Cocaine Exposure Increases Blood Pressure and Aortic Stiffness via the miR-30c-5p–Malic Enzyme 1–Reactive Oxygen Species Pathway

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See Editorial Commentary, pp 561–562

Abstract —Cocaine abuse increases the risk of cardiovascular mortality and morbidity; however, the underlying molecular mechanisms remain elusive. By using a mouse model for cocaine abuse/use, we found that repeated cocaine injection led to increased blood pressure and aortic stiffness in mice associated with elevated levels of reactive oxygen species (ROS) in the aortas, a phenomenon similar to that observed in hypertensive humans. This ROS elevation was correlated with downregulation of Me1 (malic enzyme 1), an important redox molecule that counteracts ROS generation, and upregulation of microRNA (miR)-30c-5p that targets Me1 expression by directly binding to its 3′UTR (untranslated region). Remarkably, lentivirus-mediated overexpression of miR-30c-5p in aortic smooth muscle cells recapitulated the effect of cocaine on Me1 suppression, which in turn led to ROS elevation. Moreover, in vivo silencing of miR-30c-5p in smooth muscle cells resulted in Me1 upregulation, ROS reduction, and significantly suppressed cocaine-induced increases in blood pressure and aortic stiffness—a similar effect to that produced by treatment with the antioxidant N-acetyl cysteine. Discovery of this novel cocaine-↑miR-30c-5p–↓Me1–↑ROS pathway provides a potential new therapeutic avenue for treatment of cocaine abuse–related cardiovascular disease. **(*Hypertension*. 2018;71:752-760. DOI: 10.1161/HYPERTENSIONAHA.117.10213.)** • **Online Data Supplement**

Key Words: antioxidant ■ blood pressure ■ cocaine ■ microRNA ■ reactive oxygen species

Cocaine addiction inflicts enormous health and economic costs to individuals, families, and society. Although significant research has focused on the neurobiological consequences of chronic cocaine abuse,[1–3] cocaine intake is also associated with potentially fatal cardiovascular events such as arrhythmias, myocardial infarction, and stroke.[4–7] Long-term exposure to cocaine can lead to hypertension, aortic stiffness, and atherosclerosis.[8–11] Although the clinical consequences of cocaine-induced cardiovascular toxicities are well documented, the molecular mechanisms underlying these effects of cocaine remain poorly understood.

Accumulating evidence suggests that excessive oxidative stress plays an important role in the pathogenesis of hypertension because increased levels of reactive oxygen species (ROS) have been found in aortas of hypertensive animals and humans.[12–17] Intracellular ROS is generated by oxidation of NADPH into $NADP^+$ ($NADPH+2O_2 \rightarrow NADP^+ + 2O_2^- + H^+$).[18–20] This process, however, is counteracted by antioxidants and redox molecules, such as the Me1 (malic enzyme 1), which encodes a NADP(+)-dependent enzyme that turns NADP+ into NADPH when catalyzing the oxidative decarboxylation of malate into pyruvate.[18–20] Me1 expression is regulated by microRNAs (miRNAs), the short, noncoding RNA molecules that bind to the 3′UTR (untranslated region) of target mRNAs leading to their translational repression and mRNA degradation.[21,22] MiRNA target prediction programs, such as TargetScan (targetscan.org)[23,24] and Miranda (microRNA.org),[25] have identified highly conserved binding sites for the miR-30c-5p/384-5p and miR-153-3p families in the Me1 3′UTR. Among these, the miR-30c-5p/384-5p family are predicted to have a more stable binding to the Me1 3′UTR (position 552–559) with a lower context and mirSVR score.[23–25] Although the involvement of

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miRNAs in cardiac development and pathogenesis of cardiovascular disease (CVD) has been reported,[21,22] the role of miR-30c-5p in cocaine-driven CVD is unknown.

