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RESEARCH PAPER

Enhancement of mesenteric artery contraction to 5-HT depends on Rho kinase and Src kinase pathways in the *ob/ob* mouse model of type 2 diabetes

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Background and purpose: Arteries from hypertensive subjects are reportedly hyperresponsive to 5-hydroxytryptamine (5-HT), but it remains unclear whether this is true in chronic type 2 diabetes. We have assessed responses to 5-HT shown by mesenteric arteries from type 2 diabetic *ob/ob* mice (27–32 weeks old) and have identified the molecular mechanisms involved.
Experimental approach: Contractions of mesenteric rings to 5-HT were examined *in vitro*. Activation of mesenteric RhoA, Rho kinase and Src was measured by Western blotting or by modified enzyme-linked immunosorbent assay.
Key results: Concentration-dependent contractions to 5-HT were greater in mesenteric rings from the *ob/ob* than in those from the age-matched control ('Lean') group. In each group, there was no significant change in the 5-HT-induced contractions after inhibition of nitric oxide synthase (with N^G-nitro-L-arginine), of cyclooxygenase (with indomethacin) or of protein kinase C (with chelerythrine). However inhibition of the MEK/ERK pathway (with PD98059) decreased the response to 5-HT. Although the diabetes-related enhancement of the 5-HT response was preserved with each of these inhibitors, enhancement was abolished by a Rho kinase inhibitor (Y27632) and by Src kinase inhibitors (PP1 analogue or Src kinase inhibitor I). 5-HT-induced activation of RhoA, Rho kinase and Src kinase in mesenteric arteries was greater in the *ob/ob* than in the Lean group, but the expression of RhoA, Rho kinase isoforms and Src did not differ between these groups.
Conclusions and implications: These results suggest that the enhancement of 5-HT-induced contraction in mesenteric arteries from *ob/ob* mice may be attributable to increased activation of RhoA/Rho kinase and Src kinase.
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Keywords: 5-HT; diabetes mellitus; *ob/ob* mice; Rho kinase; Src kinase

Abbreviations: 5-HT_{2A}, 5-HT receptor subtype 2A; COX, cyclooxygenase; EDHF, endothelium-derived hyperpolarizing factor; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; ERM, Ezrin/Radixin/Moesin; L-NNA, N^G-nitro-L-arginine; MEK, mitogen-activated/ERK-activating kinase; NOS, nitric oxide synthase; PERM, phospho-Ezrin/Radixin/Moesin; PKC, protein kinase C

Introduction

Across the world, the prevalence of diabetes mellitus, particularly of type 2 diabetes, has increased significantly in recent years. Although type 2 diabetes is associated with a markedly increased incidence of cardiovascular diseases (Sowers, 2004; Quinn *et al.*, 2008), the exact relationship between type 2 diabetes and cardiovascular disease is still not completely understood. Indeed, it is the subject of some dispute, partly because type 2 diabetes is often one component of an array of complex abnormalities referred to as 'metabolic syndrome', in which it is frequently accompanied by hypertension and obesity (Sowers, 2004). It is believed that an impaired ability to vasodilate and/or an enhanced responsiveness to vasoconstrictor agonists underlie the vascular dysfunction associated with diabetes (Kamata *et al.*, 1989; Hattori *et al.*, 1995; De Vriese *et al.*, 2000; Matsumoto *et al.*, 2003). Indeed, we (Matsumoto *et al.*, 2006a; 2007; 2008b; 2009) and others (Winters *et al.*, 2000; Okon *et al.*, 2003; Sena *et al.*, 2008; Weston *et al.*, 2008) have reported that abnormalities of vascular function exist in various type 2 diabetic models. The endothelial defects contributing to abnormal smooth muscle function in diabetes include a reduction in nitric oxide (NO) bioavailability and an increased release of contractile factors (De Vriese

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et al., 2000; Shi and Vanhoutte, 2008; Szabo, 2009). While many studies have examined endothelial dysfunction in diabetes, fewer studies have examined the effects of diabetes on smooth muscle dysfunction. Study of vascular muscle in diabetic subjects is important as abnormal endothelial function alone cannot account for the increased vasoconstrictor responses observed in many cardiovascular diseases (Okon *et al*., 2003; Matsumoto *et al*., 2006a; 2009).

5-Hydroxytryptamine (5-HT), a neurotransmitter with potent vasoconstrictor properties that regulates a variety of functions in the nervous and cardiovascular systems (Watts, 2005), plays important roles in cardiovascular diseases such as atherosclerosis (Henry and Yokoyama, 1980), hypertension (Watts, 2002; 2005; Budzyn *et al*., 2008; Dempsie and MacLean, 2008) and vascular inflammation (Katz *et al*., 1994). The primary targets of 5-HT in the vasculature are 5-HT receptor subtype 2A (5-HT_{2A}) receptors (nomenclature follows Alexander *et al*., 2009) and previous reports have suggested that in rat aorta (Banes *et al*., 1999) or mouse aorta (McKune and Watts, 2001), the 5-HT_{2A} receptor-mediated contraction is coupled to L-type calcium channels and phospholipase C. Moreover, the 5-HT-mediated vasoconstriction is mediated by various kinase pathways, including protein kinase C (PKC) (Banes *et al*., 1999) and tyrosine kinases (Banes *et al*., 1999; Watts, 2002). Indeed, it has been reported that in normal mesenteric arteries, 5-HT-induced contraction depends on activation of L-type calcium channels, phospholipase C and tyrosine kinases, including the extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase pathway (Watts, 2002).

Src protein tyrosine kinases and Rho kinase are potential regulators of a variety of cellular functions, apart from cellular differentiation and proliferation (Thomas and Brugge, 1997; Shimokawa and Takeshita, 2005). In vascular smooth muscle, Src tyrosine kinases have been implicated in the contractile signalling triggered by 5-HT (Alioua *et al*., 2002; Lu *et al*., 2008), as has the Rho kinase pathway (Nuno *et al*., 2007; 2009; Lu *et al*., 2008). Several reports have suggested that abnormalities of the activities of the above kinases exist in the vasculature in diabetic states (Matsumoto *et al*., 2006a; Kobayashi *et al*., 2008; Nuno *et al*., 2009). However, little is known about the reactivity to 5-HT (or about the associated molecular mechanisms, including the above kinases) shown by leptin-deficient obese *ob/ob* mice, a type 2 diabetic model (Konstantinides *et al*., 2001) at the chronic stage of diabetes.

In the present study, we investigated the changes in the 5-HT-induced contraction of the superior mesenteric artery that might occur as a result of long-term diabetes. For this, we isolated mesenteric arteries from 27- to 32-week-old *ob/ob* mice and compared their responses with those of arteries from age-matched control Lean mice. We also tried to identify some of the molecular mechanisms responsible for the differences we detected between these groups of mice.

Methods

Animals and experimental design

All animal care and experimental procedures were conducted in accordance with *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health, and *Guide for the Care and Use of Laboratory Animals* adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology, Japan) and approved by the Hoshi University Animal Care and Use Committee. Male *ob/ob* C57BL/6J mice and age-matched wild-type lean C57BL/6J mice, purchased from Jackson Laboratory (Bar Harbor, ME, USA), were housed under constant climatic conditions (room temperature 21–22°C, room humidity 50 ± 5%) and allowed a standard laboratory diet (MF; Oriental Yeast Industry, Tokyo, Japan) and water *ad libitum*. Mice were studied at 27–32 weeks of age.

Measurement of plasma glucose and insulin and blood pressure

Plasma parameters and systolic blood pressure were measured as described previously (Matsumoto *et al*., 2006b; 2008a). Briefly, the plasma glucose level was determined by the use of a commercially available enzyme kit (Wako Chemical Company, Osaka, Japan). Plasma insulin was measured by enzyme immunoassay (Shibayagi, Gunma, Japan). Plasma leptin was determined by enzyme-linked immunosorbent assay (ELISA) (Morinaga Institute of Biological Science, Yokohama, Japan). Each mouse was placed in a constant-temperature box at 37°C for a few minutes and then its blood pressure was measured by the tail-cuff method using a blood-pressure analyser (BP-98A; Softron, Tokyo, Japan).

