Contractile protein expression is upregulated by reactive oxygen species in aorta of Goto-Kakizaki rat

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Chettimada S, Ata H, Rawat DK, Gulati S, Kahn AG, Edwards JG, Gupte SA. Contractile protein expression is upregulated by reactive oxygen species in aorta of Goto-Kakizaki rat. Am J Physiol Heart Circ Physiol 306: H214–H224, 2014. First published November 8, 2013; doi:10.1152/ajpheart.00310.2013.—Although it is known that blood vessels undergo remodeling in type 2 diabetes (T2D), the signaling pathways that underlie the structural and functional changes seen in diabetic arteries remain unclear. Our objective was to determine whether the remodeling in type 2 diabetic Goto-Kakizaki (GK) rats is evoked by elevated reactive oxygen species (ROS). Our results show that aortas from GK rats produced greater force (P < 0.05) in response to stimulation with KCl and U46619 than aortas from Wistar rats. Associated with these changes, aortic expression of contractile proteins (measured as an index of remodeling) and the microRNA (miR-145), which act to upregulate transcription of contractile protein genes, was twofold higher (P < 0.05) in GK than Wistar (agematched control) rats, and there was a corresponding increase in ROS and decrease in nitric oxide signaling. Oral administration of the antioxidant Tempol (1 mmol/l) to Wistar and GK rats reduced (P < 0.05) myocardin and calponin expression. Tempol (1 mmol/l) decreased expression of miR-145 in Wistar and GK rat aorta. To elucidate the mechanism through which ROS increases miR-145, we measured their levels in freshly isolated aorta and cultured aortic smooth muscle cells incubated for 12 h in the presence of H₂O₂ (300 μmol/l). H₂O₂ increased expression of miR-145, and there were corresponding nuclear increases in myocardin, a miR-145 target protein. Intriguingly, H₂O₂-induced expression of miR-145 was decreased by U0126 (10 μmol/l), a MEK1/2 inhibitor, and myocardin was decreased by anti-miR-145 (50 nmol/l) and U0126 (10 μmol/l). Our novel findings demonstrate that ROS evokes vascular wall remodeling and dysfunction by enhancing expression of contractile proteins in T2D.

superoxide; hydrogen peroxide; microRNA; type 2 diabetes; vascular smooth muscle; aortic smooth muscle cell line; smooth muscle phenotype

TYPE 2 DIABETES (T2D), which is currently a pandemic around the world, is a major independent risk factor for cardiovascular disease (13). Indeed, vasculopathies are the most commonly observed clinical manifestation among diabetic individuals. These vascular complications include hypertension, peripheral arterial disease, atherosclerosis, and poor angiogenesis and coronary artery collateral growth (13). Accumulating evidence suggests that endothelial dysfunction is the primary cause of vascular disease in diabetes, but effective treatments for T2D-associated vascular diseases remain lacking. For example, T2D patients often have a poor outcome following thrombolytic therapy and revascularization, and they have higher rates of stent and graft failure after coronary angioplasty or coronary artery bypass grafting surgery (32).

Many factors are involved in regulating the vascular structure-function relation. A delicate balance between endothelium-derived factors and signaling pathways in the underlying vascular smooth muscle cells (VSMCs) play a pivotal role in tightly regulating the relaxation-contraction function that maintains the basal vasomotor tone of the systemic arteries. It is now thought that impaired endothelium-dependent relaxation of VSMCs and increased reactive oxygen species (ROS) within VSMCs are contributors to coronary artery dysfunction and disease in diabetes (5). We recently demonstrated that decreased bioavailability of endothelium-derived nitric oxide (NO) is a cause of vascular dysfunction in Goto-Kakizaki (GK) rats, a prototype of mild T2D (15). NO signaling not only regulates vascular tone but also inhibits platelet aggregation, monocyte adhesion, VSMC migration and phenotypic modulation, and vascular remodeling (3, 11, 30).

