Calcium homeostasis in vascular smooth muscle cells is altered in type 2 diabetes by Bcl-2 protein modulation of InsP3R calcium release channels

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Velmurugan GV, White C. Calcium homeostasis in vascular smooth muscle cells is altered in type 2 diabetes by Bcl-2 protein modulation of InsP3R calcium release channels. Am J Physiol Heart Circ Physiol 302: H124–H134, 2012. First published October 28, 2011; doi:10.1152/ajpheart.00218.2011.—This study examines the extent to which the antiapoptotic Bcl-2 proteins Bcl-2 and Bcl-XL contribute to diabetic Ca2+ dysregulation and vessel contractility in vascular smooth muscle cells (VSMCs) through their interaction with inositol 1,4,5-trisphosphate receptor (InsP3R) intracellular Ca2+ release channels. Measurements of intracellular ([Ca2+]i) and sarcoplasmic reticulum ([Ca2+]sr) calcium concentrations were made in primary cells isolated from diabetic (db/db) and nondiabetic (db/m) mice. In addition, [Ca2+]i, and constriction were recorded simultaneously in isolated intact arteries. Protein expression levels of Bcl-XL, but not Bcl-2 were elevated in VSMCs isolated from db/db compared with db/m age-matched controls. In single cells, InsP3-evoked [Ca2+]i signaling was enhanced in VSMCs from db/db mice compared with db/m. This was attributed to alterations in the intrinsic properties of the InsP3R itself because there were no differences between db/db and db/m in the steady-state [Ca2+]sr or InsP3R expression levels. Moreover, in permeabilized cells the rate of InsP3R-dependent SR Ca2+ release was increased in db/db compared with db/m VSMCs. The enhanced InsP3-dependent SR Ca2+ release was attenuated by the Bcl-2 protein inhibitor ABT-737 only in diabetic cells. Activation of ABT-737 similarly attenuated enhanced agonist-induced [Ca2+]i signaling only in intact aortic and mesenteric db/db vessels. In contrast, ABT-737 had no effect on agonist-evoked contractility in either db/db or db/m vessels. Taken together, the data suggest that in type 2 diabetes the mechanism for [Ca2+]i dysregulation in VSMCs involves Bcl-2 protein-dependent increases in InsP3R excitability and that dysregulated [Ca2+]i signaling does not appear to contribute to increased vessel reactivity.

inflammatory 1,4,5-trisphosphate receptor; Bcl-XL; pressure myography; db/db mice; ABT-737

INCREASED BLOOD VESSEL REACTIVITY TO VASOCONSTRICTORS has been reported in patients with type 2 diabetes (12, 28), as well as in animal models (19, 21, 29, 31), and likely contributes to diabetes related hypertension (41). The primary mechanisms mediating hyperreactivity are thought to be impaired endothelium-dependent smooth muscle relaxation (9) coupled with increased sensitivity of contractile proteins (38). In addition, enhanced vasoconstrictor-mediated intracellular calcium concentration ([Ca2+]i) signals in vascular smooth muscle cells (VSMCs) have been widely reported and implicated as a contributing factor to hyperreactivity (12, 15, 36, 44, 52). In addition to Ca2+ influx through plasmalemmal voltage-dependent and voltage-independent channels, receptor-mediated increases in global [Ca2+]i are highly dependent on the inositol 1,4,5-trisphosphate receptor (InsP3R) Ca2+ release channel (8, 44, 50). It is not known if diabetes impinges on InsP3R channel modulation.

InsP3R activity is exquisitely regulated by a variety of mechanisms including protein-protein interactions (reviewed in Ref. 13). We (10, 22, 48) previously identified a functional interaction between InsP3Rs and members of the Bcl-2 protein family. These proteins are well characterized as important regulators of apoptotic cell death and include both pro- and antiapoptotic members (53). The regulation of apoptosis by the antiapoptotic members Bcl-2 and Bcl-XL is complex, but it is now established that they function, in part, by modulating InsP3R-dependent [Ca2+]i, signaling (16). In single channel electrophysiological studies, Bcl-XL increased the InsP3R excitability to low levels of InsP3 concentration ([InsP3]; Refs. 22, 48), and overexpressed Bcl-2 and Bcl-XL potentiated [Ca2+]i signaling generated in response to subthreshold agonist stimulation (10, 16, 22, 48).

Intriguingly, a recent study (33) showed that expression levels of antiapoptotic Bcl-2 are upregulated in smooth muscle cells of internal mammary arteries isolated from type 2 diabetic patients. Expression levels of both Bcl-2 and Bcl-XL were also shown to be elevated in VSMCs cultured in high glucose (24, 37). Not surprisingly, these studies report increased cellular resistance to apoptotic stimuli, with important implications for vascular remodeling in diabetes pathology (42). However, given that Bcl-2 and Bcl-XL functionally regulate InsP3Rs, we postulated that these interactions could account for [Ca2+]i signaling dysregulation in diabetic VSMCs and possibly be an important component of vascular hyperreactivity. Thus, in the present study, we have explored the role of antiapoptotic Bcl-2 proteins in regulating InsP3R-dependent [Ca2+]i signaling and vessel tone in the db/db mouse model of type 2 diabetes.

