

COX-2 Up-regulation and vascular smooth muscle contractile hyperreactivity in spontaneous diabetic *db/db* mice

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Abstract

Objective: Abnormalities in vascular constriction and dilatation are associated with early diabetes and contribute to diabetic vascular complications. However, mechanisms underlying such vascular dysfunction remain to be fully elucidated. The current study tests the role of cyclooxygenase-2 (COX-2) in diabetic vascular smooth muscle dysfunction.

Methods: Small mesenteric artery and aorta are isolated from type 2 diabetic *db/db* and control mice. Isometric contractions in response to serotonin, angiotensin II, phenylephrine and high potassium are determined in small spiral mesenteric arterial or aortic strips. COX-2 mRNA and protein levels are analyzed by using DNA microarray, real-time PCR and immunoblot.

Results: Contractions induced by serotonin, angiotensin II, phenylephrine and high potassium are significantly higher in endothelium-denuded smooth muscle strips isolated from *db/db* mice than in those isolated from control mice. The contractile hyperreactivity is observed in aortic and third-order branch small mesenteric arterial smooth muscle strips. DNA microarray, real-time PCR and immunoblot analysis show that compared with control mice, COX-2 mRNA and protein are significantly increased in *db/db* mice aortic smooth muscle. The COX-2 up-regulation is temporally associated with the development of diabetes mellitus and vascular smooth muscle contractile hyperreactivity. Inhibition of COX-2 with NS-398 or SC-58125 partially—but significantly—alleviates agonist-induced but not potassium-induced contractile hyperreactivity. In addition, serum isolated from *db/db* mice induces COX-2 expression and increases thromboxane A₂ production in primary cultured vascular smooth muscle cells (VSMC). SQ-29548, a TP receptor antagonist, diminished the *db/db* mice vascular smooth muscle contractile hyperreactivity.

Conclusions: COX-2 is up-regulated and contributes at least in part to the vascular smooth muscle contractile hyperreactivity in *db/db* mice.

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Keywords: Vascular smooth muscle; Contraction; Type 2 Diabetes; Cyclooxygenase-2

1. Introduction

Sixteen million Americans have type 2 diabetes, and vascular complications are the most common causes of morbidity and mortality in diabetic patients. Among these

diabetic vascular abnormalities are alterations in blood vessel constriction and dilatation (vascular reactivity) that have been detected in type 2 diabetic patients [1–6] and type 2 diabetic animal models [7–16]. Such abnormalities in vascular reactivity can alter blood flow and peripheral vascular resistance and thereby contribute to diabetic retinopathy, nephropathy, neuropathy and increased rates of hypertension. Therefore, the vascular reactivity under type 2 diabetic conditions has been the subject of many studies. Endothelium dysfunction has been well-docu-

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mented and is recognized as an important factor in causing decreased vasodilation and increased vasoconstriction responses [17–21]. In contrast, the dysfunction of vascular smooth muscle and its contribution to the abnormal vascular reactivity under type 2 diabetic conditions is less clear and remains to be fully explored.

Cyclooxygenase (COX) is a rate-limiting enzyme that catalyzes the conversion of free arachidonic acid and O₂ into prostaglandin H₂, the committed step in the biosynthesis of prostaglandins and thromboxane. Prostaglandins and thromboxane are potent vasoconstrictors and vasodilators that play an important role in regulating vascular function. Of the prostaglandins produced in the vascular wall, prostaglandin I₂ (PGI₂) relaxes while thromboxane A₂ (TxA₂), prostaglandin F_{2α} (PGF_{2α}) and prostaglandin H₂ (PGH₂) contract vascular smooth muscle. In addition, COX is the target of commonly used non-steroidal anti-inflammatory drugs such as aspirin and ibuprofen. There are two major isoforms of COX: COX-1 and COX-2 [22]. COX-1 is constitutively expressed in many tissue and cell types. Conversely, COX-2 is not expressed in most tissues under physiological conditions, but its expression can be rapidly and markedly induced [23]. Under physiological conditions, vascular smooth muscle expresses COX-1 whereas COX-2 expression is undetectable. Under type 2 diabetic conditions, it is unknown if COX-2 expression is induced in vascular smooth muscle; if it is induced, the role COX-2 plays in vascular smooth muscle dysfunction remains unknown.

Db/db mice are a well-characterized and extensively used type 2 diabetic mouse model [24]. The syndrome in *db/db* mice is similar to that found in type 2 diabetic patients. *Db/db* mice manifest insulin resistance and compensatory hyperinsulinemia starting at 2 weeks of age. They become obese at around 4 weeks and develop hyperglycemia at around 7 weeks [25,26]. The obesity and complex diabetic mellitus in *db/db* mice is caused by an inability to respond to leptin due to a point mutation in the leptin receptor gene [27–29]. Leptin is an adipocyte-derived hormone that binds to the leptin receptor, resulting in weight loss by reducing appetite and food intake and increasing energy expenditure.

In the present study, isometric contraction measurements were combined with microarray, real-time PCR and immunoblot analysis to investigate vascular smooth muscle dysfunction under type 2 diabetic conditions. Specifically, we tested the hypothesis that COX-2 was induced in vascular smooth muscle under type 2 diabetic conditions and that the up-regulated COX-2 contributed to diabetic vascular smooth muscle dysfunction. To selectively investigate the role of vascular smooth muscle, endothelium-denuded vascular smooth muscle preparations were isolated from *db/db* and control mice. Our results show that COX-2 plays an important role in vascular smooth muscle hyperreactivity in *db/db* mice.

2. Materials and methods

2.1. Animals and materials

Female C57BL/KsJ *db/db* mice (*db/db* $-/-$) and age/gender-matched non-diabetic (*db/db* $+/?$) C57BL/KsJ control mice were purchased from Jackson Lab (Bar Harbor, ME). The mice were housed at an animal care facility at the Medical Center of the University of Kentucky that is accredited by the American Association for Accreditation of Laboratory Animal Care. Male Sprague–Dawley rats were purchased from Harlan (Indianapolis, IN). All animal protocols were approved by the Institutional Animal Care and Use Committee. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication NO. 85-23, revised 1996). The mice were fed a standard diet and RO water ad libitum until they were used at 4, 8 or 12 weeks of age. Other chemicals and reagents were purchased from Sigma (St. Louis, MO) or Fisher (Pittsburgh, PA).

2.2. Measurement of blood glucose and plasma insulin

Blood glucose was determined by using a One Touch Ultra Blood Glucose Meter with a glucose test strip (LifeScan, Inc., Milpitas, CA). The mice were then anesthetized and blood was collected. Serum was separated by centrifugation and stored at -20 °C until use. Serum insulin was measured using an ELISA kit (Crystal Chem Inc., Chicago, IL) according to the manufacturer's instructions.