We sought to understand whether the miR-30c-5p–Me1–ROS molecular axis is involved in mediating the effect of cocaine on inducing CVD. By using a cocaine abuse/use mouse model combined with aortic smooth muscle cell (SMC) cultures, we examined the inter-relationship between components of the miR-30c-5p–Me1 pathway and how modulating these components affects cocaine-induced cardiovascular phenotypes through a set of gain- and loss-of-function experiments.

Methods

The data, analytic methods, and study materials that support the findings of this study are available from the corresponding author on reasonable request.

Animals

Male C57BL/6 mice (8–10 weeks old; The Jackson Laboratory) were housed 4 animals per cage, under a regular 12/12 hour light/dark cycle with ad libitum access to food and water. Mice received a daily intraperitoneal injection of cocaine, cocaine methiodide (CM; 20 mg/kg body weight each; NIDA Drug Supply), or saline for 10 consecutive days, a treatment regimen that has been validated in multiple animal models of addiction.[26] Cocaine and CM were dissolved in 0.9% sterile saline before injection.

In parallel experiments, mice were injected 3 times on alternate days via the tail vein with 2×10^5 IU/mouse lentiviral constructs encoding a miR-30c-5p antagomir (miR-Zip30c) or no gene (empty vector control) underdriven by a SMC-specific promoter, Sm22a (Biosettia). Subsequently, the mice were injected with cocaine as above. One day prior and daily during cocaine injection, a separate group of mice received intraperitoneal injection of N-acetyl cysteine (NAC; Sigma Aldrich) at 250 mg/kg body weight dissolved in PBS. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Miami.

Blood Pressure and Aortic Stiffness

Systolic and diastolic blood pressure (BP) was measured using a noninvasive tail-cuff CODA BP monitor following the manufacturer's protocols (Kent Scientific). BP was measured 1 day before the first injection (baselines) and 1 hour (half-life of cocaine) after each daily injection in a 10-day treatment course. Pulse wave velocity (PWV) measuring aortic stiffness was determined 1 day before the first injection and 2 days after the last injection (day 12). Briefly, mice were anesthetized with 2% isoflurane through a face mask and laid on a platform in the supine position with all legs taped to electrocardiography electrodes for heart rate monitoring. Aortic arch velocity signal was assessed by placing a focused 420–40 MHz Doppler probe (Vevo 770; VisualSonics) to the right of the upper sternum, angling the sound beam to align with the direction of the aortic arch, and capturing the aortic shape velocity signal moving away from the probe. At least 3 measurements were recorded for each mouse.

Immunohistochemistry

Immediately after aortic stiffness measurements, mice were euthanized by carbon dioxide, and isolated aortas were flash frozen in optimum cutting temperature freezing medium on dry ice. Aortas were sliced using a CM 1850 microtome (Leica Biosystems) to produce 10-µm-thick sections. Cryosections were fixed in cold 4% paraformaldehyde in PBS for 10 minutes, washed with PBS, and incubated with 0.3% Triton in PBS for 10 minutes. Subsequently, sections were blocked with 5% BSA in PBS for 1 hour and incubated with rat antimouse Me1 antibody conjugated with Alexa Fluor 594 (PharMingen) overnight at 4°C. After washing with PBS, sections were incubated with Hoechst blue nuclear dye (Molecular Probes) for 1 hour at room temperature in the dark. At least 5 consecutive sections were examined for each aorta. Fluorescent signal was imaged on the Zeiss LSM710 Confocal AxioObserver Inverted Automated Microscope and quantified by Image J software (National Institutes of Health).

ROS Detection

Intracellular O_2^-, H_2O_2, and peroxynitrite ($ONOO^-$) were detected by incubating aorta cryosections with 10 µmol/L nonfluorescent dye dihydroethidium, chloromethyl-2′,7′-dichlorofluorescein diacetate (all from Sigma Aldrich), and aminophenyl fluorescein (Molecular Probes), respectively, for 5 to 30 minutes at 37°C in the dark. The fluorescent signal was imaged and quantified as described above. The level of nitrotyrosine and prostaglandin in cultured cells were detected using specific ELISA kits (Cell Biolabs) following the manufacturer's protocols.