Within smooth muscle, expression of the contractile phenotype is regulated by NO-protein kinase G (PKG) signaling (21) and by the transcription factor serum response factor (SRF) (25). SRF-dependent transcription of contractile protein genes is regulated by two cotranscription factors, myocardin and Krüppel-like factor (klf)-4, which respectively promote and stall the transcription of contractile protein genes (23). In addition, the expression of myocardin is upregulated by microRNA (miR)-145, whereas expression of klf-4 is downregulated by miR-1 and miR-145 (8). For those reasons, it is now thought that events occurring between PKG signaling (7) and the expression of either myocardin or klf-4 decide the phenotypic fate of VSMCs and hence the vessel’s structure-function relation. Although NO levels reduced in type 1 and 2 diabetes are known to cause vascular dysfunction, the consequences of reducing NO and PKG-mediated signaling on the expression of contractile proteins within the vasculature in nonobese type 2 diabetic animals and patients are not clearly known. Moreover, what happens to miRs that mediate the expression of myocardin or klf-4 and their target proteins within the arteries of nonobese type 2 diabetes has not yet been investigated. Our hypothesis, therefore, was that the NO-PKG-dependent expression of smooth muscle contractile proteins is reduced and miRs that regulate expression of myocardin and myocardin-dependent smooth muscle contractile protein genes downregulated in the arteries of mildly diabetic GK rats. This switches the

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VSMC from differentiated to dedifferentiated phenotype, and we speculate this contributes to vascular dysfunction in GK rats.

MATERIALS AND METHODS

Animal model. Male (3 mo old) euglycemic Wistar and diabetic GK rats, a nonobese model of T2D (15), were used throughout this study. Experimental protocols using animals had approval from the New York Medical College (A3362-01) and University of South Alabama (11036) Institutional Animal Care and Use Committees. Animals were maintained in accordance with institutional policies and the Public Health Service Policy on Humane Care and Use of Laboratory Animals (revised 8/2002).

Vessel isolation and functional studies. To test for physiological function and perform biochemical analysis in samples obtained from the same animal, we used aorta instead of microvessels. Aortas were isolated and vascular function was assessed as previously described (15). In brief, thoracic aortas were excised, cut into ring segments 1.5 mm in length with internal diameters of 1.0 mm, and used for measurements of isometric tension. Within a tissue bath the vessel rings were superfused with Krebs buffer solution containing (in mmol/l) 118 NaCl, 4.7 KCl, 1.5 CaCl₂, 25 NaHCO₃, 1.1 MgSO₄, 1.2 KH₂PO₄, and 5.6 glucose at 37°C, gassed with 95% air and 5% CO₂. After an equilibration period of 1 h, during which an optimal passive tension was applied to the rings (as determined from the vascular length-tension relationship), the designed experiments were performed.

miR analysis. Total RNA was extracted from rat aorta and smooth muscle cells (SMCs) using a mirNeasy kit (No. 271004, Qiagen). TaqMan miR assays ( Applied Biosystems, Foster City, CA) were used to prepare cDNA from total RNA, after which quantitative PCR was performed using TaqMan universal PCR master mix. Ct values from standard curves were then used to quantify relative expression of specific miR. All quantitative PCR reactions were performed in triplicate. Standard curves for miR-1 and miR-145 were made using synthetic miR oligonucleotides (IDT, Coralville, IA) with the following sequences: rno-miR-145: GUCCAGUUUUCCAAGAACUCU, rno-miR-1: UGAAAGUAGAAAGUAGUGUAU, rno-miR-145: UGAAAGAAGCAUGAGACUC, rno-miR-21: UAGCUCUACAGACUGAUGUGA, rno-miR-133a: UUUGCUCCUCUC-AACCAGCUC.

Western blot analysis. Proteins were extracted from frozen tissue in lysis buffer, after which Western blot analysis using the specific antibodies indicated in the individual lures was performed as previously described (2).

Nitrite measurements. Nitrite levels were measured using a colorimetric method. Aortas from Wistar and GK rats were crushed and homogenized in lysis buffer containing 50 mmol/l Tris-HCl, 150 mmol/l NaCl (pH 7.4), and 0.5% Nonidet P-40. The resultant homogenates (25 µl) were incubated for 10 min in a 96-well plate with Griess reagent 1 (50 µl of 2% sulphanilamide in 5% phosphoric acid) and Griess reagent 2 (50 µl of 0.2% N-(1-naphthyl) ethylenediamine dihydrochloride), after which absorbance was measured at 540 nm. The nitrite concentration was then calculated using a standard curve and normalized to the protein content determined using the Bradford method. The standard curve was plotted using sodium nitrite.