2.3. Isometric tension measurement

After removing connective tissue, mouse thoracic aortas and the third order branch of mesenteric arteries were cut into small spiral strips (about 3 mm in length and 1.6 mm in width for aortic strips, or 400 μ m in width for mesenteric arterial strips). Endothelium was denuded and verified by the losses of acetylcholine-induced relaxation. Isometric contractions were measured as described previously [30].

2.4. Sectioning of vascular smooth muscle tissue

The muscle strips were immediately stored in 10% formalin after the completion of the isometric contraction measurements, embedded in O.C.T. Compound (Tissue Tek, Sakura Finetek, U.S.A., Inc. Torrance, CA) and cut into 8 μ m thick cross-sections. The sections were stained with hematoxylin (Biomedica Corp., Foster City, CA). Images of the cross-sections were captured on a Spot Camera (Diagnostic Instruments) and medium smooth muscle areas were measured using Image-Pro Software (Media Cybernetics, Silver Springs, MD).

Table 1

Age-related changes in body weight, blood glucose, and serum insulin in *db/db* and control (+/?) mice

	4–5 week-old		8–9 week-old		12–13 week-old	
	Control	<i>db/db</i>	Control	<i>db/db</i>	Control	<i>db/db</i>
Body weight (g)	10.5±0.56	12.6±0.43*	18.8±0.69	40.8±0.98***	24.4±0.83	45.6±1.8***
Blood glucose (mg/dl)	198±15.3	154±8.4	190±18.2	591±9.4***	158±10.1	606±1.4***
Plasma insulin (ng/ml)	1.4±0.19	23.8±8.68*	2.6±1.05	13.6±4.29*	1.1±0.14	4.6±1.13***

Values are mean±S.E.M., $n=3$ to 10. Statistic comparisons are done between *db/db* mice and their respective age-matched controls. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

2.5. Immunoblot analysis

COX-2 and smooth muscle α -actin were detected by immunoblot as described previously (anti-COX-2: BD Biosciences, San Diego, CA; anti- α -actin: Sigma, St. Louis, MO) [31].

2.6. DNA microarray

Thoracic and abdominal aortas were harvested from 12- to 13-week-old *db/db* and control mice and immediately

placed in RNAlater solution. After carefully removing the adventitia and endothelium, RNA was extracted using an RNeasy mini-kit (Qiagen, Valencia, CA). RNA samples from three mice were pooled to obtain a sufficient quantity for one DNA array. The reverse transcription, biotin-labeling, fragmentation and hybridization to Affymetrix MGU-74A chip were carried out by the University of Kentucky Microarray Core Facility according to the Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). A total of 9 *db/db* mice and 9 control mice were used. Three pairs of DNA arrays were run.

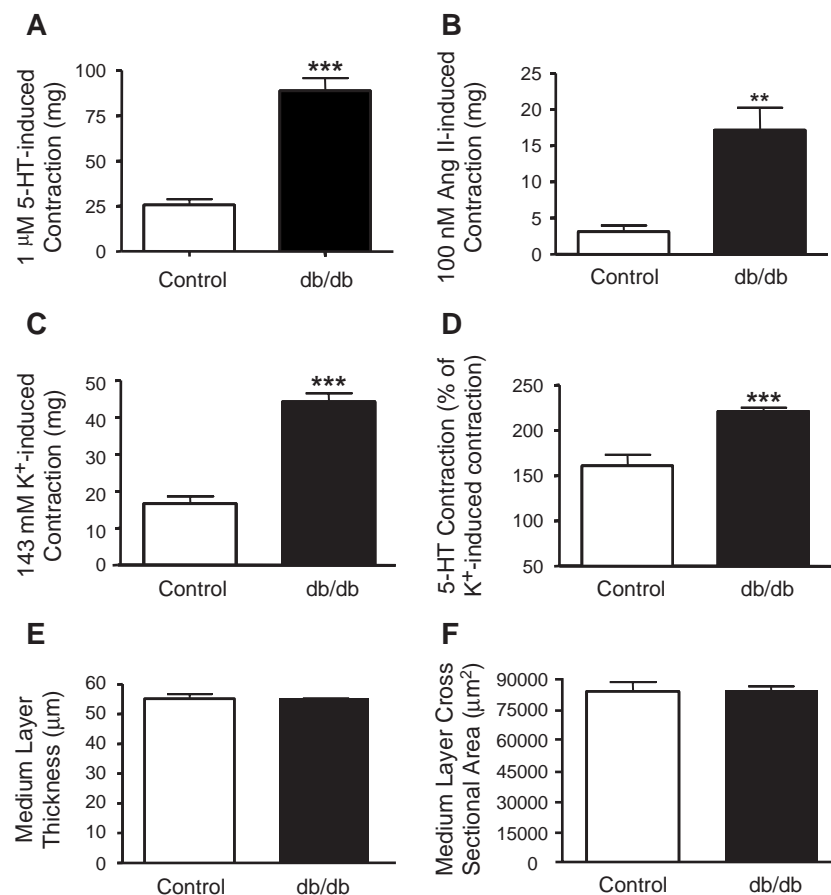


Fig. 1. Enhanced contractile responses in the absence of an increase in medium smooth muscle area in the endothelium-denuded aortic smooth muscle strips isolated from *db/db* mice. The thoracic aortas isolated from 8- to 9-week-old female *db/db* and control mice were cut into spiral strips, denuded of endothelium and stimulated with 1 μ M 5-HT, 100 nM Ang II or 154 mM potassium. Contractions were expressed as absolute force (mg, A, B, and C) or normalized to potassium-induced contractions (%). D). After completion of the contractile study, the muscle strips were fixed and the medium layer thickness (E) and cross-sectional areas (F) were determined. $N=5-7$. * $p<0.05$, ** $p<0.01$, *** $p<0.0001$.

2.7. Real-time PCR

Total RNA was isolated from aortas as described above. Genomic DNA contamination was removed by DNase DNA-free™ kit (Ambion, Austin, TX). The cDNA was synthesized by a SuperScript™ kit (Invitrogen, Carlsbad, CA) using random hexamers. The real-time PCR primers used were: 5'-AAG GCA GAG GCA GTT GGA TCT-3' (forward), 5'-CAT GGC TGG CCT AGA ACT CAC T-3' (reverse) for mouse COX-1; 5'-CCA GCA CTT CAC CCA TCA GTT-3' (forward), 5'-ACC CAG GTC CTC GCT TAT GA-3' (reverse) for mouse COX-2; 5'-AGA AAC GGC TAC CAC ATC CAA-3' (forward), 5'-GGG TCG GGA GTG GGT AAT TT-3' (reverse) for mouse 18S rRNA. The real-time PCR reactions were performed using a QuantiTect™ SYBR Green PCR kit (Qiagen, Valencia, CA) in an ABI Prism 7700 Sequence Detection System. The specificity of the PCR was verified by dissociation curve analysis, agarose gel electrophoresis, and DNA sequencing. COX-1 and COX-2 mRNAs were normalized to the 18S rRNA and relatively quantified by standard curve analysis. Two controls, one with no template and one with no reverse transcription, were included in each real-time PCR run to ensure no contamination of genomic DNA.