Measurement of isometric force

Vascular isometric force in rings cut from the superior mesenteric artery was recorded as described previously (Matsumoto *et al*., 2006b; 2008a). Mice (27–32 weeks old) were anaesthetized with diethyl ether and killed by decapitation. A section of the superior mesenteric artery was then removed and placed in ice-cold, oxygenated, modified Krebs-Henseleit solution. This solution consisted of (in mM) 118.0 NaCl, 4.7 KCl, 25.0 $NaHCO_3$, 1.8 $CaCl_2$, 1.2 NaH_2PO_4, 1.2 $MgSO_4$ and 11.0 dextrose. Each mesenteric artery was separated from the surrounding connective tissue and cut into rings (2 mm long). The ring segments were suspended by a pair of stainless-steel pins in a well-oxygenated (95% O_2/5% CO_2) bath of 10 mL Krebs-Henseleit solution at 37°C. The rings were stretched until an optimal resting tension of 0.5 g was loaded, and then allowed to equilibrate for at least 60 min. Force generation was monitored by means of an isometric transducer (model TB-611T; Nihon Kohden, Tokyo, Japan). Tension was readjusted when necessary, and the bath fluid was changed every 15 min.

For the contraction studies, 5-HT (10^{-9}–3×10^{-5} M), U46619 (10^{-9}–10^{-6} M) or phenylephrine (10^{-9}–10^{-3} M) was added cumulatively to the bath until a maximal response was achieved. After the addition of sufficient aliquots of the agonist to produce the chosen concentration, a plateau response was allowed to develop before the addition of the next dose of the same agonist. To investigate the effect on the 5-HT-induced contractile response achieved using a given drug, the ring was incubated for 30 min in the appropriate medium before the cumulative addition of agonist. The drugs

used were: 10^{-5} M indomethacin [cyclooxygenase (COX) inhibitor], 10^{-4} M N^G-nitro-L-arginine [L-NNA; nitric oxide synthase (NOS) inhibitor], 10^{-6} M chelerythrine [a non-selective PKC inhibitor], 10^{-5} M PD98059 [mitogen-activated/ERK-activating kinase (MEK)/ERK pathway inhibitor], 10^{-7}–10^{-5} M Y27632 (Rho kinase inhibitor) or one of two Src kinase inhibitors (10^{-6} and 10^{-5} M PP1 analogue or 10^{-7} and 10^{-6} M Src kinase inhibitor I). The contractile force developed by mesenteric rings is expressed in g tension·(mg tissue)$^{-1}$.

Western blotting

Protein levels were quantified using immunoblotting procedures, essentially as described before (Matsumoto *et al*., 2006b; 2007; 2008b). After incubation with or without 10^{-6} M 5-HT for 10 min, mesenteric rings (2–3 pooled vessels per sample) were collected and then homogenized in ice-cold lysis buffer. This buffer contained 50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate and 0.1% SDS containing protease and phosphatise inhibitor cocktails (Complete Protease Inhibitor Cocktail and PhosSTOP; Roche Diagnostics, Indianapolis, IN, USA). The lysate was cleared by centrifugation at 16000× *g* for 10 min at 4°C. The supernatant was collected, and the proteins were solubilized in Laemmli's buffer containing mercaptoethanol. Protein concentrations were determined by means of a bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL, USA). Samples (20 μg per lane) were resolved by electrophoresis on 10% SDS-PAGE gels, then transferred onto polyvinylidene difluoride membranes. Briefly, after blocking the residual protein sites on the membrane with ImmunoBlock (Dainippon-pharm., Osaka, Japan) or polyvinylidene difluoride blocking reagent (Toyobo, Osaka, Japan), the membrane was incubated with rabbit anti-phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) (PERM) antibody (1:1000), rabbit anti-ERM antibody (1:1000), mouse anti-Rho kinase 1 (ROCK1) antibody (1:1000), mouse anti-ROCK2 antibody (1:1000), mouse anti-RhoA antibody (1:500), rabbit anti-phospho-Src family (Tyr416) (p-Src) antibody (1:1000), mouse anti-Src antibody (1:1000), mouse anti-ERK1/2 (1:1000), mouse anti-phospho-ERK1/2 (pT202/pY204) (1:1000) or rabbit anti-5-HT_{2A} receptor antibody (1:1000) in blocking solution. Horseradish peroxidase (HRP)-conjugated, anti-mouse or anti-rabbit antibody was used at a 1:10 000 dilution in Tween PBS, followed by detection using SuperSignal (Pierce, Rockford, IL, USA). To normalize the data, we used β-actin as a housekeeping protein. The β-actin protein levels were determined after stripping the membrane and probing with β-actin monoclonal primary antibody (1:5000), with HRP-conjugated anti-mouse IgG as the secondary antibody. Specific bands were detected by chemiluminescence, according to the manufacturer's instructions, and quantified by densitometry.

RhoA activation assay

RhoA activation was measured using a modified ELISA (G-LISA; cytoskeleton, Denver, CO, USA). Mesenteric rings (2–3 pooled vessels per sample) were incubated with 10^{-6} M 5-HT for 10 min. For experiments involving Src kinase inhibition, tissue were pretreated with 10^{-6} M Src kinase inhibitor I for 30 min before the addition of 5-HT. They were flash-frozen, homogenized in lysis buffer (cytoskeleton) and centrifuged at 14 000× *g* for 5 min at 4°C. Activated GTP-bound Rho was measured by absorbance at 490 nm.

Statistical analysis

Data are expressed as means ± SEM. The contractile force developed by mesenteric rings is expressed in g tension·(mg tissue)$^{-1}$. All *n*-values for Western blotting and RhoA activation assays indicate the number of samples (each sample containing 2–3 pooled vessels). Statistical calculations for significant differences were performed by using Student's *t*-test or two-way ANOVA as appropriate. Significance was accepted at $P < 0.05$. Statistical comparisons between concentration–response curves were made using a two-way ANOVA, with Bonferroni's correction for multiple comparisons being performed *post hoc* ($P < 0.05$ again being considered significant).

Materials

5-HT hydrochloride, indomethacin, phenylephrine hydrochloride, L-NNA and monoclonal β-actin antibody were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). U46619 was from Cayman Chemical (Ann Arbor, MI, USA). Chelerythrine chloride, PD98059, PP1 analogue [4-amino-1-*tert*-butyl-3-(1′-naphthyl)pyrazolo[3,4-d]pyrimidine] and Src kinase inhibitor I [4-(4′-phenoxyanilino)-6,7-dimethoxyquinazoline] were from Calbiochem (La Jolla, CA, USA). All drugs were dissolved in saline, unless otherwise noted. U46619 was dissolved in ethanol, while indomethacin was dissolved first in a small amount of 0.1 M Na_2CO_3, then made up to the final volume with distilled water. All concentrations are expressed as the final molar concentration of the base in the organ bath. HRP-linked secondary anti-mouse or anti-rabbit antibody was purchased from Promega (Madison, WI, USA), while antibodies against Rho, ROCK1, ROCK2, ERK1/2 and phosphorylated ERK1/2 (pT202/pY204) were obtained from BD Biosciences (San Jose, CA, USA). Antibodies against phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) (PERM) and Ezrin/Radixin/Moesin (ERM) were obtained from Chemicon (Temecula, CA, USA), while antibodies against phospho-Src (Tyr416) and Src were from Cell Signaling Technology (Danvers, MA, USA). An antibody against the 5-HT_{2A} receptor was obtained from ImmunoStar (Hudson, WI, USA).

Results

General parameters

As shown in Table 1, at the time of the experiment (when the mice were 27–32 weeks old), the body weight of the *ob/ob* mice was higher than that of the age-matched non-diabetic control ('Lean') mice. The plasma glucose and insulin levels were significantly higher in *ob/ob* mice than in Lean mice. The plasma leptin level was below the limit of detection in *ob/ob* mice, but it was 8.9 ± 1.4 ng·mL^{-1} (n = 10) in Lean mice.

Table 1 Values of various parameters in *ob/ob* and Lean mice

	ob/ob *(10)*	*Lean (10)*
Body weight (g)	63.3 ± 2.7*	34.4 ± 0.8
Glucose (mM)	13.6 ± 1.4*	9.3 ± 0.3
Insulin (ng·mL^{-1})	48.2 ± 11.8*	2.0 ± 0.4
SBP (mmHg)	121.1 ± 2.5*	104.2 ± 2.3
Heart rate (beats·min^{-1})	648.1 ± 16.8	645.5 ± 18.6

Values are means ± SEM. Number of determinations is shown within parentheses.
*P < 0.05 versus Lean.
SBP, systolic blood pressure.

Systolic blood pressure was significantly higher in *ob/ob* mice than in Lean mice, although heart rate was similar between the two groups.

Contraction of mesenteric artery to 5-HT is greater in ob/ob *mice than in Lean mice*

5-HT induced concentration-dependent contractions of endothelium-intact mesenteric artery rings obtained from *ob/ob* mice and Lean mice (Figure 1A), but the contraction was significantly enhanced in the *ob/ob* group (vs. the Lean group) (Figure 1A and Table 2). In contrast, although exposure of such rings to U46619 (Figure 1B) or phenylephrine (Figure 1C) led to a concentration-dependent rise in tension in each group (*ob/ob* and Lean), there was no significant difference between these two groups in the response to either agonist.