MATERIALS AND METHODS

Animals and cell culture. Male control db/m (Dock7m+/+ LeprΔdb) mice and diabetic db/db (BKS.Cg-Dock7m+/- LeprΔdb) littermates purchased from The Jackson Laboratory were housed in the biological resource facility at Rosalind Franklin University. All animals were provided ad libitum access to food and water during the study, and blood glucose was measured using a Contour blood glucose meter and test strip (Bayer, Tarrytown, NY). Mice were killed by inhalation of a lethal dose of CO2 followed by cervical dislocation, according to the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals, and the procedures were approved by the Institutional Animal Care and Use Committee of Rosalind Franklin University of Medicine and Science. The thoracic aorta was chosen for study because it is large, easily prepared, and rich in smooth muscle cells; a factor particularly important when isolating and culturing cells for functional studies. Aortas were isolated and cleaned of fat and connective tissues, and the adventitia was removed. Vessels were then

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opened longitudinally and mechanically denuded of the endothelial layer before being cut into small lengths. These were incubated overnight in DMEM supplemented with 10% (vol/vol) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Mediatech, Manassas, VA) in a humidified 95% O2-5% CO2 atmosphere. The following day single smooth muscle cells were enzymatically dispersed by treating tissues with type-2 collagenase (1 mg/ml) and elastase (0.5 mg/ml) for 35 min at 37°C. To recapitulate the diabetic and nondiabetic states in culture, dispersed dh/db cells were seeded onto coverslips in normal glucose (5.5 mM) and db/db cells in high glucose (25 mM) containing DMEM with 10% FBS and antibiotics. After 48 h, the FBS concentration was reduced to 0.1% and experiments were carried out between days 3 and 5.

**Immunocytochemistry.** Western blot, and real-time PCR. For immunocytochemistry, cells were cultured in glass-bottomed dishes, washed with Ca2+- and Mg2+-free phosphate buffer, and fixed with 4% paraformaldehyde before permeabilization with 0.02% Triton X-100. Smooth muscle myosin heavy chain (SM-MHC) was labeled with monoclonal antibody (1:400 dilution; Abcam, Cambridge, MA) and detected by secondary labeling with green-fluorescent Alexa Fluor 488 IgG (1:100 dilution; Invitrogen, Carlsbad, CA), and nuclei were counterstained with DAPI. For Western blot, intact aortas were isolated as described above, lysates were prepared, and proteins were resolved as described previously (48). For real-time PCR, total RNA was extracted (RNeasy kit; Applied Biosystems, Carlsbad, CA) and cDNA was synthesized from 1 μg of total RNA (First-Strand cDNA synthesis kit; Affymetrix, Santa Clara, CA). Real-time quantitative RT-PCR was performed on an ABI Prism 7000 instrument using Syber Green PCR Master Mix (Applied Biosystems) and validated primer sets for Bcl-2, Bcl-xL, and reference standards (Qiagen, Germantown, MD). Fluorescent signals generated during PCR amplifications were normalized to an internal reference, the threshold cycle (Ct) was set within the exponential phase, and the relative quantitative evaluation of target gene levels was performed using the 2^−ΔΔCt method.

[Ca2+]i and sarcoplasmic reticulum calcium concentration measurements. For [Ca2+]i, cells cultured on glass coverslips were loaded with 2 μM fura-2 AM (Invitrogen) by incubation at room temperature for 45 min and mounted in a recording chamber positioned on the stage of an inverted microscope (IX71; Olympus America, Center Valley, PA). Fura-2 was alternately excited at 340 and 380 nm, and the emitted fluorescence filtered at 510 nm was collected and recorded using a CCD-based imaging system running SimplePCI software (Hamamatsu, Sewickley, PA). The chamber was continuously perfused with HBBS (Sigma-Aldrich, St. Louis, MO), pH 7.4, at room temperature. A rapid solution changer was used to switch the composition of the solution bathing the cells under study. Ratiometric data were calibrated as described previously (48). For sarcoplasmic reticulum (SR) calcium concentration ([Ca2+][SR]), cells were loaded with mag-fura-2 AM (5 μM) for 60 min at room temperature and perfused with intracellular-like medium (ICM) containing the following (in mM): 125 KCl, 19 NaCl, 10 HEPES, and 1 EGTA (pH 7.3 with KOH) and permeabilized by a 2- to 3-min exposure to ICM containing 25 μM β-escin. After being washed in regular ICM, store loading was achieved by switching to ICM solution with a free [Ca2+] adjusted to 200 nM and 1.5 mM MgATP. To induce Ca2+ release, InsP3 was applied in the same Ca2+-containing ICM solution but without MgATP to prevent reuptake. Data were acquired and calibrated as described for fura-2.