2.8. Primary cultured rat vascular smooth muscle cells (VSMC)

The procedure for isolation and culture of primary VSMC from male Sprague–Dawley rats' thoracic aorta

was adapted from a well-established method [32]. The smooth muscle identity was verified by expression of smooth muscle maker proteins: smooth muscle α -actin, myosin-heavy chains, calponin, and caldesmon (all antibodies were purchased from Sigma, St. Louis, MO) using immunocytochemistry. To test the effect of serum on COX-2 protein expression and on thromboxane A₂ production, post-confluent primary cultured cells were starved for 96 h. The starvation medium was replaced for 24 h with medium containing 10% serum isolated from *db/db* or from control mice. Then the COX-2 protein was analyzed by immunoblot. The TxB₂, stable metabolites of TxA₂, were assayed using a commercially available ELISA kit (Neogene Corp., Lexington, KY) according to the manufacturer's instructions.

2.9. Statistical analysis

Each experiment was repeated a minimum of 3 times. Data were expressed as mean \pm SEM. Statistical analysis was performed by using an unpaired *t*-test.

3. Results

3.1. Body weight, blood glucose and serum insulin in *db/db* and control mice

Body weight, non-fasting blood glucose and serum insulin were measured in 4- to 5-, 8- to 9- and 12- to 13-

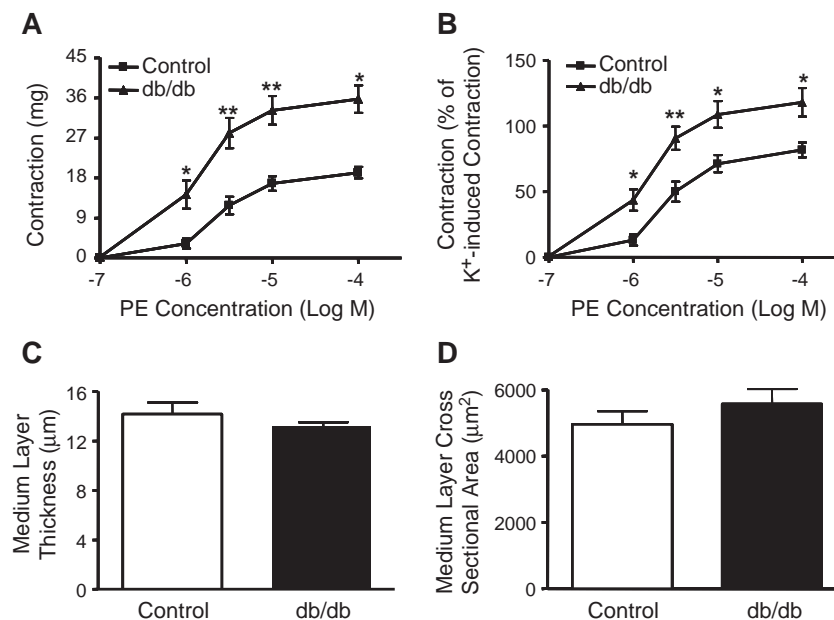


Fig. 2. Increased phenylephrine (PE)-induced contractions in the absence of an increase in medium smooth muscle area in third-order branch mesenteric arterial strips isolated from *db/db* mice. The third-order branch mesenteric arterials isolated from 12- to 13-week-old female *db/db* and control mice were cut into spiral strips and denuded of endothelium. Cumulative PE concentration–response were expressed as absolute force (mg, A) or normalized to potassium-induced contractions (% of K⁺-induced contraction, B). Then the muscle strips were fixed and the medium thickness (C) and cross-sectional area (D) were determined. *N* = 7 for *db/db* and 4 for control mice. **p* < 0.05, ***p* < 0.01.

week-old *db/db* and control mice. As shown in Table 1, at 4 weeks of age, *db/db* mice were overweight, exhibited severe hyperinsulinemia but did not have hyperglycemia. By 8 weeks of age, *db/db* mice exhibited obesity and hyperglycemia in addition to hyperinsulinemia. Similar differences remained at 12–13 weeks of age (Table 1). Consistent with literature reports [25,26], these data show that the *db/db* mice used in the present study manifest obesity, hyperinsulinemia and hyperglycemia.

3.2. Contractile responses were significantly enhanced in vascular smooth muscle tissue isolated from *db/db* mice

To determine if the contractile responses are enhanced in *db/db* mice vascular smooth muscle tissue, we determined phenylephrine (PE)- and 5-HT-induced contractions in aorta and third-order branch mesenteric artery strips.

As shown in Fig. 1A and B, maximal concentrations of 5-HT (1 μ M) and Ang II (100 nM) induced markedly higher contractions in aortic strips isolated from 8- to 9-week-old *db/db* mice than in those isolated from control mice. To test

if agonist-induced contractions were selectively enhanced, we determined potassium depolarization-induced contractions. As shown in Fig 1C, potassium (143 mM) also induced higher contractions in strips isolated from *db/db* mice than in those from control mice. Interestingly, 5-HT-induced contractions remained significantly higher in strips isolated from *db/db* mice than in those from control mice even when they were normalized to respective potassium depolarization-induced contractions (Fig. 1D).

To test a possible role for increased smooth muscle mass in the enhanced contractile response, we examined the medium thickness and medium cross-sectional area in the same aortic strips in which the isometric contractions were measured. As shown in Fig. 1E and F, no significant difference in the medium thickness and cross-sectional area were observed between strips isolated from *db/db* and control mice.