Cell Cultures and 3′UTR Reporter Assay

Mouse aorta derived SMCs were cultured following the manufacturer's protocol (Cell Biologics). SMCs were transduced by lentiviruses encoding miR30c, miR-Zip30c, or miR-scrambled control (miR-Ctr) (System Biosciences) overnight to generate stable cell lines that overexpressed or suppressed miR-30c-5p expression. Transduction efficiency was monitored by green fluorescent protein expression and transduced cells were selected by treatment with 10 µg/mL puromycin.

HEK293 cells (Sigma Aldrich) were transfected using lipofectamine 2000 reagent (Promega) with miR-30c precursor or miR-Ctr constructs (System Biosciences) along with either a wild-type 3′UTR-Me1 luciferase reporter construct or a construct in which the putative miR-30c-5p's binding sites were mutated from *TGAG*aaagctcactgctgt*TTAC* to *GAGT*aaagctcactgctgt*CATT* (GeneCopoeia). Gaussia luciferase activity in cell medium was determined 48 hours after transfection using a Centro XS^3 LB 960 Microplate Luminometer (Berthold Technologies) and normalized to the activities of secreted alkaline phosphatase.

Quantitative Reverse Transcription and Polymerase Chain Reaction

One microgram of total RNA was reversed transcribed using the SuperScript IV VILO Master Mix (Invitrogen). Me1 gene expression was measured by quantitative polymerase chain reaction on 5 ng of cDNA using the iQ SYBR Green Supermix (Bio-Rad) with specific forward (5′-gacccgcatctcaacaagga-3′) and reverse (5′-gtcgaagtcagagttcagtcgt-3′) primers. Values were normalized to the level of a house-keeping gene, Gapdh (forward: 5′-tgcatcctgcaccaccaact-3′; reverse: 5′-acgccacagctttccagagg-3′). MiR-30c-5p expression was assessed using Taqman MicroRNA Assays (Thermofisher Scientific) and normalized to the level of small nucleolar RNA, following the manufacturer's protocols.

Statistics

Five mice were included in each treatment group at each time point. For cultured cells, 3 to 6 samples (wells) were included in each treatment group with duplicate measurements for each sample, and experiments were repeated at least twice independently using different batches of cells. The sample size was selected based on results from pilot studies. All data were presented as mean±SD. Difference between 2 groups was compared by 1-way ANOVA followed by Tukey post hoc test (GraphPad Prism7) with a minimal 80% power, and significance set at $P\leq0.05$.

Results

Cocaine and CM Treatment Increases BP, Aortic Stiffness, and ROS Levels in the Aorta

Mice were treated with daily injection of saline, cocaine, or CM for 10 consecutive days. CM, a cocaine analog that does not