PKG and vasodilator phosphoprotein phosphorylation. Tissue homogenates were subjected to Western blot analysis using anti-PKG and anti-phosphorylated vasodilator phosphoprotein (VASP) antibodies (Cell Signaling) to assess PKG-catalyzed phosphorylation of VASP at Ser239 (p-VASP) (2).

Immunofluorescent staining. Frozen sections of artery were fixed in acetone, and antigen-retrieval was accomplished using 0.1% Triton X-100. After blocking the sections by incubation for 1 h in 5% normal goat serum or 5% BSA at room temperature, they were incubated with primary antibody overnight at 4°C. The sections were then washed with 1× PBS-Tween 20 and incubated with secondary antibody (anti-mouse Alexa488 or Alexa568 and anti-rabbit Alexa568 or Alexa488, Invitrogen) for 1 h at room temperature. After washing the sections again, they were stained with 4,6-diamidino-2-phenylindole (1 µg/ml) and mounted using DAKO mounting medium. Imaging was done using a Nikon-A1 confocal microscope.

Immunohistochemical staining. Frozen sections of artery were fixed in cold (4°C) acetone for 10 min. After being blocked for 10 min in 3% hydrogen peroxidase and a biotin blocking system (DAKO), the sections were incubated for 30 min, each first with the primary antibody (DAKO monoclonal mouse anti-rat Ki-67 antibody, clone MIB-5, 1:10 dilution) and then with a biotinylated horse anti-mouse IgG (rat absorbed), which served as the secondary antibody (1:100 dilution; Vector). Peroxidase-conjugated streptavidin (DAKO) was then used for detection, with diaminobenzidine serving as the chromogenic substance and hematoxylin as a counterstain. The stain was developed using a DAKO Autostainer Link 48 instrument (DAKO, Carpinteria, CA).

Dihydroethidium fluorescence. Hydroethidium, an oxidative fluorescent dye, was used as we previously described to localize superoxide production in situ (14, 15). In brief, vessels were incubated with hydroethidium (5 µmol/l; at 37°C for 60 min) and then washed three times, embedded in optimum cutting temperature medium and cryosectioned. Fluorescence images were captured using Nikon A1 confocal microscope equipped with a 60× objective lens and were analyzed using Nikon element imaging software. Ten to 15 fields/vehicle were analyzed using one image per field. The mean fluorescence intensities of ethidium-stained nuclei in the medial layer were calculated for each vessel. Thereafter, the intensity values for each animal in the group were averaged.

Chemiluminescence. ROS was determined using lucigenin and luminal chemiluminescence assay as previously described (34). In brief, aortas were homogenized in ice-cold buffer, after which the protein concentrations were determined using the Bradford method. To determine ROS, tissue homogenates (20 µl) were incubated at 37°C in the presence of 5 µM lucigenin for the detection of O₂⁻ and 10 µM luminal plus 1 µM horseradish peroxidase for the detection of H₂O₂ in a final volume of 200 µl of air-equilibrated Krebs solution buffered with 10 mM HEPES-NaOH (pH 7.4). Chemiluminescence was determined using a microplate reader (Synergy 2 from BioTek Instruments, Winooski, VT).

Statistical analysis. Values are presented as means ± SE. ANOVA and post hoc Fisher protected t-tests were used for analysis in all studies of vascular contractility. All other data were analyzed using Student’s t-tests or two-way ANOVA. Values of P < 0.05 were considered significant. In all cases, the number of experimental determinations (n) was equal to the number of rats from which aortas were harvested for this study.

RESULTS

Aortas Isolated from GK Rats Generate Greater Maximum Force than Those from Wistar Rats

Our first goal was to determine whether the aorta was remodeled and contractile function of arteries was increased in GK rats. We measured internal diameter and medial thickness of the aorta isolated from GK and Wistar rats microscopically and found that internal diameter was not different between GK (1.2 ± 0.3 mm) and Wistar (1.3 ± 0.7 mm) rats but the media of aorta from GK rats was thicker than Wistar rats (Fig. 1A). We next determined the maximum force generation of rings of endothelium-intact thoracic aorta in response to stimulation with KCl (120 and 30 mmol/l) or the thromboxane A₂ analog U46619 (100 mmol/l). We found that aortic rings from GK rats...
generated significantly more force than those from Wistar rats in response to KCl or U46619 (Fig. 1B).