To determine if the observed aortic smooth muscle contractile hyperreactivity was also present in small arteries, we isolated third-order branch mesenteric arteries from 12- to 13-week-old *db/db* and control mice. As shown in Fig. 2A, the phenylephrine concentration–response curve

Table 2
Expression levels of contraction-related genes in *db/db* and control mouse thoracic aorta

Gene category name	Accession number	Mean int in control mice	Mean int in diabetic mice	Fold change (<i>db/db</i> /control)	<i>p</i> value
<i>COXs</i>					
COX-1	M34141	696.13	511.93	0.74	NS
COX-2	M88242	283.20	840.93	2.97	*
<i>Channels and pumps</i>					
Potassium inwardly-rectifying channel (J)	AI314692	38.13	255.70	6.71	*
L-type Ca channel (alph 3)	X94404	1410.27	1841.40	1.31	NS
Potassium inwardly-rectifying channel (J 8)	D88159	2388.90	2683.23	1.12	NS
L-type Ca channel (alph 1c)	U17869	1483.70	1521.30	1.03	NS
Potassium voltage-gated channel (H)	AF012871	5804.13	5985.27	1.03	NS
Potassium voltage-gated channel, (S2)	AF008574	256.70	252.53	0.98	NS
Potassium channel (K1)	AF059576	770.60	493.37	0.64	NS
Sodium/calcium exchanger (member 1)	AF004666	4256.47	4475.53	1.05	NS
Sarco(endo)plasmic reticulum calcium ATPase (SERCA2)	AF029982	7378.37	6746.83	0.91	NS
Sarcoendoplasmic reticulum Ca ²⁺ ATPase (SERCA3b)	U49393	713.73	386.20	0.54	*
<i>Receptors</i>					
Thromboxane A2 receptor	D10849	1260.40	1867.43	1.48	NS
Adrenergic receptor, alpha 1b	Y12738	67.60	94.53	1.40	NS
Adrenergic receptor, alpha 1a	AF031431	108.90	135.93	1.25	NS
5-HT receptor (4)	Y09588	635.00	785.57	1.24	NS
5-HT receptor (5A)	Z18278	913.57	1020.27	1.12	NS
<i>Signaling molecules</i>					
ROCK2	U58513	5242.87	6223.93	1.19	NS
ROCK1	U58512	5536.33	6187.33	1.12	NS
RhoA	AF014371	11867.53	11506.83	0.97	NS
PP1C	M27073	13588.37	13023.70	0.96	NS
Thromboxane A synthase 1	L18868	741.20	714.83	0.96	NS
<i>Contractile proteins</i>					
Myosin 1c	U96723	2203.93	2587.17	1.17	NS
Alpha-actin	X13297	39011.27	40167.27	1.03	NS

Values are mean \pm S.E.M., *n*=3 for each group. Each sample consists of RNA pooled from 3 mice. Statistic comparison were carried out between *db/db* mice and their respective age-matched controls. **p*<0.01 and NS (not significant): *p*>0.01.

shifted to the left in strips isolated from *db/db* mice compared with those isolated from control mice. Potassium also induced higher contractions in strips isolated from *db/db* mice than in those from control mice (31 ± 2.2 mg vs 24 ± 2.0 mg, $p < 0.05$). The phenylephrine-induced contractions remain significantly different when normalized to their respective high potassium-induced contractions (Fig. 2B). Similar to aorta, no difference was detected in the medium thickness and cross-sectional areas between mesenteric arterial strips isolated from *db/db* and control mice (Fig. 2C and D).

In our initial experiments, we have determined both phenylephrine- and 5-HT-induced contractions in aorta and in mesenteric artery strips. Similar contractile hyperreactivity in response to phenylephrine and to 5-HT were observed in aortic as well as in mesenteric artery strips. The phenylephrine-induced contractions in aortic and the 5-HT-induced contractions in mesenteric artery strips are small in amplitude. Therefore, we used 5-HT in aorta and phenylephrine in mesenteric artery in subsequent experiments.

3.3. COX-2 mRNA and protein were up-regulated in vascular smooth muscle in *db/db* mice

To elucidate the molecular mechanisms underlying the observed vascular smooth muscle contractile hyperreactivity, the gene expression patterns in aortic smooth muscle tissue isolated from 12- to 13-week-old *db/db* and control mice were determined and compared using DNA microarray analyses (Affymetrix). In more than 12,000 genes checked, 103 genes showed an over 2-fold up- or down-regulation that was statistically significant. The expression levels of known contraction-related genes are listed in Table 2. Among them, COX-2 and the potassium inwardly-rectifying channel are significantly increased, whereas the expression of sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA3b) is significantly decreased. Since the potassium inwardly-rectifying channel mediates smooth muscle relaxation [33,34], the increased expression of potassium inwardly-rectifying channel in *db/db* mice may be a compensatory response. COX-2, a key enzyme in the synthesis of prostanoids and thromboxane, was up-regulated 3-fold in *db/db* mice aorta. In contrast, COX-1 showed a tendency to decrease (0.7-fold of the control level, Table 2 and Fig. 3A). Interestingly, genes downstream of COX-2 such as thromboxane A_2 synthase and thromboxane-prostanoid receptor (TP receptor) were not significantly different between *db/db* mice and control mice (Table 2).

To confirm that COX-2 mRNA was indeed selectively up-regulated, COX-2 and COX-1 mRNA were also quantified by using real-time PCR. Consistent with the microarray results, COX-2 mRNA was significantly increased whereas the COX-1 mRNA was not significantly different between the aortas from *db/db* and control mice (Fig. 3B). Furthermore, to determine if the increased COX-2 mRNA resulted in COX-2 protein increase, we immunoblotted the

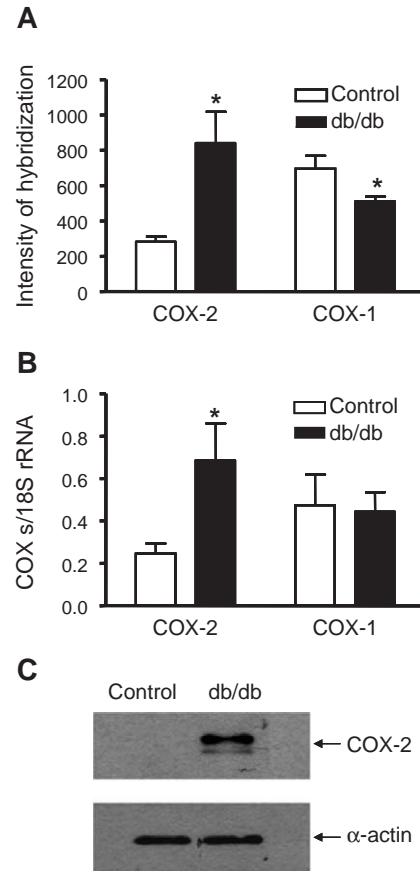


Fig. 3. COX-2 is up-regulated at the mRNA (A and B) and protein (C) level in *db/db* mice vascular smooth muscle. Total RNA was extracted from 12- to 13-week-old *db/db* and control mice. DNA microarray hybridization signal intensities are presented in A and real-time PCR qualifications of the COX-2/COX-1 mRNA were normalized to 18S rRNA and presented in B. A representative immunoblot of COX-2 protein is presented in C. $N=5-7$. * $p < 0.05$.

COX-2 protein in aortas isolated from *db/db* and control mice. In the aortas isolated from control mice, COX-2 protein was hardly detectable (Fig. 3C). Conversely, the COX-2 protein was markedly induced and readily detectable in the aortas from *db/db* mice.