**Flash photolysis of caged-InsP3.** Cells were loaded with the membrane-permeable caged InsP3 compound d-2,3-O-isopropylidene-6-O-(2-nitro-4,5-dimethoxy)benzyl-nyso-inositol 1,4,5-trisphosphate-hexakis (propionoxymethyl) ester (ci-IP3/PM; SiChem, Bremen, Germany) by incubation with 1 μM for 60 min at room temperature. InsP3 was photoreleased by brief pulses (5–100 ms) of ultraviolet light (350–400 nm) delivered uniformly throughout the image field. The application of ultraviolet pulses precluded the use of fura-2 as a [Ca2+]i indicator; therefore, cells were coloaded with the longer wavelength dye fluo-4 AM (5 μM) and fluorescence data (F) normalized to basal levels (F0).

**Intact vessel measurements.** Sections of proximal thoracic aorta (~3 mm) or mesenteric artery (3rd order branches ~2 mm) were dissected and placed in ice-cold physiological salt solution with the following composition (in mM): 145 NaCl, 4.7 KCl, 1.2 NaH2PO4, 1.17 MgSO4, 2 pyruvic acid, 0.02 EDTA, 3 MOPS, 2 CaCl2, 5 glucose, and 1% BSA (pH 7.4 with NaOH). The adventitia was removed, the endothelium was mechanically denuded, and the vessel was transferred to an organ chamber (Living Systems Instrumentation, Burlington, VT) and cannulated with the perfusion pressure set by an electronic pressure servo system (Living Systems Instrumentation). The luminal solution contained 10 μM fura-2 AM, and the preparation was incubated for 1 h at room temperature and 10-mmHg transmural pressure to enable fura-2 loading. Vessels were then pressurized to 80 mmHg and equilibrated for 30 min at room temperature and a further 30 min at 37°C. Viability was assessed with 80 mM KCl, and the failure of acetylcholine (1 μM) to induce dilation was taken as confirmation of endothelium removal. The calibration parameters for fura-2 were acquired by first exposing the vessel to Ca2+-free physiological salt solution containing 10 μM ionomycin to record the minimum fluorescence ratio (Rmin) followed by the addition of 20 mM CaCl2 to determine the maximum (Rmax). Background autofluorescence measurements were made in the presence of 50 mM MnCl2 and 10 μM ionomycin, and the values were subtracted from fluorescence measurements to calculate the fluorescence ratio (Rfura2). Due to difficulties in determining the Kd for fura-2 in intact tissues, ratio values were not converted into [Ca2+] but rather normalized according to the following equation: Rf = (Rfura2 – Rmin)/(Rmax – Rmin) (45).

**Analysis and statistics.** In all experiments, data were pooled from multiple trials carried out on cells or tissues originating from at least two animals and summarized as means ± SE. Differences between means were assessed using the Student’s t-test for paired or unpaired comparisons. For multiple comparisons, a one-way ANOVA with Fisher’s least significant difference post hoc analysis was employed. For all tests, the differences between means were accepted as statistically significant at the 95% level (P < 0.05).

**RESULTS**

**Expression level of Bcl-xL is increased in the db/db aorta.** The db/db mouse model is well characterized and shares many cardiovascular phenotypes with human type 2 diabetes. The disease develops due to a point mutation in the leptin receptor gene, a key regulator of food intake, energy expenditure, and body weight. Importantly, littermates heterozygous for the leptin receptor mutation (dh/db) do not develop the disease and serve as controls. In younger animals (~4 wk), the db/db mice are physically indistinguishable from their dh/db littermates; however, by 10 wk db/db mice are markedly obese and hyperglycemic and beyond 12 wk display vascular hyperreactivity (15, 18, 19, 21) and hypertension (2, 39, 43). In the present study, the expression levels of Bcl-xL and Bcl-2 were defined in aortas isolated from control and diabetic mice at ages 4 and 14 wk. Consistent with previous reports, the 4-wk-old dh/db and dh/db mice had similar body weights, and the db/db animals displayed a slightly elevated blood glucose level. However, at 14 wk the db/db mice were almost twice as heavy with a 5.5-fold greater blood glucose compared with aged-matched dh/db controls (Table 1). As assessed by Western blot, the expression levels of both Bcl-xL and Bcl-2 were similar in 4-wk-old db/m and db/db samples (Fig. 1A). Bcl-2 concentrations were also found to be similar in db/m and db/db at 14 wk; however, Bcl-xL showed a twofold larger expression level in the db/db (Fig. 1B). Expression levels were also similar in 4-wk-old db/m and db/db (Fig. 1C).
_InsP3R MODULATION BY Bcl-2 PROTEINS IN DIABETIC VSMCs_