In another experiment, aortic rings were contracted using two different doses of KCl. The rings were first primed using KCl (120 mmol/l), then washed with normal Krebs solution, and recontracted with KCl (30 mmol/l). This was followed by washout with normal Krebs. After 15–20 min, normal Krebs buffer was replaced with Ca\(^{2+}\)-free Krebs buffer containing the Ca\(^{2+}\)-chelating agents EGTA (0.5 mmol/l) and BAPTA (0.2 mmol/l), and the rings were allowed to equilibrate in the Ca\(^{2+}\)-free solution for 20 min. Thereafter, we determined the force generation elicited by U46619 (100 nmol/l). We found that under Ca\(^{2+}\)-free conditions, U46619 elicited greater (\(P < 0.05\)) contractions in aortas from GK rats (Fig. 1C).

Additionally, we found that U46619-elicited contraction of Wistar and GK rat aorta under Ca\(^{2+}\)-free conditions was
inhibited by Y-27632 (10 μmol/l), Rho kinase inhibitor, by 95.1 ± 4.9 and 44.5 ± 9.1%, respectively. Thereafter, in the presence of U46619 when Ca²⁺ (0.09 –1.5 mmol/l) was added back to the bath solution in dose-dependent fashion, the resultant dose-effect curve was shifted significantly upward with aortas from GK rats, compared with Wistar rats (Fig. 1D).

ROS Are Increased in Aortas from GK Rats

Many studies, including ours, have shown that levels of NADPH oxidase- and mitochondria-derived ROS are increased in the vascular tissue of diabetics (34) and are known to alter vascular function. Consistent with that finding, we observed that ROS levels, as indicated by dihydroethidium (DHE) fluorescence, were increased approximately twofold (P < 0.05) in the medial layer of aortas from diabetic GK rats (Fig. 2, A and B). Tempol treatment (for 3 wk) decreased DHE fluorescence in Wistar + Tempol (45 ± 8 nuclei/μm²) vs GK + Tempol (26 ± 4 nuclei/μm²) rat aorta. Similarly, lucigenin chemiluminescence assays showed that ROS were ~2.5-fold higher (P < 0.05) in aortic homogenates prepared from GK rats than Wistar rats (Fig. 2C). On the other hand, incubating the homogenates with Tempol (1 mmol/l; Fig. 2C), a spin trap that scavenges superoxide and hydrogen peroxide (H₂O₂) and prevents formation of hydroxyl radicals (41), or pegalated-SOD (100 U/ml; Wistar, 150 ± 75; and GK,: 853 ± 145 arbitrary units) and pegalated-catalase (300 U/ml; Fig. 2C) decreased (P < 0.05) lucigenin chemiluminescence in GK samples. On those lines, ebselen (100 μmol/l), a glutathione peroxidase mimetic that detoxifies H₂O₂ (16), reduced (P < 0.05) lucigenin chemiluminescence in GK samples by 52.8 and 55.7%, respectively, as compared with untreated samples and almost completely abolished (89.1 and 62.4%, respectively) ROS production in aortic homogenates from Wistar rats. Furthermore, we found that GK rat aorta produced ~267 μmol/l H₂O₂ more than the Wistar rat aorta (Fig. 2D).

PKG Expression and Activity Is Reduced in Aortas from GK Rats

Endothelial function is compromised in diabetes. Potentially, hyperglycemia could remodel the intimal layer of the arteries and inhibits endothelial NO biosynthesis. We found there no was difference in the appearance/morphology of lumen surface between GK and Wistar rats. We next estimated nitrite levels, PKG expression as well as activity based on VASP phosphorylation at S239, and determined peroxinitrite by nitrotyrosine staining. We found that nitrite levels were decreased (Fig. 3A) and nitrotyrosine staining (Fig. 3B) was unchanged in GK compared with Wistar rat aorta. On those lines, PKG expression (Fig. 3, C and D) and p-VASP levels were lower in aortas from GK than Wistar rats (Fig. 3, E and F).