Differences in NOS and COX-derived substances do not account for enhanced 5-HT-induced contraction in mesenteric arteries from ob/ob *mice*

As 5-HT can stimulate release of NO, which modulates contraction in smooth muscle (Lamping *et al*., 1999), we wondered whether a difference in NO synthesis might account for the above difference between *ob/ob* and Lean mice. Responses to 5-HT were measured after inhibition of NOS (using L-NNA, 10^{-4} M). The 5-HT-induced contraction tended (but not significantly) to be increased by L-NNA in both the *ob/ob* and the Lean group (Figure 2A and Table 2). However, under such acute inhibition of NOS, the magnitude of the difference in 5-HT-induced contractions between *ob/ob* and Lean mice was maintained.

As COX-derived substances modulate vascular tone, they could account for the above diabetes-related difference (Matsumoto *et al*., 2007; 2008b). We therefore examined whether acute inhibition of COX might abolish this difference. The 5-HT-induced contraction was not significantly altered by indomethacin (10^{-5} M) in either the *ob/ob* or Lean group (Figure 2B and Table 2). Further, as was the case for acute NO deficiency (Figure 2A), the contraction to 5-HT in indomethacin-treated mesenteric arteries remained greater in the *ob/ob* group than in the Lean group. These data demonstrate that although NO and COX-derived substances modulate contractions to 5-HT, changes in their release do not account for the differences between *ob/ob* and Lean mice in the 5-HT-induced contractions of mesenteric arteries.

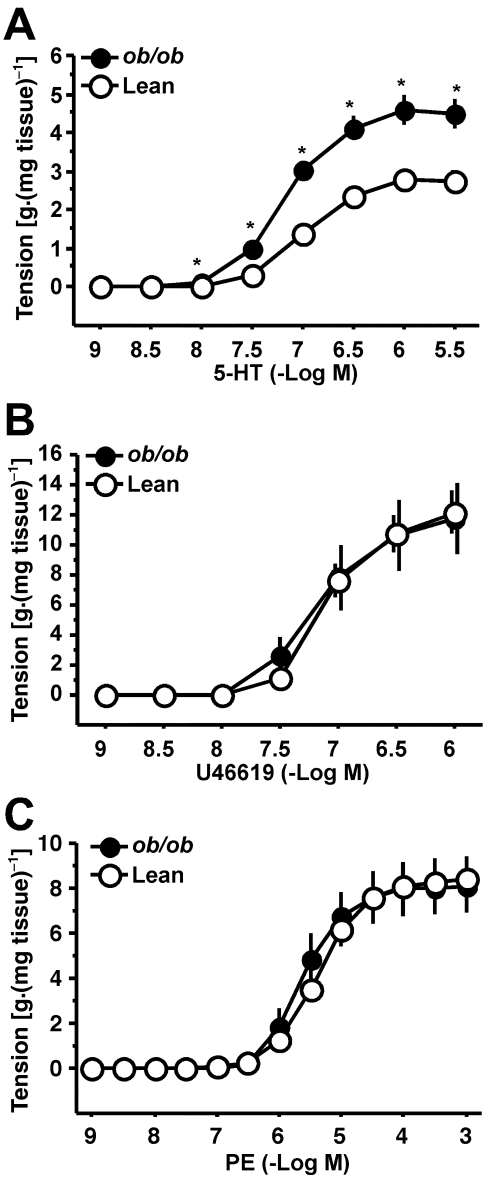

Figure 1 5-Hydroxytryptamine (5-HT)-induced contractile response is enhanced in mesenteric artery rings from *ob/ob* mice. (A) Concentration–response curves for 5-HT (n = 12). (B and C) Concentration–response curves for U46619 (n = 6) (B) and PE (n = 4) (C) showing that the mesenteric artery contractions to these agonists did not differ between *ob/ob* mice and Lean mice. Experimental values are expressed in g tension·(mg tissue)$^{-1}$ (means ± SEM). *P < 0.05 versus Lean. The dose–response curves for 5-HT shown in (A) are shown again as controls in Figures 2, 3, 5 and 7.

PKC and MEK/ERK pathways do not account for greater 5-HT-induced contractions in arteries from ob/ob *mice*

Published evidence suggests that vasoconstrictors activate several ancillary pathways that modulate the primary contractile response (Touyz and Schiffrin, 2000; Somlyo and Somlyo, 2003). Among the many pathways activated, those involving PKC (Banes *et al*., 1999), mitogen-activated protein

Table 2 Maximal responses and EC_{50} values for 5-HT-induced contraction of mesenteric rings from *ob/ob* and Lean mice

Treatment	ob/ob *mice*		*Lean mice*	
	Max. response [g tension·(mg tissue)$^{-1}$]	*–log EC_{50}*	*Max. response [g tension·(mg tissue)$^{-1}$]*	*–log EC_{50}*
Untreated	4.61 ± 0.38 (12)‡	7.17 ± 0.02 (12)‡	2.85 ± 0.22 (12)	6.94 ± 0.04 (12)
L-NNA (10^{-4} M)	5.02 ± 0.44 (5)	7.19 ± 0.10 (5)	3.33 ± 0.27 (5)	7.13 ± 0.04 (5)
Indomethacin (10^{-5} M)	5.19 ± 1.21 (4)	7.07 ± 0.05 (4)‡	3.04 ± 0.34 (4)	6.82 ± 0.07 (4)
Chelerythrine (10^{-6} M)	4.21 ± 0.62 (5)	7.14 ± 0.08 (5)‡	2.78 ± 0.41 (5)	6.83 ± 0.09 (5)
PD98059 (10^{-5} M)	4.35 ± 0.41 (5)‡	6.87 ± 0.08 (5)*	2.24 ± 0.46 (5)	6.58 ± 0.18 (5)†
Y27632 (10^{-7} M)	2.95 ± 0.24 (5)*	6.98 ± 0.04 (5)*	2.86 ± 0.29 (5)	6.87 ± 0.04 (5)
Y27632 (10^{-6} M)	1.59 ± 0.21 (5)*	6.82 ± 0.09 (5)*	1.29 ± 0.23 (5)†	6.75 ± 0.07 (5)
Y27632 (10^{-5} M)	0.91 ± 0.13 (5)*	6.45 ± 0.03 (5)*	0.67 ± 0.16 (5)†	6.40 ± 0.05 (5)†
PP1 analogue (10^{-6} M)	2.88 ± 0.20 (6)*	6.98 ± 0.08 (6)*	3.04 ± 0.14 (6)	6.81 ± 0.03 (6)
Src kinase inhibitor I (10^{-7} M)	3.13 ± 0.18 (4)	6.96 ± 0.04 (4)*	2.78 ± 0.40 (4)	6.82 ± 0.05 (4)

Values are means ± SEM. Number of determinations is shown within parentheses.
*$P < 0.05$ versus untreated *ob/ob* group.
†$P < 0.05$ versus untreated Lean group.
‡$P < 0.05$ versus corresponding Lean group.
5-HT, 5-hydroxytryptamine; L-NNA, N^G-nitro-L-arginine.

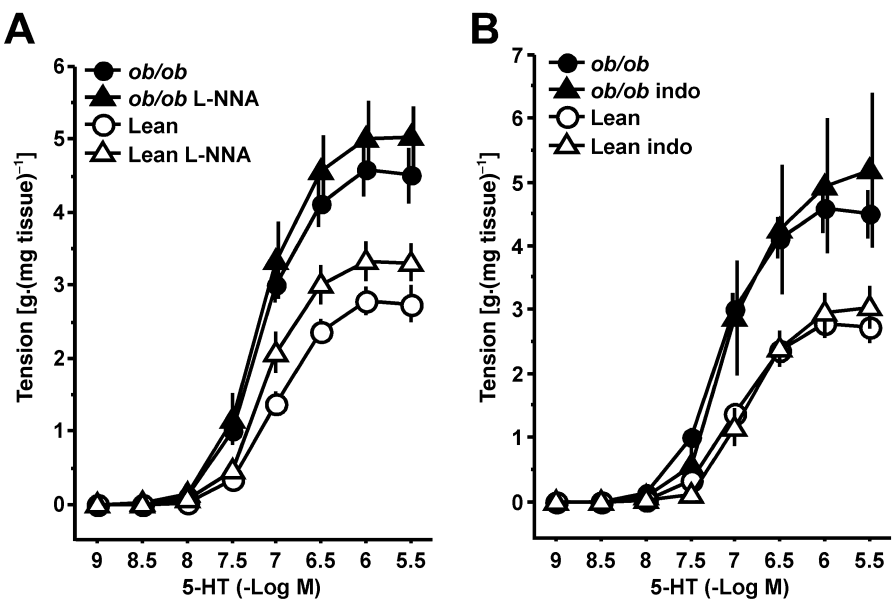

Figure 2 (A) 5-Hydroxytryptamine (5-HT)-induced contractions of mesenteric artery rings from *ob/ob* and Lean mice in the presence and absence of an inhibitor of nitric oxide synthase, N^G-nitro-L-arginine (L-NNA, 10^{-4} M for 30 min). L-NNA tended (not significantly) to increase the contraction in rings from either *ob/ob* ($n = 5$) or Lean ($n = 5$) mice. (B) 5-HT-induced contractions of mesenteric artery rings from *ob/ob* and Lean mice in the presence and absence of an inhibitor of cyclooxygenase, indomethacin (10^{-5} M for 30 min). Indomethacin did not significantly alter the contraction in rings from either *ob/ob* ($n = 4$) or Lean ($n = 4$) mice. Data are presented as means ± SEM.