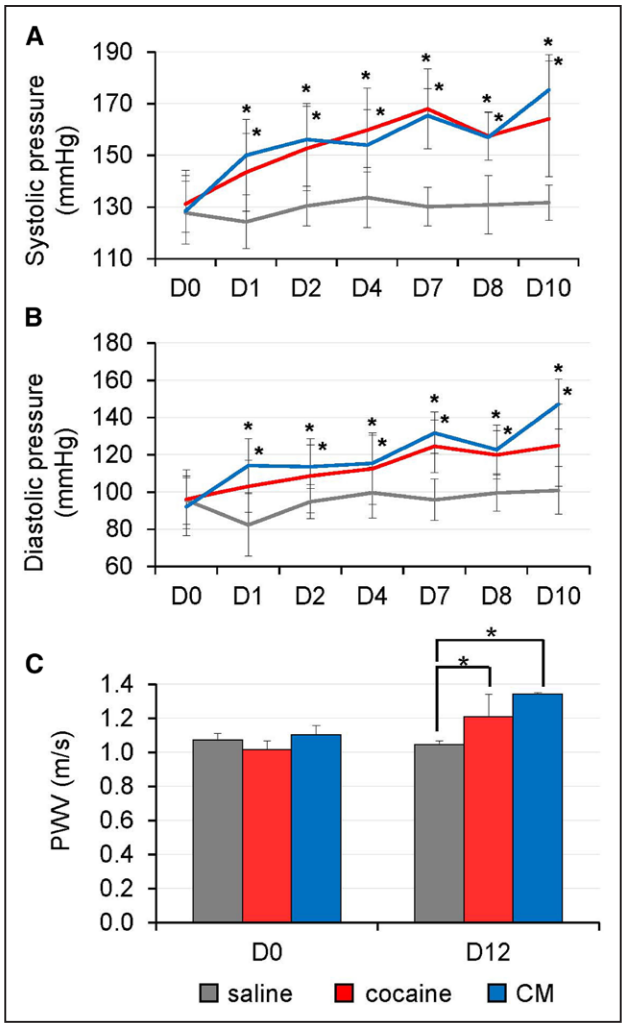

Figure 1. Cocaine and cocaine methiodide (CM) treatment increases blood pressure (BP) and aortic stiffness in mice. C57/BL6 mice (n=5) were injected with cocaine, CM, or saline each day for 10 consecutive days. **A** and **B**, Cocaine- or CM-treated mice showed elevated systolic and diastolic BP compared with saline-treated mice with the BP steadily increasing over the treatment course (**P*<0.05 vs saline at each time point). **C**, Aortic stiffness (measured by pulse wave velocity [PWV]) was elevated in the cocaine- or CM-treated mice 2 d after the last injection (**P*<0.05 vs saline at D12).

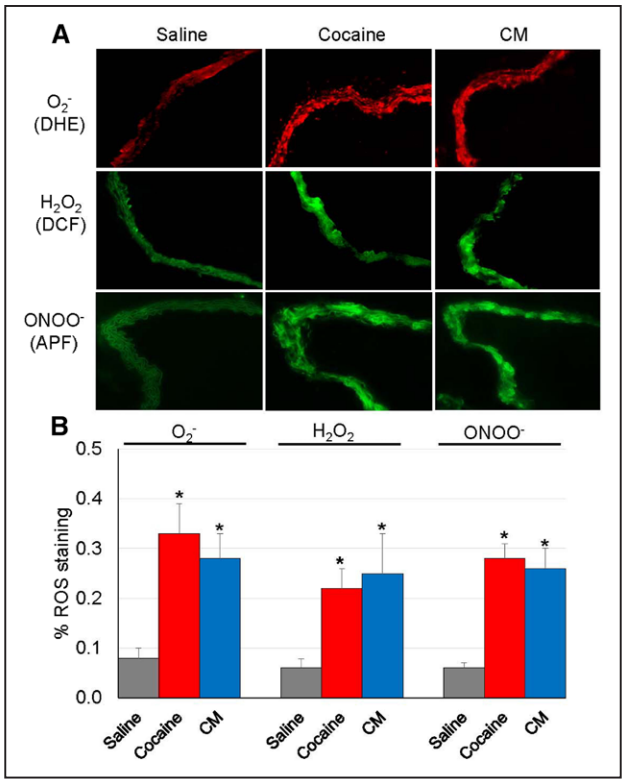

Figure 2. Cocaine and cocaine methiodide (CM) treatment leads to increased reactive oxygen species (ROS) levels in the mouse aorta. Aortas were obtained from mice (n=5) that received repeated injections of cocaine, CM, or saline. **A**, Intracellular O_2^-, H_2O_2, and $ONOO^-$ were examined by incubating aorta cryosections with dihydroethidium (DHE), chloromethyl-2′,7′-dichlorofluorescein diacetate (CM-H2DCFDA), and aminophenyl fluorescein (APF) dye, respectively. **B**, Fluorescent signal was quantified using Image J software. Cocaine or CM treatment significantly increased ROS levels compared with saline treatment (**P*<0.05 vs saline in each ROS).