**Fig. 3.** Nitric oxide (NO) and phosphorylated vasodilator phosphoprotein (p-VASP) levels in aortas from GK and Wistar rats. A: nitrite (NOx) levels were measured using a colorimetric assay. B: peroxinitrite formation was measured using nitrotyrosine staining. C–F: Western blot showing aortic levels of protein kinase G (PKG) and p-VASP in Wistar and GK rats. A representative blot from among 3 experiments is shown. β-Actin served as a loading control. Summary data showing that aortic expression of PKG and p-VASP normalized by β-actin in GK than Wistar rats (n = 4 to 5 in each group). Significance was calculated using Student’s t-test. T-Vasp, total VASP.
Expression of Contractile Proteins Is Increased in Aortas from GK Rats

Because NO-PKG signaling is known to upregulate contractile protein gene transcription, we used immunofluorescence microscopy and immunoblotting to assess the expression of contractile proteins in aortas from Wistar and GK rats. Unexpectedly, we found that expression of smooth muscle myosin heavy chain (SM-MHC) (Fig. 4A, top) and SM22α (Fig. 4A, middle) was higher in aortas (medial layer) from GK than Wistar rats. Likewise, expression of myocardin, a co-transcription factor that regulates SM-MHC and SM22α expression in VSMCs, was also higher in GK rats (Fig. 4A, bottom). Immunoblot analysis showed that aortic expression of calponin was about fourfold (P < 0.05) higher in GK rats than Wistar rats (Fig. 4, B and C). By contrast, there were no differences in the expression levels of GAPDH, an enzyme involved in glucose metabolism, or β-actin, a cytoskeleton protein, between the two rat types (Fig. 4B and D). Nor was there a difference in the aortic expression of Ki-67 (Fig. 4E), a mitogenic marker that is commonly used to determine cell proliferation, between GK and Wistar rats.

Expression of Contractile Proteins in Aortas from GK Rats Is Increased by ROS

To determine whether the contractile protein expression is increased by elevated ROS seen in GK rats, we added Tempol...
Expression of miR-145 and miR-1 Increases in Aortas from GK Rats

Because miR-145 and miR-1 are known to upregulate contractile protein gene expression, we next measured miR expression in aortic tissue from Wistar and GK rats (Fig. 6A). We found that levels of both miR-145 and miR-1 were increased (P < 0.05) in GK rat aortas (Fig. 6, A–C). miR-145/143 and miR-1/133a are bicistronic miR clusters. Therefore, we estimated miR-143 and miR-133a levels in Wistar and GK rat aorta (Fig. 6A), and found that miR-143 levels were slightly increased (Wistar: 0.87 ± 0.11 and GK: 1.22 ± 0.17 pg/100 µg total RNA; P < 0.05, n = 7) but miR-133a levels were unchanged (Wistar: 0.42 ± 0.02 and GK: 0.35 ± 0.03 pg/100 µg total RNA; not significant, n = 7) in GK compared with Wistar rat aorta. Additionally, we found that miR-21 levels were slightly but significantly elevated (Wistar: 0.95 ± 0.10 and GK: 1.31 ± 0.09 pg/100 µg total RNA; P < 0.05, n = 7) in GK compared with Wistar rat aorta.

To determine whether this rise reflected the elevated ROS seen in GK rats, we measured miR levels in aortic tissue from rats treated with and without Tempol (1 mmol/l) for 3 wk. We found that Tempol administration reduced (P < 0.05) myocardin and calponin levels compared with untreated rats. We found that Tempol administration reduced (P < 0.05) myocardin and calponin levels compared with untreated rats. We found that Tempol decreased medial thickness by 150–175 µm and U46619-elicited contraction of GK rat aorta in presence of extracellular Ca²⁺ (Fig. 5D).

Expression of miR-145 Is Increased in Aorta and Aortic SMCs by H₂O₂ via MEK1/2 Pathway

To further determine whether ROS increases miR expression, we incubated freshly isolated rat aorta with H₂O₂ (300 µmol/l) in vitro for 12 h and measured miR-145 and miR-1 levels. H₂O₂ (300 µmol/l) increased miR-145 and decreased miR-1 (Fig. 7).