kinase (Banes *et al*., 1999; Watts, 2002) and Rho/Rho kinase (Shimokawa and Takeshita, 2005; Nuno *et al*., 2007) have been shown to play roles in smooth muscle contraction. First, we examined the effects of the broad-spectrum PKC inhibitor chelerythrine (10^{-6} M) on 5-HT-induced contractions (Figure 3A and Table 2). Chelerythrine did not alter the 5-HT-induced contraction in mesenteric arteries from either *ob/ob* or Lean mice. Moreover, the magnitude of the difference between the *ob/ob* and Lean groups was maintained in the presence of chelerythrine (Figure 3A and Table 2).

5-HT-induced contraction is partly mediated by the MEK/ERK pathway in several vessels (Banes *et al*., 1999; Watts, 2002). However, although such contractions were weaker (*ob/ob* group) or tended to be weaker (Lean group) in mesenteric arteries after inhibition of the MEK/ERK pathway (using PD98059, 10^{-5} M), the magnitude of the difference between the *ob/ob* and Lean groups was maintained (Figure 3B and Table 2). In addition, to assess the activity of the MEK/ERK pathway we compared the levels of phosphorylated ERK1/2 (Figure 4). Western immunoblots were obtained from mesenteric arteries under 5-HT stimulation (10^{-6} M for 10 min). There was no significant difference in the level of phosphorylated ERK1/2 between the *ob/ob* and Lean groups (Figure 4). The above data demonstrate that alterations in the PKC and

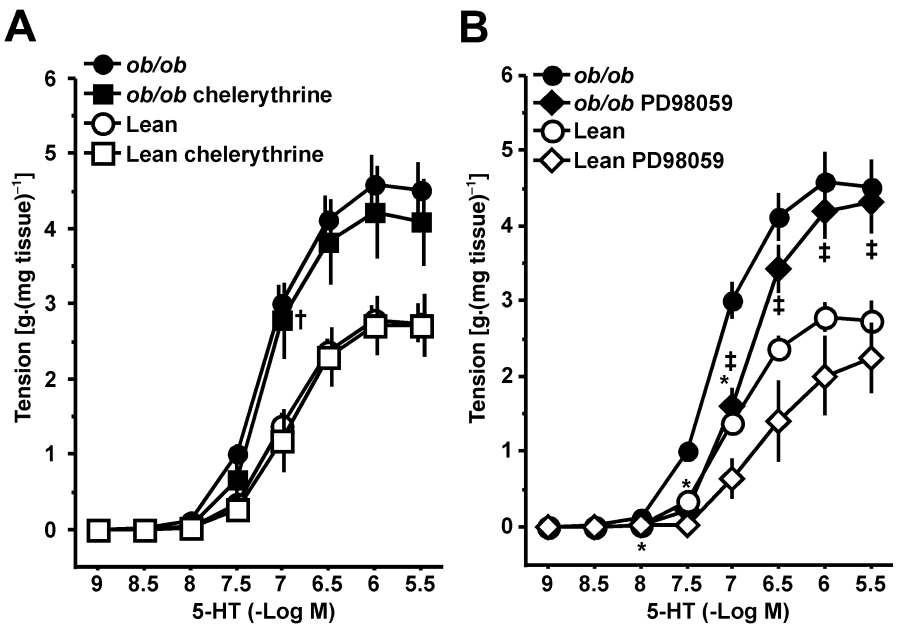

Figure 3 (A) Effects of a PKC inhibitor (chelerythrine, 10^{-6} M for 30 min) on the 5-HT-induced contraction of mesenteric artery rings obtained from *ob/ob* and Lean mice. Chelerythrine did not alter the contraction in either the *ob/ob* ($n = 5$) or Lean ($n = 5$) group, and the response remained greater in the *ob/ob* than in the Lean group (†$P < 0.05$, *ob/ob* with chelerythrine vs. Lean with chelerythrine). (B) Effects of a MEK/ERK pathway inhibitor (PD98059, 10^{-5} M for 30 min) on 5-HT-induced contraction of mesenteric artery rings obtained from *ob/ob* and Lean mice. PD98059 reduced (*ob/ob*) or tended to reduce (Lean) the contraction in each group [*ob/ob* ($n = 5$) and Lean ($n = 5$) mice (*$P < 0.05$, *ob/ob* vs. *ob/ob* PD98059)], but the response remained greater in the *ob/ob* than in the Lean group (‡$P < 0.05$, *ob/ob* with PD98059 vs. Lean with PD98059). Data are presented as means ± SEM. 5-HT, 5-hydroxytryptamine; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated/ERK-activating kinase; PKC, protein kinase C.

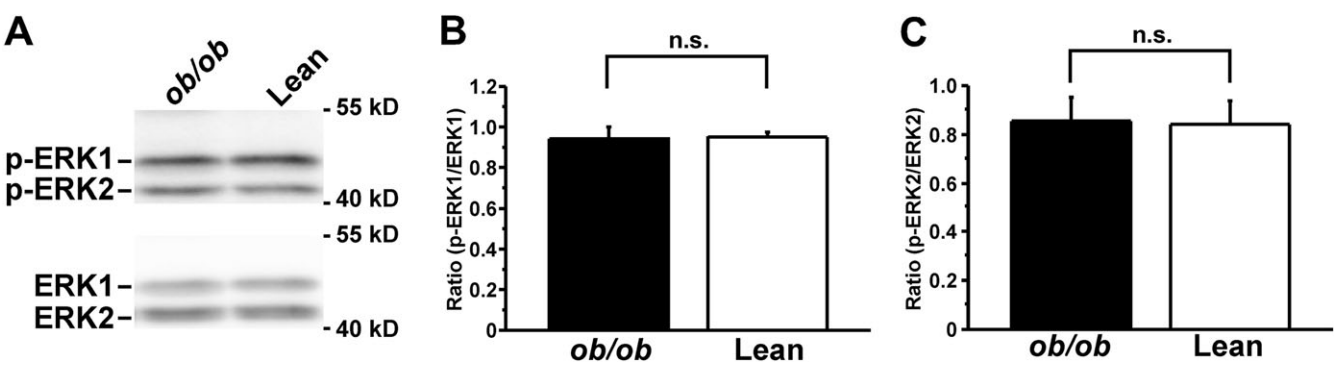

Figure 4 Analysis of phosphorylated extracellular signal-regulated kinase (ERK) and ERK protein expressions in 5-hydroxytryptamine (5-HT)-treated mesenteric arteries. (A) Representative Western blots for phosphorylated ERK1/2 and ERK1/2. (B and C) ERK1 (B) or ERK2 (C) bands were quantified by densitometry, and ratios were calculated for the optical density of phosphorylated ERK1/2 over that of the corresponding ERK1/2. Data are represented as the mean ± SEM ($n = 4$). n.s., not significant.

MEK/ERK pathways do not account for the observed difference between *ob/ob* and Lean mice in the mesenteric artery contraction to 5-HT.

Contraction of mesenteric arteries mediated by Src tyrosine kinase and activation of Src kinase is greater in ob/ob *mice than in Lean mice*

In vascular smooth muscle, Src tyrosine kinases have been implicated in the contractile signalling pathway triggered by 5-HT (Banes *et al*., 1999; Lu *et al*., 2008). We therefore assessed the effect of two Src kinase inhibitors [PP1 analogue (10^{-6} or 10^{-5} M for 30 min) and Src kinase inhibitor I (10^{-7} or 10^{-6} M for 30 min)] on the 5-HT-induced contraction (Figure 5 and Table 2). In the *ob/ob* group, PP1 analogue (Figure 5A) and Src kinase inhibitor I (Figure 5C) each dose-relatedly inhibited the contraction. In the Lean group, neither 10^{-6} M PP1 analogue (Figure 5B) nor 10^{-7} M Src kinase inhibitor I (Figure 5D) affected the 5-HT-induced contraction, but it was completely blocked by a higher dose of either inhibitor. It should be noted that at the lower concentrations used, treatment with either inhibitor abolished the *ob/ob* versus Lean difference in the 5-HT-induced contraction (Figure 5 and Table 2). These data suggest that the enhancement of 5-HT-induced contractions observed in mesenteric arteries from *ob/ob* mice was mediated by an increase in Src kinase activity.