**Table 1. Age-related differences in body weight and blood glucose between db/m and db/db mice**

<table>
<thead>
<tr>
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<th>db/m</th>
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<tr>
<td><strong>4 wk</strong></td>
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<tr>
<td>Body weight, g</td>
<td>19.6 ± 0.4 (n = 4)</td>
<td>20 ± 0.6 (n = 4)</td>
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<tr>
<td>Blood glucose, mg/dl</td>
<td>93.3 ± 3.6 (n = 4)</td>
<td>116.8 ± 6.1*(n = 4)</td>
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<tr>
<td><strong>14 wk</strong></td>
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<tr>
<td>Body weight, g</td>
<td>27.4 ± 0.8 (n = 6)</td>
<td>49.8 ± 2* (n = 6)</td>
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<tr>
<td>Blood glucose, mg/dl</td>
<td>134.7 ± 7.6 (n = 6)</td>
<td>759 ± 72.1* (n = 6)</td>
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Values are means ± SE (n = number of animals). *P < 0.05, statistical comparisons between db/m and db/db within age group by t-test.

assessed by real-time PCR in an independent group of 12- to 14-wk-old db/m and db/db mice (Fig. 1C). In agreement with the Western blot data, mRNA levels of Bcl-XL were upregulated. Interestingly, Bcl-2 levels were also significantly upregulated in the db/db, highlighting dissociation between Bcl-2 expression at the mRNA and protein level. Collectively, these data provide further confirmation of the link between diabetes and upregulated Bcl-2 protein expression in vascular tissues (24, 33, 37).

**Primary smooth muscle cell culture: validation of the experimental model and regulation of Bcl-XL expression by glucose.** A suitable cell culture model was required to explore the role of Bcl-XL in regulating Ca^{2+} signaling. Primary cells were cultured following established methodologies to maintain enzymatically dispersed single myocytes in culture (see MATERIALS AND METHODS and Ref. 14). Smooth muscle cells are generally not terminally differentiated and cultured cells undergo a time-dependent switch from a contractile to a proliferative phenotype (30). The phenotypic state of cultured cells was assessed by visualizing the expression levels of SM-MHC. Several molecular markers of the contractile phenotype have been characterized; however, SM-MHC is regarded as the most discriminating since it is exclusively expressed in differentiated smooth muscle (25, 27). We assessed the expression levels of SM-MHC in our cultured db/m and db/db cells after 3, 5, and 7 days in culture using immunocytochemistry. At 3 and 5 days, robust labeling was observed in >95% of cells; however, after 7 days the fluorescence intensity of SM-MHC labeling was noticeably reduced, suggesting that dedifferentiation and phenotypic switching begins after 5 days in culture (Fig. 2A). Importantly, dedifferentiation has also been associated with remodeling of Ca^{2+} signaling proteins; this includes a downregulation of the SR-localized ryanodine receptor (RyR) Ca^{2+} release channel (4). The presence of RyR can be easily assessed using whole cell Ca^{2+} imaging experiments by applying caffeine, a potent RyR agonist, that evokes Ca^{2+} release from the SR (11). Previous studies (4) have demonstrated that during dedifferentiation the loss of RyR expression, assessed by Western blot and RT-PCR, closely correlates with the complete loss of responsiveness to caffeine. We tested the response to caffeine in our 5-day-old cultures and found that caffeine evoked a robust [Ca^{2+}]_i response in >85% of cells. In addition, there was no difference in the [Ca^{2+}]_i transient amplitude between cultured cells and freshly isolated myocytes (Fig. 2, B and C).

The expression levels of Bcl-XL after 5 days in culture were assessed using Western blot analysis of both db/m and db/db cells treated with media containing 5.5 mM glucose (normal), 25 mM (diabetic) glucose, or 5.5 mM glucose and mannitol (osmotic control; Fig. 2D). Consistent with expression levels determined in freshly isolated tissues, Bcl-XL expression was significantly higher in db/db compared with db/m cells, when cultured in high and normal glucose, respectively. There was no significant difference between Bcl-XL levels in db/m and db/db cells when both were cultured in high glucose, consistent with previous studies (24, 37) reporting a link between Bcl-2 protein expression and hyperglycemia. Although not significant, Bcl-XL levels in db/db cells were also lower when cultured in normal glucose compared with high glucose (Fig. 2E). Taken together, these data demonstrate that cells isolated and cultured for 5 days retain the differentiated, contractile phenotype and that culturing db/m and db/db cells in normal and high glucose concentrations, respectively, recapitulates the difference in Bcl-XL expression levels seen in freshly isolated tissues.

**Fig. 1.** Antiapoptotic Bcl-XL is upregulated in aortas from db/db mice compared with db/m controls. A and B: Western blots of Bcl-2 and Bcl-XL protein in aortic smooth muscle lysates from 4-wk-old (A) and 14-wk-old (B) diabetic (db/db) and nondiabetic (db/m) mice. Independent samples were run in each lane, and 3 representative samples of db/m and db/db are shown from the same gel. A vertical white strip between lanes shows were a lane has been cropped out to illustrate more samples run on the gel. C: Densitometry analysis from the Western blots was performed using ImageJ analysis software and normalized to β-tubulin (n = number of animals; *P < 0.05, unpaired t-tests).