Additionally, to find out whether H₂O₂ increased miR in the SMCs, we incubated aortic SMCs for 12 h in the presence of Tempol (1 mmol/l) and H₂O₂ (300 µmol/l). We found that Tempol (1 mmol/l) decreased [control (Con), 0.48 ± 0.02 pg/100 µg total RNA; and Tempol, 0.36 ± 0.02 pg/100 µg total RNA; P < 0.05, n = 9] and H₂O₂ increased (Fig. 8A) the expression levels of miR-145 compared with untreated cells. As p38 MAPK and ERK1/2 pathways regulate miR’s expression and because p-ERK1/2-to-total ERK ratio is increased (P < 0.05; n = 4) in GK (2.33 ± 0.064) compared with Wistar (1.87 ± 0.17) rat aorta, we determined the effect of U0126 (10 µmol/l), widely used MEK1/2 inhibitor, on basal and H₂O₂ (300 µmol/l)-induced expression of miR-145 and its target proteins myocardin. Interestingly, U0126 had no significant effect on basal miR levels but decreased (P < 0.05) H₂O₂-induced expression (Fig. 8) and not miR-1 (Con, 0.019 ± 0.002; H₂O₂, 0.012 ± 0.004; and H₂O₂ + U0126, 0.021 ± 0.003 fg/100 µg total RNA; not significant, n = 7–9) after 12 h. Furthermore, immunofluorescent microscopy revealed that H₂O₂ increased nuclear levels of myocardin (Fig. 8, B and C). On the contrary, H₂O₂ decreased cytosolic levels of myocardin (Con, 0.0005205 ± 9.563e-005, n = 22; and H₂O₂, 0.0002124 ± 4.881e-005; n = 15). H₂O₂-induced increase of nuclear myocardin expression was decreased (P < 0.05) by U0126 (Fig. 8, C).

Fig. 5. Aortic expression of contractile proteins in GK and Wistar (W) rats treated with Tempol (T, Temp, Tem). A–C: myocardin and calponin expression in aorta from GK rats treated with Tempol (1 mmol/l). D: comparison of U46619 (100 nmol/l)-induced contraction of aortic ring from GK rats untreated and treated with Tempol (n = 4 to 5 in each experiment). *P < 0.05 between Wistar and GK; #P < 0.05 between WK and treatment groups. Statistical significance between GK and Wistar rat aortas was determined using 2-way ANOVA and post hoc Bonferroni’s test. Con. control.
and C), and by anti-miR-145 (50 nmol/l; 0.0085/0.0002) pretreatment.

Additionally, in some studies, p38 MAPK (SB202190; 10 μmol/l) and JNK (SP200169; 10 μmol/l) inhibitors were found not to affect H₂O₂-induced miR-145 expression.

DISCUSSION

Our findings demonstrate that generation of ROS, the expression of contractile proteins, and miR-145 levels were increased in aortas from GK rats, a model of mild T2D. The increase in ROS-induced contractile proteins presumably underlies the remodeling of the T2D aorta that led to enhanced force generation. Our other significant findings suggest that increases in ROS augment miR-145 expression and nuclear levels of myocardin in VSMCs.

Studies have shown that ROS reduce the biological activity of NO and inactivate soluble guanylate cyclase, thereby inhibiting NO-induced relaxation of vascular smooth muscle in diabetic animal models and VSMCs (15, 34, 40). Consistent with those earlier findings, our present results clearly demonstrate that ROS levels were increased by two- to threefold in the medial smooth muscle layer of aortas (detected by DHE staining) from GK rats, as well as in homogenates of whole aorta (estimated by lucigenin chemiluminescence) from the T2D animals. This elevation in ROS would be expected to reduce nitrite and PKG activity and so inhibit vascular smooth muscle relaxation (30, 40).