enter the brain, was used to corroborate the non–central nervous system effect of cocaine. There were no differences in baseline (day 0) BPs between treatment groups. However, a significant increase in systolic (Figure 1A) and diastolic (Figure 1B) BP was observed in cocaine- and CM-treated animals throughout the treatment course compared with saline treatment. Comparable baseline levels of PWV were also found across the groups before the initiation of treatment (Figure 1C). However, PWV was significantly elevated in cocaine- and CM-treated mice at day 12 compared with saline treatment.

Staining for intracellular ROS in aorta cryosections revealed that cocaine and CM treatment significantly increased the levels of O_2^-, H_2O_2, and $ONOO^-$ compared with saline treatment (Figure 2A and 2B). There were no significant differences between cocaine- and CM-treated mice in terms of BP, PWV, and ROS levels.

Cocaine and CM Exposure Affects the Expression of miR-30c-5p and Me1 in the Aorta

Quantitative reverse transcription and polymerase chain reaction was performed to assess the expression of Me1 and its predicted regulatory miRNA, miR-30c-5p, in the mouse aorta. Significantly reduced Me1 mRNA expression but elevated miR-30c-5p expression was detected on cocaine or CM treatment compared with saline treatment (Figure 3A). Immunohistochemistry staining of aorta cryosections also confirmed a 67% and 58% decrease in Me1 protein expression following cocaine and CM exposure, respectively, compared with saline (Figure 3B and 3C).

3′UTR reporter assays were performed in HEK293 cells to determine whether miR-30c-5p can directly silence Me1 expression. As shown in Figure 3D, the luciferase activity of the wild-type Me1-3′UTR was significantly decreased by miR-30c-5p but not miR-Ctr in a dose-dependent manner. In contrast, the luciferase activity of the mutant (with disrupted miR-30c-5p–binding site) Me1-3′UTR was not affected by miR-30c-5p, suggesting that Me1 is a direct target of miR-30c-5p.

MiR-30c-5p–Me1 Axis Mediates Cocaine-Induced ROS Elevation in Mouse Aortic SMCs

To determine whether modulating the miR-30c-5p–Me1 pathway alters ROS levels, SMCs were transduced with