3.4. The vascular smooth muscle contractile hyperreactivity in *db/db* mice was temporally associated with type 2 diabetic mellitus and COX-2 up-regulation

To test a possible causative relationship between diabetes mellitus and vascular smooth muscle hyperreactivity, we first investigated the time course of vascular smooth muscle hyperreactivity. As shown in Fig. 4A, at 4–5 weeks of age, when the *db/db* mice were pre-diabetic, 5-HT induced contractions only showed a small increase in aortas isolated from *db/db* mice. At 8–9 or 12–13 weeks of age, when the *db/db* mice exhibit severe obesity, hyperglycemia and hyperinsulinemia, dramatic differences in 5-HT-induced contractions were detected between strips isolated from *db/db* and control mice (Fig. 4C and E). The EC_{50} s were

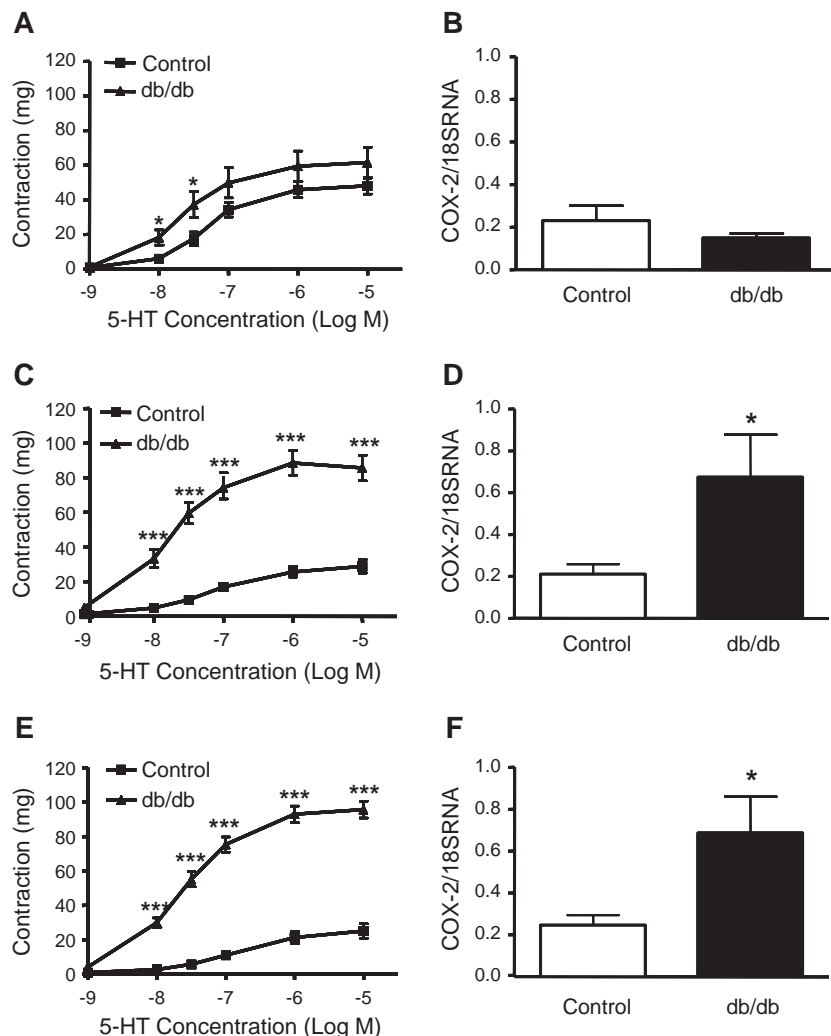


Fig. 4. Age-dependent contractile hyperreactivity (A, C, E) and COX-2 mRNA up-regulation (B, D, F) in *db/db* mice aortic smooth muscle tissue. Cumulative 5-HT concentration-response curves (A, C, E) and real-time PCR quantification of COX-2 mRNA (B, D, F) were carried out in two separate sets of endothelium-denuded aortic strips isolated from different ages of *db/db* and control mice. A & B: 4–5 weeks; C & D: 8–9 weeks; E & F: 12–13 weeks. $n=5-7$. * $p<0.05$, ** $p<0.01$, *** $p<0.0001$.

-7.7 ± 0.27 ($n=10$) in 8- to 9-week-old *db/db* mice and -7.1 ± 0.25 ($n=10$, $p<0.0001$) in control mice. The maximal responses were 96.0 ± 19.6 mg ($n=10$) in 8- to 9-week-old *db/db* mice and 28.9 ± 10.74 mg ($n=10$, $p<0.0001$) in control mice. Interestingly, 5-HT-induced maximal contractions increased with the age of *db/db* mice but the maximal contractions decreased as the age in control mice increased (Fig. 4A and C); this suggests that the aging of the mice did not account for the observed vascular smooth muscle hyperreactivity. In addition, we tested if leptin directly affects vascular smooth muscle contractility as the leptin receptor is expressed in smooth muscle. Acute leptin treatment (50 nM, 30 min) neither causes vascular smooth muscle contraction nor affects phenylephrine-induced contractions in mesenteric artery strips isolated from normal control mice (data not shown).

To test a possible association between COX-2 up-regulation and vascular smooth muscle hyperreactivity, we determined the time course of COX-2 mRNA up-regulation

and compared it with the time course of diabetes mellitus and vascular smooth muscle contractile hyperreactivity. As shown in Fig. 4 and Table 1, at 8–9 and 12–13 weeks of age, the COX-2 mRNA expression level in *db/db* mice aorta showed a marked increase (Fig. 4D and F) when diabetic mellitus were severe (Table 1) and the vascular smooth muscle contractile hyperreactivity was dramatic (Fig. 4C and E). In contrast, at 4–5 weeks of age, COX-2 mRNA levels were comparable in *db/db* and control mice aortas when *db/db* mice were pre-diabetic (Table 1) and the vascular smooth muscle contractile responses were only slightly increased in response to low concentration of 5-HT (Fig. 4A).