Fig. 7. H₂O₂ increases expression of miR-145 and miR-1 in rat aorta. Total RNA was extracted from freshly isolated rat aorta untreated or treated for 12 h with H₂O₂ (300 μmol/l; n = 5), after which expression of miR-145 was assessed using 2-step RT-PCR (in triplicate). Expression of miR-145 (A) and miR-1 (B) was increased and decreased, respectively, by H₂O₂. Significance was calculated using Student’s t-test.
Vascular inflammation, monocyte adhesion to smooth muscle, platelet coagulation, hyperproliferation, and VSMC migration have all been shown to contribute to vascular remodeling in diabetes (6, 12, 17, 19, 31). Apart from controlling vaso-motor function, NO and cGMP-PKG signaling also regulates VSMC inflammation, phenotype, proliferation, and migration (1, 4, 21). Activation of PKG via NO-cGMP signaling inhibits cell migration and proliferation and increases expression of contractile proteins (21). Given that endothelium-derived NO-mediated relaxation (15) and nitrite/PKG/p-VASP levels (Fig. 3) were downregulated in aortas from GK rats, we anticipated that aortic expression of contractile proteins would be lower and expression of cell proliferative proteins would be higher in GK than Wistar rats. To our surprise, however, aortic expression of myocardin, SM-MHC, SM22α, and calponin was significantly increased in GK rats, compared with Wistar rats. There was no Ki67+ cell in GK as well as Wistar rat aortas. This indicates that VSMCs were not in mitotic phase or proliferating in rat aorta at the time of the experiment. Although these findings rule out that increase of contractile protein expression in GK rat aorta was due to increase in cell numbers, there is a plausibility that proliferation could have occurred earlier and there could be more VSMCs in the medial layer of GK rats compared with Wistar rats that could account for the increased levels of contractile proteins. However, it is highly unlikely that cell proliferation, if turned on, in diabetes would abruptly stop, and hence there is alternate route/mechanism through which the contractile protein expression is potentially increased in GK rat aortas. The expression of transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) is increased in diabetes (26, 29, 37). Given that these two mediators are known to control the phenotype of VSMCs [e.g., TGF-β promotes expression of contractile proteins (36)], we therefore speculate that they may have contributed to the increases in myocardin, SM-MHC, SM22α, and calponin expression observed in GK rats. Consistent with that idea, activation of TGF-β signaling reportedly increases miR-145 expression via SRF-myocardin in cultured human coronary artery SMCs (24). Conversely, myocardin expression is upregulated by miR-145 (8). Since anti-miR-145 suppressed H2O2-induced nuclear expression of myocardin protein, it is safe to suggest that miR-145 promoted myocardin expression in aortic SMCs. Elevation of myocardin also promotes transcription of SM-MHC and other contractile protein expression.

Fig. 8. H2O2 increases expression of miR-145 and on nuclear levels of myocardin in A7r5 cells. Total RNA was extracted from A7r5 cells untreated or treated for 12 h with H2O2 (300 μmol/l) or H2O2 + U0126 (10 μmol/l), after which expression of miR-145 was assessed using two-step-RT-PCR (in triplicate). A: expression of miR-145 was increased by H2O2. Cotreatment with U0126 and H2O2 inhibited miR-145 expression. B: representative confocal images of myocardin (green) staining in A7r5 cells untreated or treated for 12 h with H2O2 (300 μmol/l), H2O2 (300 μmol/l) + U0126 (10 μmol/l), and H2O2 (300 μmol/l) + anti-miR-145 (50 nmol/l). Nuclei were stained with DAPI (blue). C: summary data showing that nuclear myocardin was increased by H2O2 treatment. Data were collected from A7r5 cells (4 to 5 fields) cultured on coverslips (n = 5 in each group). *P < 0.05 between Wistar and GK; #P < 0.05 between Wistar and GK vs. treatment groups.
genes. MiR-1 and miR-145 that controls cardiac and smooth muscle myocyte differentiation in turn reduces expression of klf-4, a suppressor of the SRF-myocardin complex (8), thereby increasing transcription of contractile protein genes (9). Interestingly, we observed that aortic miR-145 levels were higher in GK than Wistar rats and that expression of miR-145 and myocardin, but not miR-1, could be reduced by treating the rats with Tempol, an antioxidant. Consistently, expression of miR-21, which is upregulated by H₂O₂ in cultured aortic SMCs (20), was slightly increased in GK compared with Wistar rat aorta. Collectively, these results suggest that miR-145 and miR-1 expression was differentially regulated and that expression of myocardin-dependent contractile proteins calponin and SM-MHC, which potentiate vasoconstriction (10), is increased via a ROS-miR-145 pathway in arterial smooth muscle. Since GK rat aorta generated significantly higher force than Wistar rat aorta in Ca²⁺-free condition and in presence of Rho kinase inhibitor that almost completely inhibited U46619-induced contraction of Wistar rat aorta, we suggest that increase in the ROS-induced SM-MHC expression additionally contributed to increase force generation in Ca²⁺- and Rho kinase-independent manner in aorta from GK rats.