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InsP₃R-dependent \([\text{Ca}^{2+}]_i\) transients are potentiated in diabetic VSMCs. InsP₃-dependent \([\text{Ca}^{2+}]_i\) signaling was assessed in cultured VSMCs isolated from \(\text{db/m}\) and \(\text{db/db}\) aortas. To directly evaluate the IP₃R activity, \([\text{Ca}^{2+}]_i\) was monitored in freshly isolated cells. In freshly isolated cells, \(\text{db/m}\) and \(\text{db/db}\) cells were observed to have similar \([\text{Ca}^{2+}]_i\) transient amplitude of the caffeine-evoked response (means ± SE). No difference was observed between the responses recorded in \(\text{db/m}\) and \(\text{db/db}\) cells (\(P > 0.05\), unpaired t-test). In freshly isolated cells, \(\text{db/m}\) and \(\text{db/db}\) responded similarly to caffeine (\(P > 0.05\) unpaired t-test). In freshly isolated cells, \(\text{db/m}\) and \(\text{db/db}\) responded similarly to caffeine (\(P > 0.05\) unpaired t-test).

**Fig. 2.** Primary cells in culture retain a contractile phenotype and Bcl-XL expression levels for up to 5 days. A: immunocytochemistry showing primary cells cultured for 3, 5, or 7 days, labeled with smooth muscle myosin heavy chain (SM-MHC; green), counterstained with DAPI (blue), and visualized using epifluorescence microscopy. B: bar graphs summarizing the peak \([\text{Ca}^{2+}]_i\) in response to cyclopiazonic acid, suggesting that the SR \([\text{Ca}^{2+}]_i\) pool size was similar in both cell types (Fig. 3, C and D). We next examined the expression levels of all three InsP₃R isoforms in freshly isolated aorta smooth muscle. As assessed by Western blot, the protein levels of types 1, 2, and 3 InsP₃R were not significantly different between \(\text{db/m}\) and \(\text{db/db}\) (Fig. 3E). Therefore, the enhanced InsP₃-dependent \([\text{Ca}^{2+}]_i\) release observed in the diabetic was most likely due to modulation of the InsP₃R channel itself rather than an alteration in channel numbers or SR \([\text{Ca}^{2+}]_i\) store content.

**InsP₃R excitability is enhanced in diabetic VSMCs.** Direct measurements of InsP₃-dependent SR \([\text{Ca}^{2+}]_i\) flux were carried out to further assess the InsP₃R excitability in \(\text{db/m}\) and \(\text{db/db}\) VSMCs. This was achieved by monitoring the \([\text{Ca}^{2+}]_i\) using the low affinity \([\text{Ca}^{2+}]_i\) indicator mag-fura-2 (10). Mag-fura-2 compartmentalizes in the SR as well as in the cytoplasm; therefore, the plasma membrane was permeabilized to remove cytoplasmic indicator, and the cells were perfused with a \([\text{Ca}^{2+}]_i\)-free solution to allow passive depletion of SR \([\text{Ca}^{2+}]_i\) store. As depicted in Fig. 4A, after equilibration in \([\text{Ca}^{2+}]_i\)-free solution, \([\text{Ca}^{2+}]_i\) uptake into the store was induced by switching the perfusate to 1.5 mM MgATP and 200 mM free \([\text{Ca}^{2+}]_i\) containing solution. Once a steady state had been reached, the
solution was switched to one containing InsP₃ without MgATP to induce depletion of the SR. This was a unidirectional measure of Ca²⁺ flux since the MgATP required for sarco(endoplasmic reticulum Ca²⁺-ATPase (SERCA) pump function was not present during InsP₃ addition. When InsP₃ was removed and MgATP re-added, store filling was restored to the original steady state and repeated cycles of depletion and refilling were possible in db/m and db/db cells without rundown of either the release or uptake capacities. An example of this is shown in Fig. 4A.

In both db/m and db/db cell types, 10 μM InsP₃ (saturating for the mammalian channel; Ref. 13) evoked a faster rate of Ca²⁺ release compared with subsaturating InsP₃ concentrations. Importantly, the rate of release evoked by subsaturating concentrations appeared faster in the db/db compared with db/m (Fig. 4B). Since the flow of Ca²⁺ is unidirectional, the rate of release is a measure of Ca²⁺ flux across the SR through the InsP₃R and thus an index of InsP₃R channel activity (5). The initial rate of [Ca²⁺]SR decrease was fit with a single exponential function to determine the rate constant and plotted against InsP₃ concentration (Fig. 4C). As can be seen from Fig. 4C, the SR Ca²⁺ release flux in response to subsaturating (1 μM) [InsP₃] was approximately threefold faster in the db/db compared with db/m. Of note, the absolute magnitude of Ca²⁺ release at each InsP₃ concentration was not significantly different between db/m and db/db (Fig. 4D). Also of note, there was no statistically significant difference between db/m and db/db cells with regard to the steady-state filling capacity or the rate of Ca²⁺ uptake into the SR store (Fig. 4E). These results suggest that altered SR store content or SERCA expression/modulation are unlikely to contribute to the changes in global Ca²⁺ signaling seen with diabetes. Taken together, these data show that the excitability of the InsP₃R is increased in the diabetic mouse cells.

