma-Aldrich, 1 mg per kg body weight per day), methysergide (Sigma-Aldrich, 3 mg per kg body weight per day) and ketanserin (Sigma-Aldrich, 1 mg per kg body weight per day) were administered daily for 7 d from G6 to G12 by intraperitoneal injection. BrdU (Sigma-Aldrich) was administered in drinking water bottles at 1 mg ml^{-1} for 4 d for nonpregnant mice and for pregnant mice from G9 to G13. Tryptophan-free diet, histidine-free diet, low-tryptophan diet and the equivalent control diet (Baker amino acid diet) were purchased from Labdiet. The control diet contained 0.2% tryptophan and 0.4% histidine, and the low-tryptophan diet contained 0.04% tryptophan.

Glucose and insulin tolerance and insulin secretion tests were performed on nonpregnant female or pregnant mice at G13. Mice were fasted overnight and injected intraperitoneally with glucose (2 g per kg body weight) or insulin (0.75 U per kg body weight), and blood glucose concentration was measured at the times shown in Figures 2 and 3 from tail vein blood with a portable glucometer (FreeStyle flash blood glucose monitoring system, Abbott Diabetes Care). For insulin secretion tests, mice were fasted for 6 h and injected intraperitoneally with glucose (2 g per kg body weight), and blood was sampled from retroorbital plexus with a heparinized microvette (Sarstedt AG & Co.) at 0 and 30 min after glucose injection. Plasma insulin concentration was determined with the Linco Mouse Insulin ELISA kit (Millipore).

Pancreatic islets were isolated from female mice by collagenase digestion, hand-picked and pooled. Counting of BrdU-positive beta cells and morphometric analyses were performed by serially sectioning the entire pancreas followed by staining and counting every twentieth section. A minimum of 3,000 insulin-positive cells were counted per pancreas.

Human studies. Human pancreatic tissue was obtained at autopsy at the UCSF Medical Center and Hirosaki University–affiliated teaching hospitals with the informed consent of the legal next-of-kin in accordance with the guidelines of the UCSF Committee on Human Research and Hirosaki University Institutional Review Board. Nine age-matched nonpregnant female and 5 male control pancreata were used for comparison. These cases had no history of diabetes. Human pancreas tissues were all fixed in formalin and stored in paraffin blocks from which sections were obtained for conventional H&E staining and immunohistochemistry.

Statistical analyses. All statistical data are presented as means ± s.e.m. Statistical significance was determined by Student's *t* test.

Additional methods. Details of tissue processing and staining and RT-PCR assays are provided in the **Supplementary Methods**.