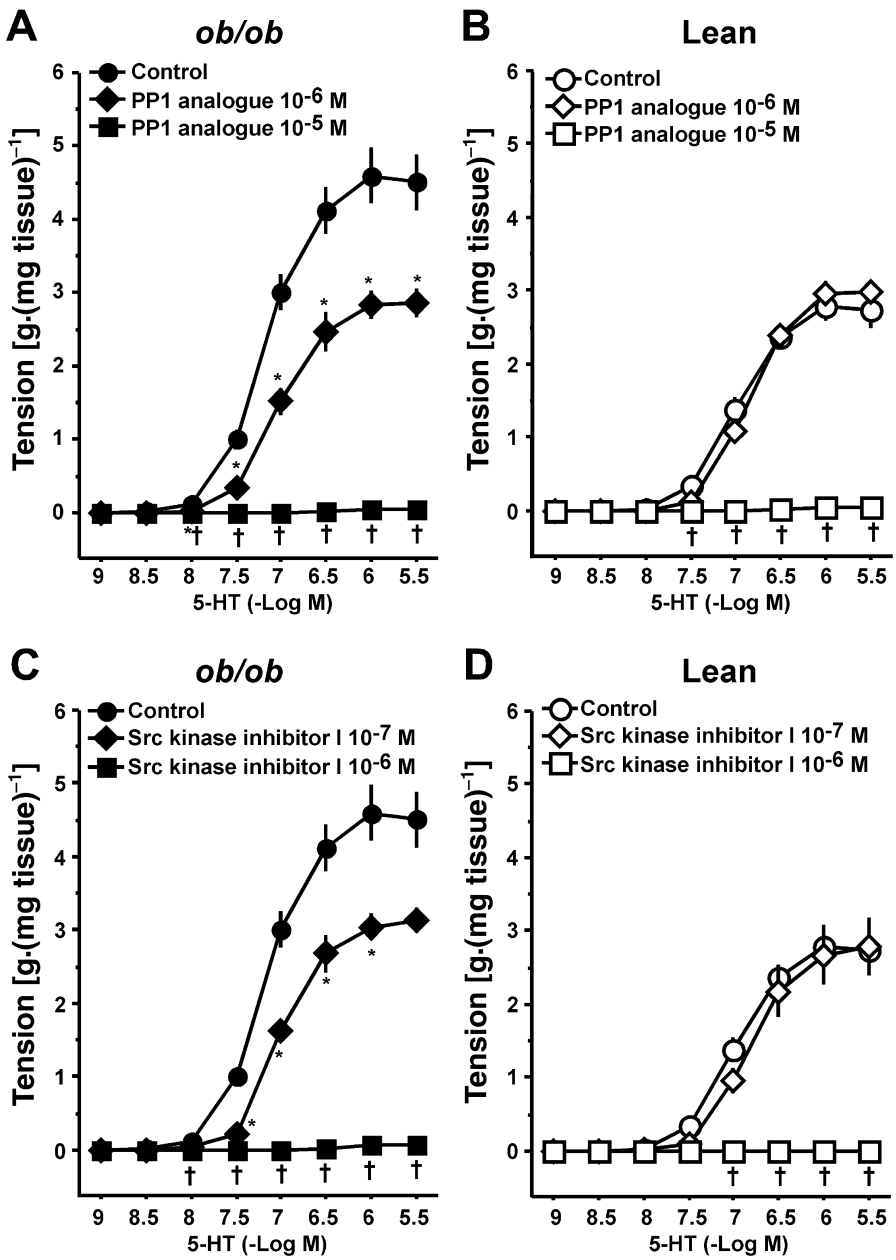

Figure 5 Effects of Src kinase inhibitors (PP1 analogue, 10^{-6} or 10^{-5} M for 30 min; Src kinase inhibitor I, 10^{-7} or 10^{-6} M for 30 min) on 5-hydroxytryptamine (5-HT)-induced contraction of mesenteric artery rings obtained from *ob/ob* (A and C) and Lean (B and D) mice. (A) PP1 analogue dose-relatedly reduced the contraction in the *ob/ob* group [*$P < 0.05$, control vs. PP1 analogue 10^{-6} M ($n = 6$); †$P < 0.05$, control vs. PP1 analogue 10^{-5} M ($n = 6$)]. (B) PP1 analogue abolished the contraction in the Lean group when applied at 10^{-5} M [†$P < 0.05$, control vs. PP1 analogue 10^{-5} M ($n = 6$)], but had no effect at 10^{-6} M ($n = 6$). (C) Src kinase inhibitor I dose-relatedly reduced the contraction in the *ob/ob* group [*$P < 0.05$, control vs. Src kinase inhibitor I 10^{-7} M ($n = 4$); †$P < 0.05$, control vs. Src kinase inhibitor I 10^{-6} M ($n = 4$)]. (D) Src kinase inhibitor I abolished the contraction in the Lean group when applied at 10^{-6} M [†$P < 0.05$, control vs. Src kinase inhibitor I 10^{-6} M ($n = 4$)], but had no effect at 10^{-7} M ($n = 4$). Data are presented as means ± SEM.

Src kinase activity is up-regulated by tyrosine phosphorylation at Tyr416 in the activation loop of the kinase domain (Xu *et al*., 1999). To assess Src kinase activity, we examined the levels of phosphorylated Src (Tyr416) (p-Src). Western immunoblots were obtained from mesenteric arteries under 5-HT stimulation (10^{-6} M for 10 min) (Figure 6). Although total Src expression in mesenteric arteries was similar between *ob/ob* and Lean mice (Figure 6C), the p-Src level was significantly greater in the *ob/ob* than in the Lean group (Figure 6B). These data suggest that an increase in Src kinase activity due to increased phosphorylation at Tyr416 may contribute to the enhancement of 5-HT-induced contraction seen in mesenteric arteries from *ob/ob* mice.

Contraction of mesenteric arteries mediated by Rho kinase is greater in ob/ob *mice than in Lean mice*

5-HT activates RhoA and its effector Rho kinase to regulate calcium sensitivity and vascular smooth muscle contraction

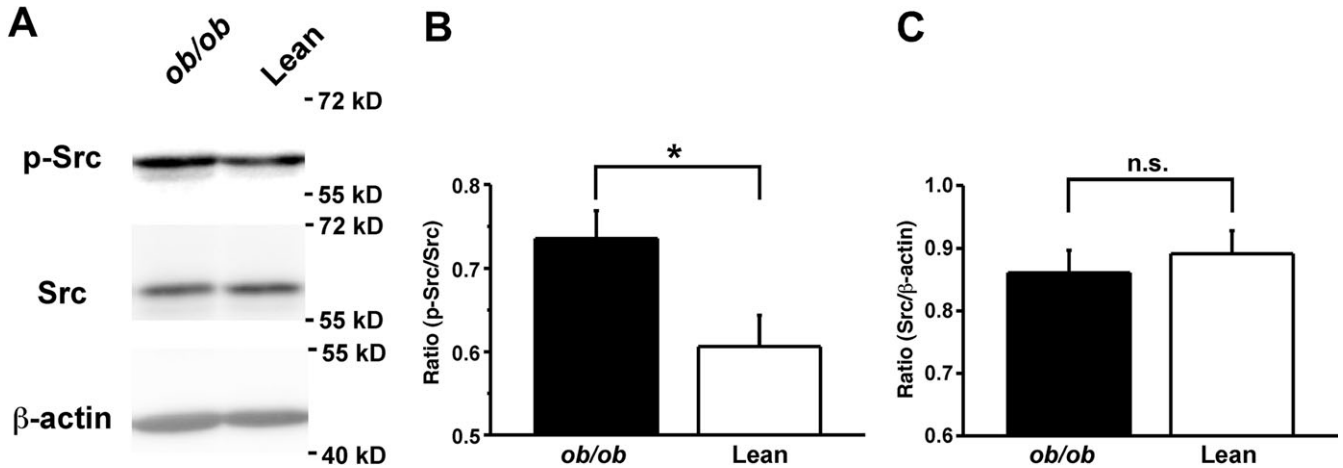

Figure 6 Src kinase activation by 5-hydroxytryptamine (5-HT). (A) Phosphorylated Src (at Tyr416) (p-Src) and total Src levels in mesenteric arteries from *ob/ob* and Lean mice under 5-HT stimulation (10^{-6} M for 10 min). (B) The p-Src level was greater in the *ob/ob* ($n = 4$) than in the Lean ($n = 4$) group (*$P < 0.05$). (C) Expression level of Src (normalized to β-actin) was not different between the *ob/ob* and Lean groups (each, $n = 4$). Data are presented as means ± SEM. n.s., not significant.