Bcl-2 protein inhibition attenuates InsP₃R channel excitability in diabetic VSMCs. Previous studies have shown that increased antiapoptotic Bcl-2 protein expression enhances InsP₃R excitability and modulates [Ca²⁺], signaling. Can the Bcl-X₁ upregulation observed in diabetic VSMCs account for the augmented InsP₃R activity seen in these cells? The ability of different Bcl-2 family members to heterodimerize with one another is central to their role in regulating apoptosis (53). The interactions occur between the hydrophobic binding pocket of one Bcl-2 protein and the amphipathic helical region of another. The compound ABT-737 was identified as a target of the Bcl-2 hydrophobic pocket, using a combination of NMR screening, structure-based design, and chemical synthesis, and has been shown to bind very specifically and with high affinity to Bcl-2 and Bcl-X₁, preventing protein-protein interactions between the Bcl-2 molecule and its interacting partners (46). In our model of Bcl-2-InsP₃R interactions, the hydrophobic binding pocket of Bcl-2/Bcl-X₁ binds to amphipathic helical domains within the InsP₃R carboxyl terminus (22, 48); therefore, ABT-737 is predicted to disrupt the interaction between Bcl-X₁ and the InsP₃R. Control responses to 1 μM InsP₃ were first evoked, and InsP₃ was then washed out and [Ca²⁺]SR allowed to recover; the same cells were then exposed to ABT-737 (0.5 μM) for 10–15 min, and InsP₃ was applied again in the presence of inhibitor (Fig. 5A). In db/m cells, the SR Ca²⁺ release rate under control conditions was not changed by incubation with ABT-737; however, the Ca²⁺ release rate in
db/db cells was significantly reduced in the presence of ABT-737 (Fig. 5B). Notably, ABT-737 reduced the db/db SR Ca\(^{2+}\) release rate to levels comparable to those observed in the db/m control. This observation supports a model in which Bcl-x\(_L\)-InsP\(_3\)R interactions are causative of the augmented Ca\(^{2+}\) signaling observed in the diabetic.

Bcl-2 protein inhibition reduces receptor-mediated [Ca\(^{2+}\)]\(_i\) signaling in intact vessels from diabetic animals without affecting hyperreactivity. We next assessed whether or not Bcl-x\(_L\)-dependent modulation of the InsP\(_3\)R channel activity could contribute to diabetic contractile hyperreactivity. Hyperreactivity has been described in both large conduit arteries as well as smaller resistance vessels (12, 19, 21, 28, 29, 31); therefore, experiments were carried out on both aortic and mesenteric vessels. Importantly, Bcl-x\(_L\) expression levels are also upregulated in mesenteric arteries isolated from db/db compared with db/m (see Fig. 7G). Isolated arteries were mounted for simultaneous monitoring of [Ca\(^{2+}\)]\(_i\), and diameter under constant transmural pressure. Application of the InsP\(_3\)R-dependent agonist phenylephrine (PE) evoked increases in [Ca\(^{2+}\)]\(_i\), and vessel constriction. Once this response had peaked, vessels were washed in normal external buffer (15–20 min) until both [Ca\(^{2+}\)]\(_i\) and diameter returned to basal levels. In each preparation, the response to three PE concentrations (0.01, 0.1, and 1 \(\mu\)M) was tested in this way. The preparations were then incubated with ABT-737 (0.5 \(\mu\)M) for 1 h, and the concentration response to PE was repeated. Representative traces depict the effect of 0.1 \(\mu\)M PE in the presence and absence of ABT-737 in aortas (Fig. 6, A and B) and mesenteric vessels (Fig. 7, A and B). In both db/m and db/db aortic and mesenteric vessels, an 80 mM K\(^+\) challenge evoked similar increases in [Ca\(^{2+}\)]\(_i\), and constriction that were not affected by incubation in ABT-737 (Figs. 6 and 7, C and D). In contrast, the magnitudes of the [Ca\(^{2+}\)]\(_i\) increase and vessel constriction evoked by PE concentrations of 0.1 and 1 \(\mu\)M were significantly greater in db/db compared with db/m vessels (Fig. 6 and 7, E and F). In db/m vessels, ABT-737 had no effect on either the [Ca\(^{2+}\)]\(_i\), or the constriction response to PE (Figs. 6 and 7, E and F, middle). In the db/db, however, ABT-737 significantly attenuated the [Ca\(^{2+}\)]\(_i\), increase evoked by PE (0.1 and 1 \(\mu\)M) without affecting the vasoconstriction response (Figs. 6 and 7, E and F, right). Therefore, during agonist-dependent signaling, the augmented [Ca\(^{2+}\)]\(_i\), response observed in the diabetic can be attributed to Bcl-x\(_L\)-InsP\(_3\)R interactions but this signal does not contribute to the increased contractility in either aortic or mesenteric arteries.