3.5. Inhibition of COX-2 activity alleviated vascular smooth muscle contractile hyperreactivity

To further test a possible causative relationship between the COX-2 up-regulation and vascular smooth muscle hyperreactivity, we investigated the effect of COX-2 in-

inhibition on 5-HT-induced vascular smooth muscle contractile hyperreactivity. We first determined the effects of indomethacin, a potent inhibitor of COX-1 and COX-2, on the contractile hyperreactivity. As shown in Fig. 5C, indomethacin significantly inhibited the 5-HT-induced contractions. To further investigate the contribution of COX-2 to the contractile hyperreactivity, we used two structurally different selective COX-2 inhibitors, NS-398 and SC-58125. The IC_{50} of NS-398 and SC-58125 for ovine or murine COX-2 are over 200- to 1400-fold lower than the IC_{50} for COX-1 [35,36] and have been used to selectively inhibit COX-2 in a system similar to the current study [37–39]. Thirty min pre-incubation with 3 μ M NS-398 (Fig. 5A) or 10 μ M SC-58125 (Fig. 5B) significantly alleviated the contractile hyperreactivity to 5-HT in aortic strips isolated from *db/db* mice without significantly affecting the contractions in strips isolated from control mice. Interestingly, neither NS-398 nor SC-58125 significantly inhibited K^+ -

induced contractions (NS-398: 12 ± 5.2 vs. 8 ± 3.5 in control and 41 ± 7.6 vs. 36 ± 8.5 in *db/db* mice; SC-58125: 14 ± 7.1 vs. 14 ± 9.8 in control and 41 ± 5.1 vs. 40 ± 5.1 in *db/db* mice).

To determine if COX-2 up-regulation is selectively involved in 5-HT-induced contractile hyperreactivity in *db/db* mice, we determined the effects of the COX-2 inhibitor NS-398 on Ang II- and PE-induced contractions. As shown in Fig. 6, NS-398 (3 μ M, 30 min pre-incubation) significantly inhibited Ang II- and PE-induced hyperreactivity in *db/db* mice aorta and mesenteric artery strips, respectively.

3.6. COX-2 up-regulation in primary cultured vascular smooth muscle cells (VSMC) by diabetic serum increased TxA_2 production

In the whole animal, multiple factors may up-regulate COX-2. To test if diabetic serum can directly act on smooth

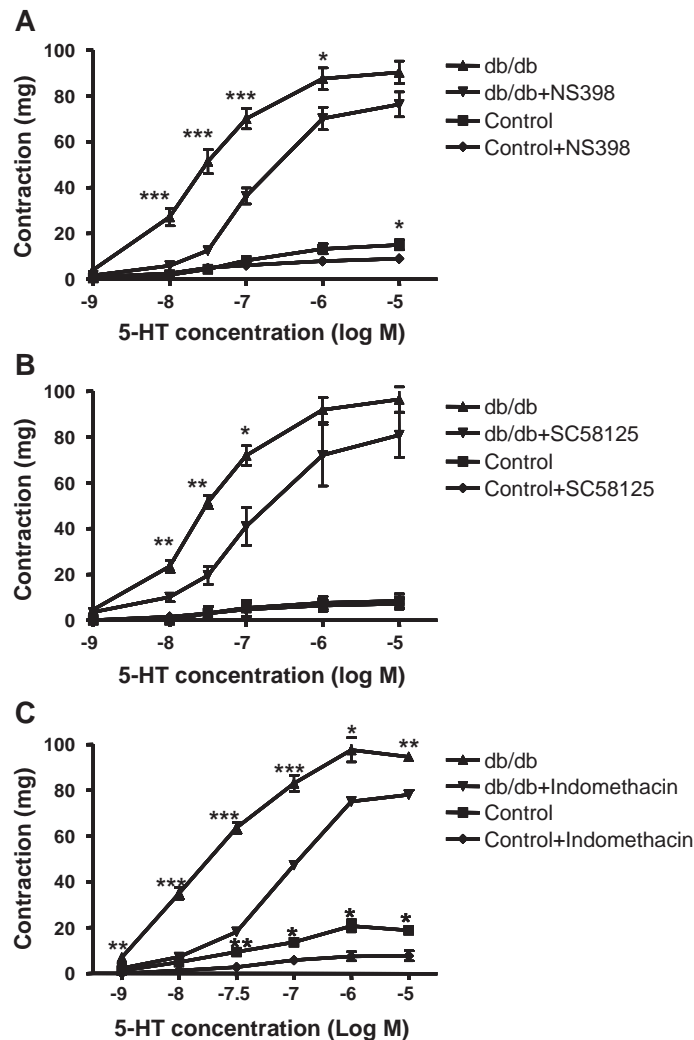


Fig. 5. Inhibition of COX-2 diminishes the vascular smooth muscle contractile hyperreactivity in response to 5-HT. Cumulative 5-HT concentration–response curves were determined in endothelium-denuded aortic strips isolated from 12- to 13-week-old *db/db* or control mice. Then the strips were incubated with 3 μ M NS398 (A) or 10 μ M SC58125 (B) or 10 μ M indomethacin (C) for 30 min, and the cumulative 5-HT concentration–response curves were obtained again in the presence of the inhibitors. $n=5-7$. * $p<0.05$, ** $p<0.01$, *** $p<0.0001$.

muscle cells to up-regulate COX-2, we incubated primary cultured VSMC with serum isolated from 12 to 13-week-old *db/db* or control mice. Serum isolated from diabetic *db/db* mice induced much higher COX-2 protein expression than serum isolated from control mice in VSMC (Fig. 7A).

To test if the COX-2 up-regulation is associated with TxA₂ production increase, we measured TxB₂ (a stable metabolite of TxA₂) in cells after incubation with diabetic serum. Accompanied with the COX-2 up-regulation (Fig. 7A), diabetic serum dramatically increased TxB₂ production (Fig. 7B).

To directly demonstrate that TxA₂ can potentiate agonist-induced smooth muscle contractions, we determined the contractions induced by low concentrations of U-46619 alone (1 nM, a TP receptor agonist), 5-HT alone (10 nM) and U-46619 plus 5-HT. As shown in Fig. 7C, 1 nM U-46619 induced a small contraction but it significantly enhanced 5-HT-induced contractions.

3.7. TP receptor mediates part of the vascular smooth muscle contractile hyperreactivity in *db/db* mice

To test the possibility that increased production of the contractile prostaglandins TxA₂/PGH₂ by COX-2 up-regulation mediates the contractile hyperreactivity, we determined the effects of SQ-29548, a TP receptor antagonist, on 5-HT- and PE-induced contractions. As shown in Fig. 8, SQ-29548 (10 μM, 30 min pre-incubation) partially but significantly inhibited 5-HT- and phenylephrine-induced contractile hyperreactivity in *db/db* mice aortic and mesenteric artery strips.