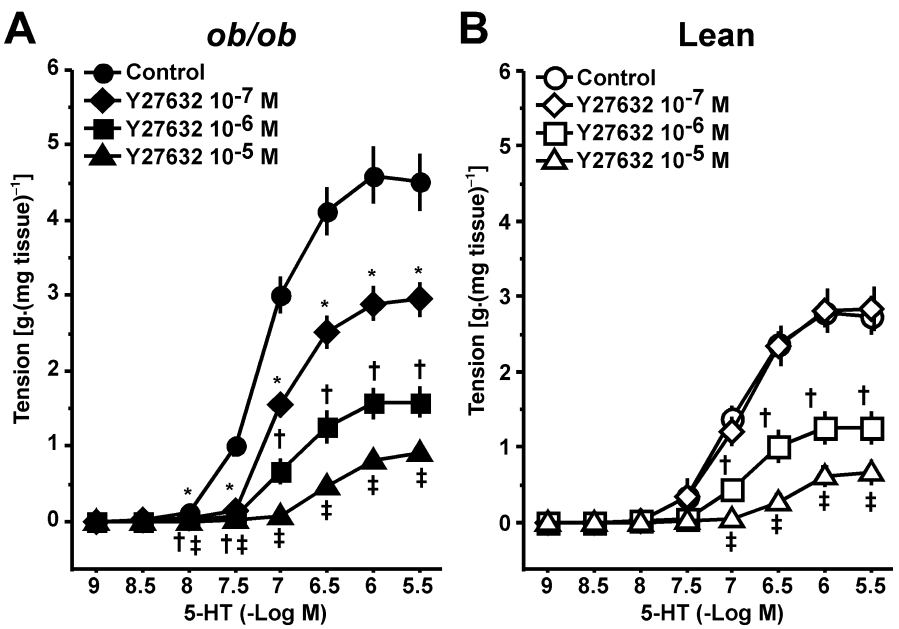

Figure 7 Effects of a Rho kinase inhibitor (Y27632, 10^{-7}–10^{-5} M for 30 min) on the 5-hydroxytryptamine (5-HT)-induced contraction of mesenteric artery rings obtained from *ob/ob* (A) and Lean (B) mice. (A) Y27632 dose-dependently reduced the contraction in the *ob/ob* group [*$P < 0.05$ control vs. Y27632 10^{-7} M ($n = 5$); †$P < 0.05$ control vs. Y27632 10^{-6} M ($n = 5$); ‡$P < 0.05$, control vs. Y27632 10^{-5} M ($n = 5$)]. (B) In the Lean group, the contraction was significantly reduced by Y27632 (10^{-6} and 10^{-5} M), but not by 10^{-7} M Y27632 ($n = 5$) [†$P < 0.05$, control vs. Y27632 10^{-6} M ($n = 5$); ‡$P < 0.05$, control vs. Y27632 10^{-5} M ($n = 5$)]. Data are presented as means ± SEM.

(Nuno *et al*., 2007). We therefore assessed the effect of the Rho kinase inhibitor Y27632 (10^{-7}–10^{-5} M for 30 min) on the 5-HT-induced contraction (Figure 7 and Table 2). In the *ob/ob* group, Y27632 dose-dependently inhibited this contraction (Figure 7A). In the Lean group, Y27632 (10^{-6} and 10^{-5} M) dose-relatedly inhibited the 5-HT-induced contraction, but 10^{-7} M Y27632 had no effect (Figure 7B). It should be noted that at each concentration, Y27632 treatment abolished the difference in the 5-HT-induced contraction between the *ob/ob* and Lean groups (Figure 7 and Table 2). These data suggest that the enhancement of the 5-HT-induced contraction observed in the *ob/ob* group is mediated by an increase in Rho kinase activity.

Activation of Rho kinase by 5-HT is greater in mesenteric arteries from ob/ob *mice than in those from Lean mice*

To assess Rho kinase activity, we measured the level of phosphorylated ezrin, radixin and moesin (PERM), the substrate for Rho kinase (Shimokawa and Takeshita, 2005). Western immunoblots were obtained from mesenteric arteries after 5-HT stimulation (10^{-6} M for 10 min) (Figure 8A). Although

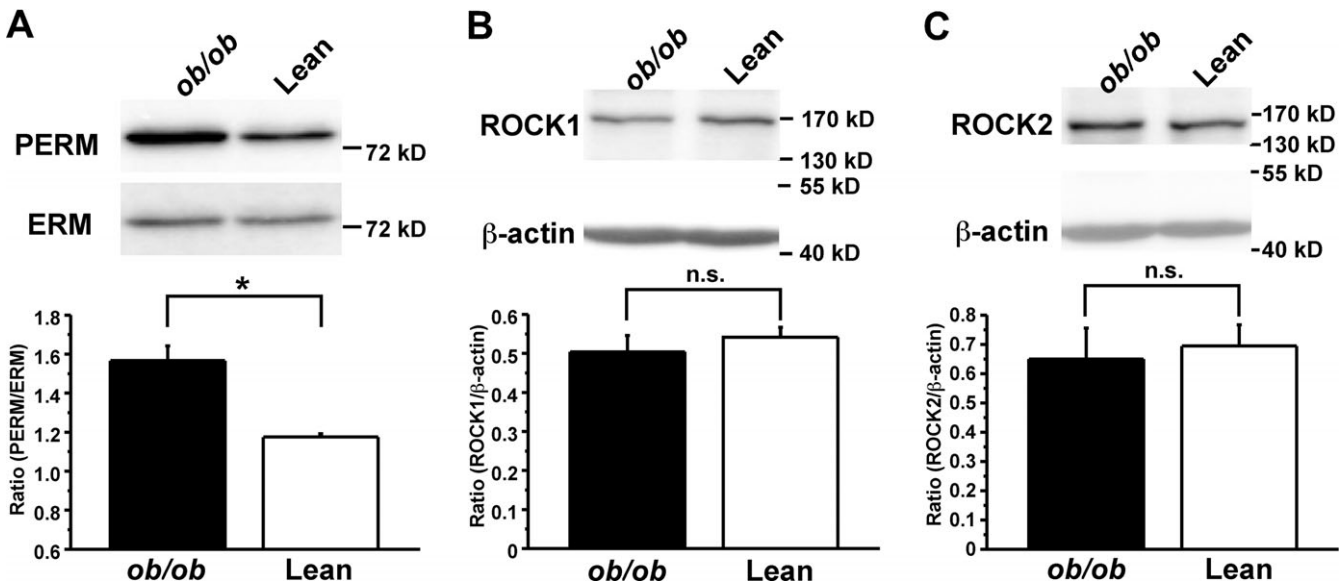

Figure 8 (A) Phosphorylated Ezrin/Radixin/Moesin (PERM) and total ERM levels in mesenteric arteries from *ob/ob* and Lean mice after 5-hydroxytryptamine stimulation (10^{-6} M for 10 min). The PERM level was greater in the *ob/ob* ($n = 4$) than in the Lean ($n = 4$) group (*$P < 0.05$). (B and C) Protein expressions of Rho kinase (ROCK) isoforms in mesenteric arteries from *ob/ob* and Lean mice. Western blots are shown for ROCK1 (B) and ROCK2 (C), and also for β-actin. Top, representative Western blots. Bottom, bands were quantified by densitometry. There was no difference in the level of either ROCK1 or ROCK2 between the *ob/ob* and Lean groups (each, $n = 4$). Data are presented as means ± SEM. n.s., not significant.

total ERM expression in mesenteric arteries was similar between the *ob/ob* and Lean groups (normalized to β-actin; data not shown), the level of PERM was significantly greater in the former than in the latter group (Figure 8A).

To determine whether the increase in Rho kinase activity was due to an increase in the expression of Rho kinase, the expressions of ROCK1 and ROCK2 (Shimokawa and Takeshita, 2005) were compared between *ob/ob* mice and Lean mice. Our Western blot analysis revealed that the expression of each isoform was similar between *ob/ob* and Lean mice (Figure 8B and C). The above data suggest that the enhancement of the 5-HT-induced activation of Rho kinase seen in *ob/ob* mice is not attributable to a difference in the basal expression of Rho kinase, between these two strains of mice.