DISCUSSION

The current study assessed the effects of diabetes-dependent upregulation of antiapoptotic Bcl-2 proteins on [Ca\(^{2+}\)]\(_i\), signaling and contraction in vascular smooth mus-
Bcl-XL is known to be regulated by the cellular milieu, and it is well established that levels of PKC signaling contribute to the observed differences between diabetic and nondiabetic myocytes. While altered expression levels of Ca2+ signaling proteins in vascular tissues have been reported in both type 1 and type 2 models of diabetes (3, 32, 35, 40), as well as defects in agonist receptor-dependent InsP3 production (1, 35), similar modifications are unlikely to account for the observations made in the current study. We show that InsP3R protein levels are the same in db/m and db/db and that the steady-state [Ca2+]iSR, assessed either by directly measuring the store filling capacity in permeabilized cells, or indirectly during SERCA inhibition, is unchanged in diabetic cells. It could also be argued that remodeling of RyrR-dependent [Ca2+]i, signaling contributes to the observed differences between db/m and db/db by a mechanism of increased Ca2+-induced Ca2+ release. However, it is unlikely that Ca2+-induced Ca2+ release is a component of the Sr Ca2+ release flux in our permeabilized cell experiments since the free [Ca2+]i was clamped at 200 nM, well below the threshold for Ryr activation (11). Furthermore, [Ca2+]i, transient amplitudes evoked by caffeine (an RyrR agonist) are comparable in diabetic and nondiabetic myocytes.

Fig. 5. Enhanced InsP3-dependent Sr Ca2+ release in db/db cells is attenuated by the Bcl-2 inhibitor ABT-737. A: representative recordings of [Ca2+]iSR in db/m (left) and db/db (right) cells in response to 1 μM InsP3 in the absence and presence of ABT-737 (0.5 μM). Stores were filled by preapplication of 200 nM Ca2+ and 1.5 mM MgATP and then challenged with InsP3. After washout and refilling, InsP3 was reapplied in the presence of 0.5 μM ABT-737. B: bar graphs showing the [Ca2+]iSR release rates in response to 1 μM InsP3 recorded in the absence and presence of ABT-737. Data are summarized as means ± SE for db/m (n = 16; 3 animals) and db/db (n = 12; 2 animals) animals (*p < 0.05, paired t-test).

Bcl-XL promotes InsP3R gating by directly binding to the carboxyl terminus of the channel to allosterically increase its sensitivity to InsP3-dependent activation (22, 48). Thus we postulated that InsP3R-dependent [Ca2+]i, signals would be similarly modified by diabetes-driven Bcl-XL overexpression. We now report that photorelease of low [InsP3] evokes larger [Ca2+]i, transients in db/db compared with db/m cells, and in permeabilized cells the rate of Sr Ca2+ release in response to submaximal [InsP3], an index of InsP3R channel activity, is also greater in db/db cells. While altered expression levels of Ca2+ signaling proteins in vascular tissues have been reported in both type 1 and type 2 models of diabetes (3, 32, 35, 40), as well as defects in agonist receptor-dependent InsP3 production (1, 35), similar modifications are unlikely to account for the observations made in the current study. We show that InsP3R protein levels are the same in db/m and db/db and that the steady-state [Ca2+]iSR, assessed either by directly measuring the store filling capacity in permeabilized cells, or indirectly during SERCA inhibition, is unchanged in diabetic cells. It could also be argued that remodeling of RyrR-dependent [Ca2+]i, signaling contributes to the observed differences between db/m and db/db by a mechanism of increased Ca2+-induced Ca2+ release. However, it is unlikely that Ca2+-induced Ca2+ release is a component of the Sr Ca2+ release flux in our permeabilized cell experiments since the free [Ca2+]i was clamped at 200 nM, well below the threshold for Ryr activation (11). Furthermore, [Ca2+]i, transient amplitudes evoked by caffeine (an RyrR agonist) are comparable in diabetic and nondiabetic myocytes.

Exposure to the Bcl-2 inhibitor ABT-737 reduces InsP3R-dependent Sr Ca2+ release in db/db cells, supporting our hypothesis that InsP3R activity is modified in the diabetic as a consequence of Bcl-2-protein interaction. Our interpretation assumes that ABT-737 can specifically disrupt these interactions. This has recently been demonstrated using biochemical pull-down experiments as well as functional electrophysiology assays by Kevin Foskett’s group at the University of Pennsylvania (unpublished observations, personal communication). Importantly, we show here that ABT-737 does not affect Sr Ca2+ release in db/m cells, demonstrating that the effects of ABT-737 are not due to
direct inhibition of the InsP$_3$R and suggesting that Bcl-2 proteins do not impinge on [Ca$^{2+}$]$_i$ signaling in the normal, nondiabetic cell. A caveat of employing ABT-737 is that it is equally likely to disrupt binding of both Bcl-2 and Bcl-XL to the InsP$_3$R (46). Even though we demonstrate that only Bcl-XL is upregulated in the diabetic, it remains a possibility that overexpression alone may not be the primary mechanism for promoting InsP$_3$R interactions and that other factors could enable Bcl-2 proteins to more effectively engage the InsP$_3$R. This is not without precedent, indeed, functional aspects of Bcl-2 and Bcl-XL, including localization and interactions with binding partners, are modulated by phosphorylation and reactive oxygen species (23, 34). Further studies will be required to address the possible influence of posttranslational modifications on Bcl-2 protein-InsP$_3$R interactions. Nevertheless, we (10, 22, 48) have shown previously that both Bcl-2 and Bcl-XL can have similar effects on InsP$_3$R behavior.