4. Discussion

4.1. Vascular smooth muscle hyperreactivity in type 2 diabetes

Increased vascular contractile responses to various agonists or transmural pressure have been reported in

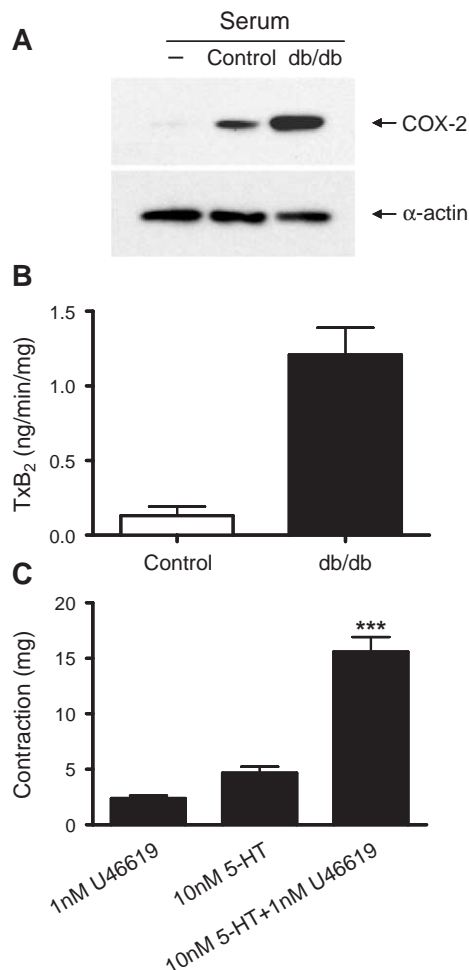


Fig. 7. Serum isolated from *db/db* mice induces COX-2 protein expression (A) and increases thromboxane A₂ (TxA₂) production (B). Post-confluent primary cultured rat aortic smooth muscle cells were starved for 96 h and then incubated with medium containing 10% serum isolated from diabetic *db/db* or from control mice. (A) The COX-2 protein expression was analyzed by immunoblot. (B) The medium TxB₂ was assayed using an ELISA kit according to the manufacturer's instructions. (C) U-46619, a TP receptor agonist, potentiates 5-HT-induced contraction in aortic smooth muscle strips. The thoracic aortic strips isolated from control mice were stimulated with 1 nM U46619 or 10 nM 5-HT or both. *n* = 4–8. ****p* < 0.001.

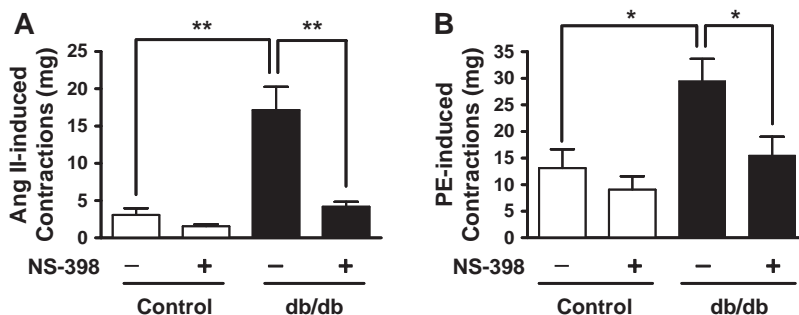


Fig. 6. Inhibition of COX-2 diminishes the vascular smooth muscle contractile hyperreactivity in response to angiotensin II and phenylephrine. (A) control and *db/db* mice aortic strips were treated with 3 μM NS-398 or vehicle for 30 min. Then 100 nM angiotensin II (Ang II)-induced contractions were obtained. (B) 10 μM phenylephrine-induced contractions were determined in endothelium-denuded third order branch mesenteric artery strips isolated from 12- to 13-week-old *db/db* or control mice. Then the strips were incubated with 3 μM NS-398 for 30 min, and the phenylephrine-induced contractions were obtained again in the presence of the NS-398. *n* = 4–6. **p* < 0.05, ***p* < 0.01.

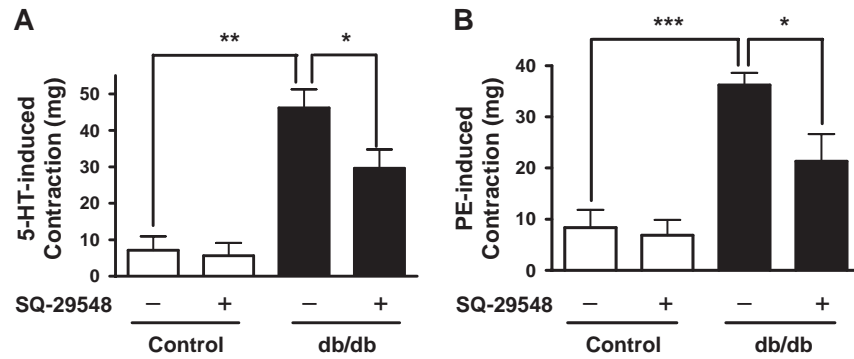


Fig. 8. Blocking TP receptor by SQ-29548 diminishes 5-HT- and phenylephrine-induced vascular smooth muscle contractile hyperreactivity in *db/db* mice. Aortic (A) or third order branch mesenteric artery strips (B) isolated from control or *db/db* mice were stimulated by 30 nM 5-HT or 10 μ M PE. Then the strips were incubated with 10 μ M SQ-29548 for 30 min and the 5-HT and PE responses were obtained again in the presence of SQ-29548. $n=4-7$. ** $p<0.01$, *** $p<0.0001$.

thoracic aorta [10–12,14] and mesenteric arteries [13,40] isolated from *db/db* mice when compared with those isolated from control mice. In addition to endothelium dysfunction, vascular smooth muscle dysfunction has been implicated in vascular hyperreactivity. In endothelium-intact vascular preparations, the contractile responses after blockage of the endothelium-derived nitric oxide (NO) production remain significantly higher in tissue isolated from *db/db* mice than in that from control mice [14]. This suggests that the basal eNOS activity is not altered in *db/db* mice. Furthermore, the ACh-induced endothelium-dependent relaxation is only slightly attenuated in tissue isolated from *db/db* mice compared with that from control mice [12]. Such a small difference in endothelium-dependent relaxation could not account for the dramatic increases in vascular contractile responses. By using endothelium-denuded vascular smooth muscle preparations, the present study provides clear evidence that compared to control mice, vascular smooth muscle isolated from *db/db* mice is indeed hyperreactive in response to agonist and high-potassium stimulations. However, our results do not exclude the possibility that long-term endothelium dysfunction under diabetic conditions can alter gene expression patterns in vascular smooth muscle and consequently cause contractile hyperreactivity.