Activation of RhoA by 5-HT is greater in mesenteric arteries from ob/ob *mice than in those from Lean mice*

5-HT activates the small GTPase RhoA and its effector Rho kinase through a G protein-coupled receptor to regulate the calcium sensitivity of contractile proteins and muscle contraction (Nuno *et al*., 2007; 2009). RhoA serves as a molecular switch in the transduction from extracellular stimuli to the induced changes in the intracellular signalling pathways regulating muscle contractions, organization of the actin cytoskeleton, cell adhesion and motility (Somlyo and Somlyo, 2003). To determine whether a diabetes-induced increase in RhoA activation might account for the increase in Rho kinase activity, we measured RhoA activation in mesenteric arteries after 5-HT stimulation (10^{-6} M for 10 min) using a modified ELISA (Figure 9A). Such RhoA activation was significantly greater in mesenteric arteries from *ob/ob* mice than in those from Lean mice (Figure 9A).

One possible explanation for this finding might be that the expression of RhoA is greater in *ob/ob* mice. However, Western blot analysis revealed that RhoA expression was similar between the two groups of mice (Figure 9B and C). Thus, although both RhoA activation and Rho kinase activation were greater in 5-HT-stimulated mesenteric arteries from *ob/ob* mice than in those from Lean mice, these increases were not related to increases in their expression levels.

To test whether the 5-HT-induced RhoA activation was mediated via increased Src kinase activity, we measured the effect of Src kinase inhibitor I on the 5-HT-induced RhoA activation. As shown in Figure 9A, in mesenteric arteries from *ob/ob* mice, such RhoA activation was significantly suppressed by this inhibitor, while in those from Lean mice, such activation tended (not significantly) to be reduced by the same inhibitor.

Expression of 5-HT$_{2A}$ receptors in mesenteric arteries is similar between ob/ob *and Lean mice*

Conceivably, an increase in the expression of the 5-HT receptors that mediate mesenteric artery contractions could underlie the enhanced vasoconstriction observed in *ob/ob* mice. Because the contraction induced by 5-HT in the mouse aorta is mediated primarily by the 5-HT$_{2A}$ receptor (McKune and Watts, 2001; Nuno *et al*., 2007), we estimated the expression levels of this receptor in mesenteric arteries by immunoblotting. As shown in Figure 10, this expression was similar between the *ob/ob* and Lean groups. Thus, a difference in the 5-HT$_{2A}$ receptor expression level does not account for the enhanced mesenteric vasoconstriction seen in *ob/ob* mice.

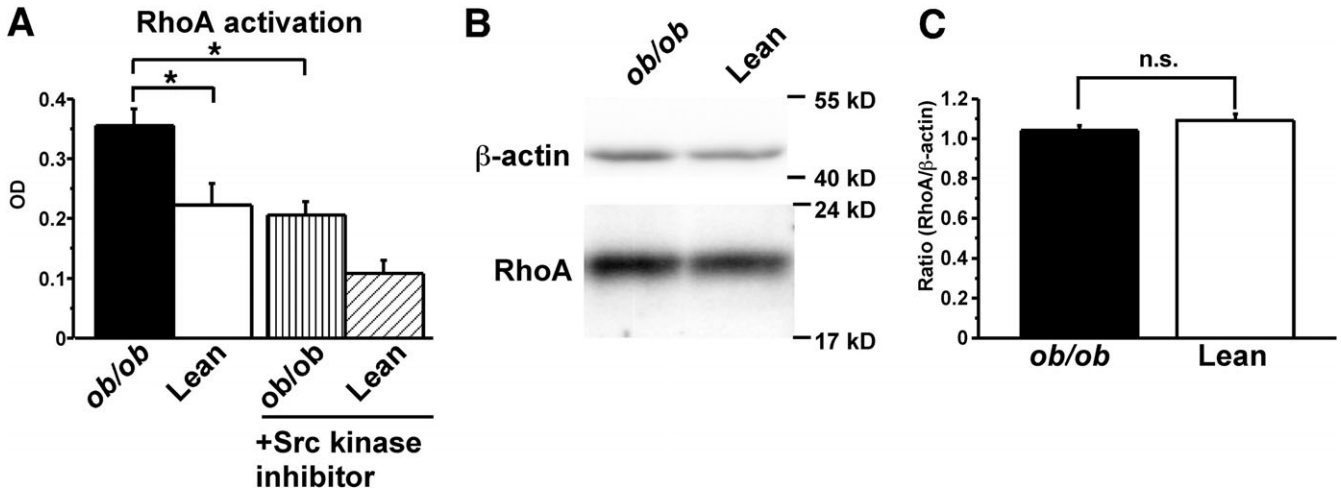

Figure 9 RhoA activation and expression in mesenteric arteries from *ob/ob* and Lean mice. (A) RhoA activation (modified enzyme-linked immunosorbent assay) under 5-hydroxytryptamine stimulation (10^{-6} M for 10 min) in the absence (each $n = 8$) and presence (each $n = 4$) of Src kinase inhibitor I (10^{-6} M). *$P < 0.05$ vs. *ob/ob* group. (B) Representative immunoblots for RhoA and β-actin. (C) Expressions of RhoA over β-actin in mesenteric arteries from *ob/ob* and Lean mice. The expression ratio was not different between the *ob/ob* and Lean group (each $n = 4$). OD, optical density.

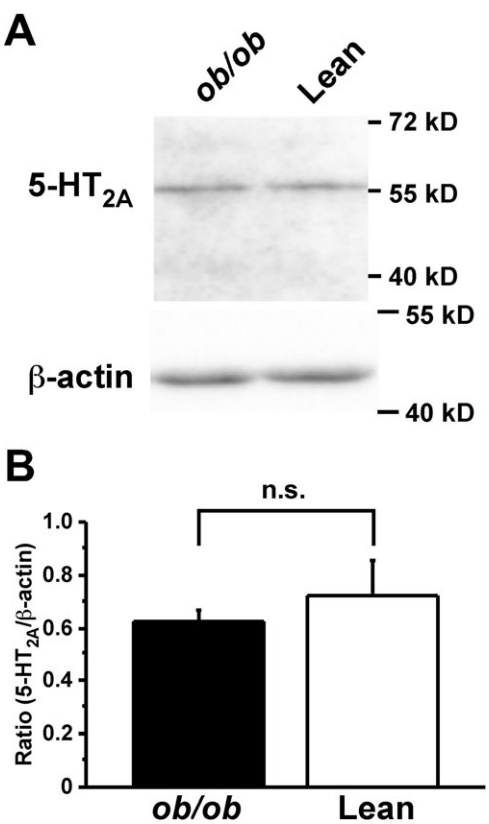

Figure 10 (A) Expressions of the 5-HT_{2A} receptor and β-actin in mesenteric arteries from *ob/ob* and Lean mice. (B) The 5-HT_{2A} receptor expression level (normalized to β-actin) was not different between the *ob/ob* and Lean groups (each, $n = 4$). Data are presented as means ± SEM. 5-HT, 5-hydroxytryptamine; 5-HT_{2A}, 5-HT receptor subtype 2A; n.s., not significant.

Discussion

We made several new findings in this study. First, 5-HT-induced vasoconstriction of superior mesenteric arteries is greater in type 2 diabetic obese *ob/ob* mice at the chronic stage (27–32 weeks old) than in age-matched non-diabetic control Lean mice. Second, the above diabetes-associated alteration is not attributable to a difference between such mice in the modulation by NOS or COX-derived products. Third, expression of the 5-HT_{2A} receptor in mesenteric arteries was similar between the two groups of mice, suggesting that the enhanced vasoconstriction to 5-HT seen in *ob/ob* mice was attributable to alterations in pathway(s) downstream of its receptor in smooth muscle. Fourth, the 5-HT-induced activations of RhoA, Rho kinase and Src kinase were greater in mesenteric arteries from *ob/ob* mice than in those from Lean mice, and inhibition of Rho kinase or Src kinase abolished the observed diabetes-related difference in 5-HT-induced vasoconstriction. These results strongly suggest that changes in the RhoA/Rho kinase and/or Src kinase pathways underlie the enhancement of 5-HT-induced mesenteric vasoconstriction seen in type 2 diabetic *ob/ob* mice.

Several studies from different laboratories have examined 5-HT-induced vasoconstriction in various arteries from animal models of diabetes. However, the vascular reactivity to 5-HT has been variously reported to be decreased (Head *et al.*, 1987), increased (Hattori *et al.*, 1995; Nuno *et al.*, 2009) or unchanged (Agrawal and McNeill, 1987) during the development of diabetes. The reasons for this discrepancy are not yet clear, but are generally attributed to the wide variations in the experimental conditions used.