To test our hypothesis that elevated ROS increases miR-145 expression in smooth muscle, we examined the effect of H₂O₂ on miR-145 and miR-1 levels and their target protein, myocardin, in freshly isolated aorta from Wistar rat or cultured aortic SMCs. Incubating aorta or aortic SMCs for 12 h in the presence of 300 μmol/l H₂O₂, a concentration found in GK rat aortas (see RESULTS) and often observed under pathophysiological conditions (28, 35), significantly increased expression of miR-145 as well as nuclear myocardin levels. Conversely, the antioxidant Tempol reduced expression of miR-145. These findings, which were similar to what we found in Wistar and GK rats, demonstrate that endogenous basal ROS production supported miR-145 expression and that ROS exert twofold effects on the mechanisms that regulate contractile protein synthesis in aortic SMCs: they increase expression of miR-145, which elevates myocardin, and they simultaneously facilitate the increase of nuclear myocardin. Our results are opposite to the previous findings showing miR-145 (detected by microarray analysis) decreased in cultured aortic SMCs isolated from control rat aortas and incubated with H₂O₂ (200 μmol/l) for 6 h (20). We attribute these differences to the different: pathologies, doses of H₂O₂, duration of incubation periods, and methodologies employed between our studies. Nonetheless, our findings are consistent with studies that have shown that ROS regulate the expression of some miRs involved in angiogenic pathways (33) and miR-21 involved in inhibiting apoptosis (20) in VSMCs. Furthermore, p38 MAPK and ERK1/2 pathways control miR expression (39). A recent study has shown that the miR-133a-1/miR-1–2 and miR-133a-2/miR-1–1, two bicistronic miR clusters expressed specifically in cardiac and skeletal muscle (22), are also present in VSMCs and miR-133a, but not miR-1, is downregulated via the PDGF-induced MEK-ERK pathway (38).

Since U0126, a MEK1/2 inhibitor that inhibits ERK phosphorylation in rat VSMCs (18), significantly decreased H₂O₂-induced miR-145 and myocardin, but not miR-1, expression in aortic SMCs. Since U0126 decreased miR-145 and myocardin expression after 12 and 48 h, respectively, this indicates MEK-ERK pathway activated by H₂O₂ increased miR-145 that subsequently elevated myocardin expression. Coincidentally, ROS and H₂O₂ also elicit contraction of coronary arteries by activating MEK-ERK pathway (27). We therefore suggest that increase in H₂O₂-induced MEK1/2 activity selectively upregulated miR-145 and myocardin expression, and that they make conditions conducive for the synthesis of contractile proteins evoked by increased ROS in VSMCs and for the remodeling of smooth muscle in GK rats.

Vascular complications are common in T2D. Endothelial dysfunction leading to lower levels of endothelium-derived NO signaling has been implicated as a probable cause of vasoconstriction in diabetes, although other studies have reported that arteries are also structurally remodeled in T2D. Our novel

Fig. 9. Schematic illustration. Overall effect of ROS signaling on miRs and contractile proteins in diabetic arteries. Broken arrows demonstrate a novel ROS/H₂O₂-dependent mechanism increased miR-145 expression that upregulated the contractile protein expression in the type 2 diabetic rat aorta. Solid arrows represent known information. Black and gray colored arrows indicate positive and negative regulation, respectively. SRF, serum response factor; ROCK, RhoA-Rho kinase; MLC-P, myosin light chain phosphorylation; [Ca²⁺⁺], intracellular calcium concentration.
findings demonstrate that elevated ROS selectively increases expression of miR-145 that upregulates expression of contractile proteins in GK rat aorta. The resultant ROS-mediated overexpression of contractile proteins in conduit arteries, which along with decreased NO signaling, contributes to VSMC contraction observed in non-obese T2D (Fig. 9).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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