Multiple mechanisms including alterations in receptors, signaling mechanisms, channels, contractile proteins and smooth muscle mass may contribute to the observed enhancement of agonist- and K^+ -induced contractions in arterial smooth muscle tissue isolated from *db/db* mice. K^+ -depolarization initiates smooth muscle contraction mainly by increasing cytoplasmic Ca^{2+} whereas agonists induce smooth muscle contraction by increasing cytoplasmic Ca^{2+} and by increasing the sensitivity of the contractile apparatus to Ca^{2+} (Ca^{2+} -sensitization). The enhancements of both K^+ -induced and agonist-induced contractions, even when the agonist-induced contractions were normalized to their respective K^+ -induced contractions, suggest dysfunction in mechanisms regulating intracellular Ca^{2+} as well as Ca^{2+} -sensitization in *db/db* mice. For example, the significant decrease in the sarcoendoplasmic reticulum Ca^{2+} -ATPase

mRNA (SERCA 3b) detected by microarray, if paralleled by a decrease in protein function, may prolong the cytoplasmic free Ca^{2+} increase and contribute to high K^+ - and agonist-induced contractile hyperreactivity. Further, different agonists may initiate contraction by activating divergent signaling pathways, and activation of two or more receptors may induce synergistic force output. For example, 5-HT and U-46619 caused synergistic contractions (Fig. 6C). And finally, autocrine and/or paracrine mechanisms also are altered and can contribute to the observed hyperreactivity (see below).

4.2. Involvement of COX-2 in the vascular hyperreactivity in *db/db* mice

COX activity may contribute to the cause underlying vascular hyperreactivity in *db/db* mice as indomethacin (an inhibitor of COX-1 and COX-2) [12,40] and SQ29548 (a TP receptor antagonist) [40] significantly alleviate the hyperreactivity in endothelium-intact preparations. However, the specific isoform of COX involved (COX-1 vs. COX-2) and the source of COX (endothelium vs. smooth muscle cells) remains unclear. Furthermore, the underlying mechanisms (transcriptional and/or post-translational) responsible for COX activity up-regulation in *db/db* mice remain unclear.

Our data provide several lines of evidence suggesting that transcriptional up-regulation of COX-2 in vascular smooth muscle contributes to the vascular smooth muscle hyperreactivity in *db/db* mice. First, the combination of DNA microarray, real-time PCR and immunoblot analysis clearly shows that COX-2 (but not COX-1) is up-regulated at the mRNA and protein level in vascular smooth muscle tissue isolated from *db/db* mice (Figs. 3 and 4). Second, the time course of COX-2 mRNA up-regulation mostly coincides with the appearances of vascular smooth muscle hyperreactivity (Fig. 4) and diabetic mellitus (Table 1) in *db/db* mice. Third, selective inhibition of COX-2 activity with NS398 and SC58125 significantly alleviates the agonist-induced vascular smooth muscle contractile hyperreactivity in strips isolated from *db/db* mice, whereas this type of selective inhibition does not significantly affect the

contractions in strips isolated from control mice (Fig. 5). However, the inhibition is incomplete, suggesting mechanisms in addition to COX-2 up-regulation contribute to the contractile hyperreactivity. Taken together, these data suggest that the selective COX-2 up-regulation in aortic vascular smooth muscle tissue is partially responsible for the vascular smooth muscle contractile hyperreactivity in *db/db* mice.

In addition to COX-2 up-regulation, other mechanisms also are likely involved in the observed vascular smooth muscle contractile hyperreactivity. Two pieces of evidence support this notion. First, the 5-HT-induced contractile responses in tissue isolated from *db/db* mice remain significantly higher than in that isolated from control mice after COX-2 inhibition (Fig. 5). Secondly, at 4–5 weeks of age, the small but significant increase in low concentrations of 5-HT-induced contractions (Fig. 4A) is not associated with the COX-2 mRNA increase (Fig. 4B).

4.3. COX-2 up-regulation in vascular smooth muscle cells (VSMC) of *db/db* mice

In VSMC, COX-2 can be rapidly and markedly up-regulated by various stimulations, including serum [41–43], interleukin-1 β [44–46], EGF [41,43], Ang II [47–49] and thrombin [41]. In addition, high glucose [44,50] and Advanced Glycation End Products (RAGE) [51] have been shown to up-regulate or potentiate interleukin-1 β -induced up-regulation of COX-2. At 8 weeks of age, when *db/db* mice demonstrate vascular smooth muscle contractile hyperreactivity, changes are observed in multiple serum components. Among these changes are increases in glucose, insulin, triglyceride, total cholesterol, LDL and vLDL; decreases in HDL, and altered cytokine and growth factor levels [52]. Indeed, several of the potential COX-2 up-regulators including high glucose, increased TNF α [53] and other cytokines [54], activation of the rennin–angiotensin system [55], increased EGF [56,57], and increased RAGE [58,59] are detected in diabetic patients and in diabetic animal models. That supports the *in vivo* significance of the COX-2 up-regulation in vascular dysfunction in diabetes. It is therefore conceivable that high glucose in combination with these multiple changes in diabetic serum up-regulates COX-2. Indeed, we find that serum isolated from *db/db* mice is much more potent than the serum isolated from control mice in inducing COX-2 protein expression in VSMC (Fig. 6A). However, further studies are required to clarify the exact contribution of each serum component to COX-2 up-regulation in *db/db* mice.

4.4. Mechanisms mediate up-regulated COX-2-induced vascular smooth muscle hyperreactivity

We (Fig. 6B) and others [45,47] show that COX-2 up-regulation is associated with significantly increased production of the potent vasoconstrictor TxA₂ in VSMC. This

suggests that the increased production of contractile prostanoids such as TxA₂, PGF_{2 α} and PGH₂ is one potential mechanism mediating COX-2-induced vascular smooth muscle hyperreactivity. By autocrine and paracrine mechanisms, the vasoconstrictive prostanoids can enhance the vascular smooth muscle contractions in response to stimuli (Fig. 6C). Indeed, blockade of the TP receptor by SQ-29548 diminished the vascular smooth muscle hyperreactivity (Fig. 8). Up-regulated COX-2 may also cause vascular smooth muscle hyperreactivity by increasing oxidative stress [60] or decreasing NO production by down-regulated iNOS [45,61].

Although COX-1 and COX-2 exhibit similar biochemical activity in converting arachidonate to PGH₂ *in vitro* [62,63], the ultimate function and prostanoids they produce *in vivo* may be different due to differential regulation of COX-1 and COX-2, tissue distribution, cellular localization and availability of the down-stream prostanoid synthases [64,65]. Therefore, it is conceivable that functionally more contractile prostanoids are produced by the up-regulated COX-2 under diabetic conditions than what COX-1 produces under physiological conditions.

4.5. Possible pathological significance and perspective

Type 2 diabetes is one of the most serious long-term health problems in the United States, with enormous social and economic consequences. Cardiovascular disease is a major complication and the leading cause of premature death among people with diabetes. The present study shows that transcriptional up-regulation of COX-2 in vascular smooth muscle is partially responsible for vascular hyperreactivity in *db/db* mice. As abnormal vascular smooth muscle responses may contribute to the etiology of diabetic vascular complications, our study raises the possibility that the selective inhibition of vascular smooth muscle COX-2 activity may help prevent vascular complications in type 2 diabetes.

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