Increased vascular contractile responses to various agonists have been reported in type 2 diabetic vessels (Winters *et al.*, 2000; Okon *et al.*, 2003; Matsumoto *et al.*, 2009). The leptin-deficient *ob/ob* mouse, which is used as a model of type 2 diabetes, exhibits obesity, hyperinsulinemia and insulin

resistance (Konstantinides *et al*., 2001). Previous reports have demonstrated that abnormalities of vascular function are present in several arteries in *ob/ob* mice (Winters *et al*., 2000; Okon *et al*., 2003). Here, we used such mice at the chronic stage of diabetes (27–32 weeks old) because long-term diabetic conditions are associated with severe diabetic complications involving cardiovascular dysfunction, and because no previous study has investigated whether 5-HT-induced contraction is abnormal in mesenteric arteries in the established phase of diabetes. Regarding the phenylephrine-induced contraction in mesenteric arteries from *ob/ob* mice, Okon *et al*. (2003) found that this contraction was increased in the second branches of mesenteric arteries from 8-week-old *ob/ob* mice (vs. those from age-matched control mice). On the other hand, Winters *et al*. (2000) reported that the phenylephrine-induced contraction in mesenteric microvessels was similar between control and *ob/ob* mice at 3–6 months of age, a result consistent with the finding made in the present study (in vessels from mice 27–32 weeks old). The discrepancy among these studies may be explained by differences in the duration of the diabetes.

In addition to endothelial dysfunction, vascular smooth muscle dysfunction has been implicated in diabetes-related vascular hyperreactivity. In our endothelium-intact vascular preparations, although the 5-HT-induced contraction tended to be slightly potentiated by a NOS inhibitor in mesenteric arteries from both groups, the contractile response seen after blockade of NOS remained stronger in tissues isolated from *ob/ob* mice than in those from Lean mice (Figure 2). In addition, several reports have suggested that altered COX activities may contribute to the underlying vasomotor abnormalities in diabetic animal models (Matsumoto *et al*., 2007; 2008b). However, our data suggest that the observed difference between *ob/ob* and Lean mice in the 5-HT-induced contraction did not result from any alteration of COX activities because the 5-HT-induced contraction was not modified by indomethacin treatment in either group of mice. Although both the ACh-induced endothelium-dependent relaxation and the endothelium-derived hyperpolarizing factor (EDHF)-mediated relaxation were reduced in mesenteric arteries from *ob/ob* mice versus those from Lean mice (our unpublished observations), the above results strongly suggest that alterations in NOS- and COX-derived substances do not account for the clear increase in the vascular contractile response to 5-HT seen in *ob/ob* mice. Indeed, this is partly supported by the previous finding that the presence of the endothelium profoundly depresses 5-HT-induced contractions in the rabbit isolated basilar artery, and that this phenomenon can be fully accounted for by the endothelial release of NO, with neither EDHF nor COX products playing a significant role (Trezise *et al*., 1992).

A novel, intriguing and potentially important finding made in this study was that the enhancement of 5-HT-induced contraction seen in mesenteric arteries from *ob/ob* mice may be secondary to marked augmentations of Rho kinase and Src kinase signalling in these arteries. Although 5-HT-induced contraction has been reported to be mediated by various kinases in smooth muscle (Banes *et al*., 1999; McKune and Watts, 2001; Watts, 2002; Nuno *et al*., 2007; Lu *et al*., 2008), the above interpretation is supported by four lines of evidence. First, in our pharmacological studies we found that both Rho kinase inhibition and Src kinase inhibition decreased the 5-HT-induced contraction in mesenteric arteries from each group of mice. Notably, when we used low concentrations of such inhibitors (i.e. those with no inhibitory effects on 5-HT-induced contraction in Lean mice), the 5-HT-induced contraction in the *ob/ob* group was reduced to the level seen in the Lean mice. Moreover, the residual 5-HT-induced contractions (i.e. the contraction resistant to a given concentration of inhibitor) were similar between the *ob/ob* and Lean groups. Use of a PKC inhibitor did not affect the 5-HT-induced contraction in either group, and although a MEK/ERK pathway inhibitor reduced the 5-HT-induced contraction, the contraction remaining in the presence of the latter inhibitor was greater in *ob/ob* mice than in Lean mice. Second, 5-HT-induced ERK activation was similar between the *ob/ob* and Lean groups. Third, 5-HT-induced RhoA and Rho kinase activations were increased in *ob/ob* mice, but this was not associated with increased basal expressions of RhoA and Rho kinases (i.e. ROCK1 and ROCK2). This is supported, for instance, by evidence that the enhanced aortic contraction to 5-HT seen in male (vs. female) mice is attributable to an increase in RhoA/Rho kinase activation in the smooth muscle that is independent of any difference in the basal expression of RhoA or Rho kinase (Nuno *et al*., 2007). Fourth, phosphorylation of Src (Tyr416) positively regulates Src kinase activity (Xu *et al*., 1999), and the increase in such phosphorylation under 5-HT stimulation was greater in *ob/ob* than in Lean mice (Figure 6). To judge from these results, the increased 5-HT-induced contraction seen here in *ob/ob* mice may be attributable to increased Rho kinase and Src kinase signalling.

The major finding in this study was that the mechanisms underlying the enhancement of the 5-HT-induced contraction in mesenteric arteries from *ob/ob* mice may involve augmentation of the Src kinase and RhoA/Rho kinase pathways. In coronary artery smooth muscle, the sphingosylphosphorylcholine-dependent regulation of contraction appears to be linked to Src upstream of Rho kinase, because Src autophosphorylation is resistant to Y27632 (Nakao *et al*., 2002). Further, Montezano *et al*. (2008) found that the increased RhoA activation induced by angiotensin II plus aldosterone in vascular smooth muscle cells was largely suppressed by a c-Src inhibitor. Moreover, Lu *et al*. (2008) reported that the idea that Src activation is an early mechanism upstream of Rho kinase in the 5-HT-induced aortic contractile pathway was supported by the finding that the Rho kinase inhibitor Y27632 did not inhibit Src kinase activity, a conclusion consistent with the localization of c-Src at the myocyte plasmalemma. In the present study, the 5-HT-induced RhoA activation was reduced by a Src kinase inhibitor (Figure 9C). Taking the above evidence and our data together, we suggest that Src activation may be an early mechanism upstream of Rho kinase in the 5-HT-induced contractile pathway in mouse mesenteric arteries.

An accumulating body of evidence indicates that 5-HT is involved in diabetic complications. For example, blood 5-HT concentrations are elevated in diabetics (Malyszko *et al*., 1994), and there is evidence that the pathogenesis of diabetic nephropathy involves the 5-HT_{2A} receptors on mesangial cells

(Kasho *et al*., 1998). In small-scale clinical trials on diabetic patients (Takahashi *et al*., 2002), sarpogrelate hydrochloride, a selective 5-HT_{2A} receptor antagonist, has been reported to have a renoprotective effect. Moreover, the severity of hindlimb ischaemia in patients with peripheral arterial disease, the prevalence of which is higher in diabetics than in non-diabetics (Doggrell, 2003), depends in part upon the ability to mobilize collateral vessels, and recruitment of collateral vessels has been shown to be compromised by an excessive vasoconstrictor reactivity to 5-HT in various models of hindlimb ischaemia (Doggrell, 2003). Further, Janiak *et al*. (2002) demonstrated that 5-HT receptor blockade improved distal perfusion after lower limb ischaemia in the fatty Zucker rat, a strain characterized by obesity, hyperglycaemia, hyperinsulinemia and hyperlipidaemia. These pieces of evidence suggest that the regulation of 5-HT signalling and function could represent an important therapeutic target in case of diabetic vasculopathy. A further point of interest is that the present *ob/ob* mice, at the chronic stage of diabetes, exhibited an elevated systolic blood pressure. 5-HT plays an important role in hypertension (Watts, 2002; 2005), and treatment with a 5-HT receptor antagonist (Nagatomo *et al*., 2004) can reduce blood pressure in hypertensive patients and models. As yet, it remains unclear whether there is a direct relationship between hypertension and the abnormalities of 5-HT-mediated contraction seen in our diabetic *ob/ob* mice. Further work will be required on this point; for example, on time-related changes in blood pressure and in blood 5-HT levels, and in the vascular responsiveness to 5-HT, in animals in a developing diabetic state. Taking the above evidence and the present data together, we suggest that manipulations of 5-HT signalling, for instance by inhibition of Rho kinase and/or Src kinase activities, could have important potential for the future treatment of diabetic vasculopathy and the associated hypertension.

In conclusion, the present study provides evidence that increased activities of Rho kinase and Src kinase may make particularly important contributions to the vascular hyperreactivity to 5-HT displayed by type 2 diabetic *ob/ob* mice at the chronic stage, which exhibit hypertension. As abnormal vascular smooth muscle responses may contribute to the aetiology of diabetic vascular complications, our study raises the possibility that manipulations of 5-HT signalling, for example by inhibition of vascular Rho kinase and/or Src kinase activities, may help prevent vascular complications developing in type 2 diabetes.

Acknowledgements

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Conflict of interest

The authors state no conflict of interest.

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