In pressurized whole artery preparations, we report that [Ca$^{2+}$]$_i$ transient amplitude and vessel constriction in response to PE is dramatically enhanced in both conduit (aorta) and resistance (mesenteric) vessels isolated from db/db animals. Here, vasoconstrictor agonist binding and InsP$_3$R activation are requirements because db/m and db/db respond similarly when challenged with high K$^+$ concentration. It is also notable that when Bcl-2 proteins are inhibited in the diabetic by preincubation with ABT-737 the [Ca$^{2+}$]$_i$ transients evoked by PE are attenuated to levels comparable to those observed in the nondiabetic. These data are entirely consistent with the effects of ABT-737 on [Ca$^{2+}$]$_{SR}$ release flux in single cells and further strengthen the hypothesis that [Ca$^{2+}$]$_i$ signaling dysregulation in the diabetic is Bcl-2-InsP$_3$R dependent. Elevated [Ca$^{2+}$]$_i$ is the primary stimulus for smooth muscle contraction, yet despite a dramatic reduction in the amplitude of the PE-evoked [Ca$^{2+}$]$_i$ transients in diabetic aortic and mesenteric arteries, ABT-737 had no effect on vessel contractility under these conditions. It is unlikely that ABT-737 itself impinges on contractile ability, either directly or through modification of Ca$^{2+}$ sensitivity, since it has no effect on the K$^+$-evoked Ca$^{2+}$ and contractile response. A similar dissociation between [Ca$^{2+}$]$_i$ and tension has also been observed previously in mesenteric arteries from type 1 diabetic rats in response to norepinephrine (8). We now show for the first time, in a model of type 2 diabetes, that enhanced [Ca$^{2+}$]$_i$ responses during agonist stimulation do not contribute to enhanced contraction. Importantly, we show that this occurs in both conduit and resistance vessels by the same mechanism.

Our findings raise two important questions. 1) What is the likely mechanism of increased reactivity in the diabetic, and

![Figure 6. ABT-737 attenuates enhanced agonist-induced [Ca$^{2+}$]$_i$ signaling in intact db/db aortas without affecting contractility. A and B: [Ca$^{2+}$]$_i$ and vessel diameter were monitored simultaneously in pressurized aortas. Representative recordings from db/m (A) and db/db (B) depict the response to 0.1 μM phenylephrine (PE) alone and in the presence of ABT-737 (0.5 μM). C and D: summary plots of the means ± SE amplitude of the [Ca$^{2+}$]$_i$ transient (C) and vessel constriction (D) in response to 80 mM KCl. There was no difference between db/m and db/db in either the [Ca$^{2+}$]$_i$ or contractile response under control conditions and in the presence of ABT-737 (P > 0.05, ANOVA). E and F: summary plots of the means ± SE amplitude of the [Ca$^{2+}$]$_i$ transient (E) and vessel constriction (F) for the PE concentration ([PE]) indicated. Data are expressed as a percentage of the response to 80 mM KCl recorded in the same preparation and statistical comparisons between db/m (n = 3 animals) and db/db (n = 3 animals) at each [PE] are indicated (*P < 0.05, ANOVA).](http://ajpheart.physiology.org/doi/10.1152/ajpheart.00218.2011)
what role, if any, does enhanced [Ca\textsuperscript{2+}]i signaling play in diabetic vascular complications? First, removal of the endothelial layer in the current study rules out reduced endothelial-dependent relaxation as a contributor to hyperreactivity; therefore, we speculate that increased Ca\textsuperscript{2+} sensitivity of the contractile proteins is the most likely mechanism. Indeed, this is a well documented feature of receptor-mediated signaling and known to be amplified in diabetes (38). Second, our conclusions in no way rule out an important role for enhanced InsP\textsubscript{3}R sensitivity in diabetic vascular pathology. Ca\textsuperscript{2+} signaling is fundamental in modulating numerous cellular processes including many of those involved in diabetic vascular complications. For example, alterations in the rates of apoptosis and proliferation are central to vascular remodeling (42), and InsP\textsubscript{3}R-dependent signaling has been well documented to play a prominent role in regulating both processes (16, 47, 49). Thus further studies will be required that specifically address the possible role of InsP\textsubscript{3}R-Bcl-2 interactions in vascular remodeling.

In summary, we have defined a novel mechanism to account for dysregulated [Ca\textsuperscript{2+}]i homeostasis in VSMCs in type 2 diabetes. In this model, InsP\textsubscript{3}R sensitivity is enhanced through increased interactions with Bcl-2 proteins.

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DISCLOSURES
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Author contributions: G.V.V. and C.W. conception and design of research; G.V.V. and C.W. performed experiments; G.V.V. and C.W. analyzed data;
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