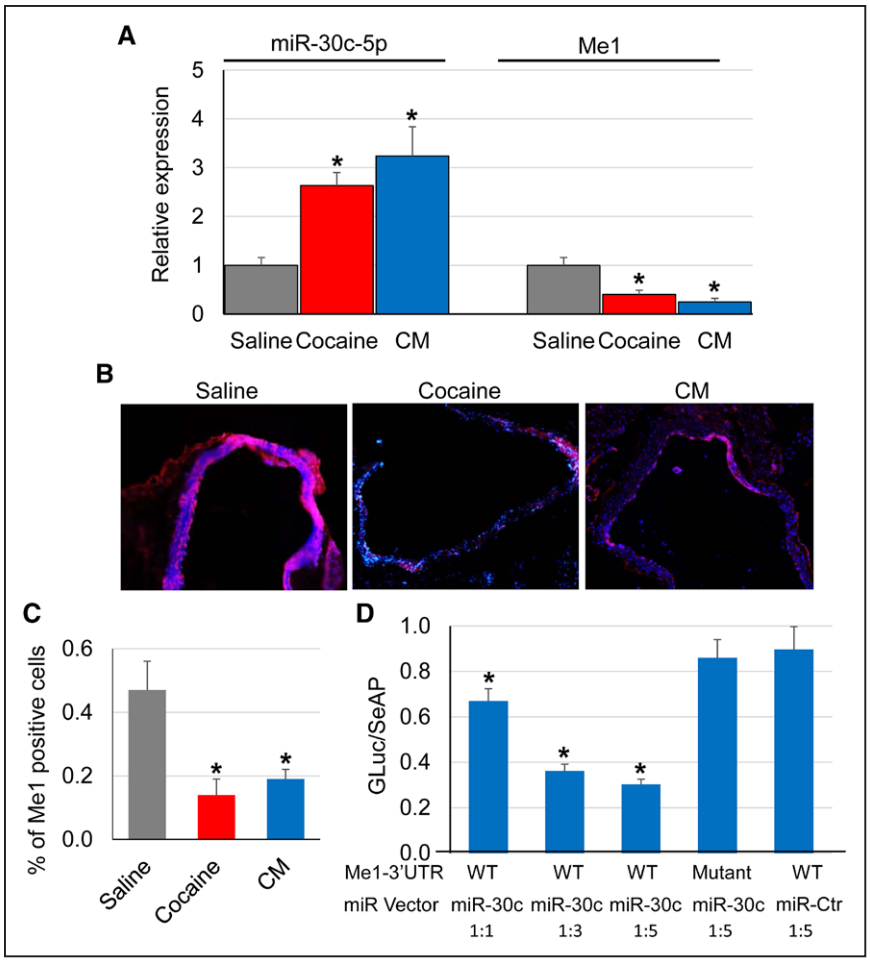

Figure 3. Cocaine and cocaine methiodide (CM) treatment increases miR-30c-5p expression but decreases Me1 (malic enzyme 1) expression in the mouse aorta. Total RNAs were extracted from aortas of mice (n=5) after injection with cocaine, CM, or saline for 10 consecutive days. **A**, Quantitative reverse transcription and polymerase chain analyses showed significantly increased miR-30c-5p levels and decreased Me1 expression (normalized to small nucleolar RNA and Gapdh, respectively) by cocaine treatment (*P<0.05 vs saline). **B** and **C**, Immunohistochemistry analysis showed cocaine- and CM-induced decreases in Me1 protein expression compared with saline. The relative number of Me1-positive cells was quantified using Image J software (*P<0.05 vs saline). **D**, 3′UTR (untranslated region) reporter assays showed that transfection with miR-30c-5p but not miR-scrambled control (miR-Ctr) decreased luciferase activities (normalized to secreted alkaline phosphatase activities) of reporter constructs containing the wild type (WT), but not mutant (with disrupted binding sites for miR-30c-5p), 3′UTR of Me1 gene in a dose-dependent manner (*P<0.05 vs WT Me1-3′UTR+miR-Ctr at 1:5).

lentiviral vectors that express miR-30c-5p, miR-Zip30c, or miR-Ctr. Overexpression of miR-30c-5p led to a significant decrease in Me1 levels compared with miR-Ctr–treated cells (Figure 4A and 4B). Conversely, SMCs with suppressed miR-30c-5p levels by miR-Zip30c had elevated Me1 expression relative to miR-Ctr (Figure 4A and 4B). Cocaine treatment further potentiated the miR-30c-5p–induced Me1 reduction in a dose-dependent manner (Figure 4B). In contrast, cocaine exposure produced no significant changes in Me1 expression in miR-Zip30c–transduced SMCs (Figure 4B), suggesting that cocaine induces its effect on Me1 expression through miR-30c-5p.

ROS detection showed that overexpression of miR-30c-5p in SMCs increased prostaglandin (an indicator of peroxides) levels compared with miR-Ctr–treated cells (Figure 4C). Cocaine had an additive effect on prostaglandin upregulation in miR-30c-5p–transduced cells (Figure 4C). In contrast, miR-Zip30c treatment reduced prostaglandin to levels below that seen in miR-Ctr–treated cells, and cocaine exposure failed to increase prostaglandin levels in miR-Zip30c–treated cells (Figure 4C). In addition, increased staining for H_2O_2 was observed in cells overexpressing miR-30c-5p, whereas reduced H_2O_2 staining was seen in miR-Zip30c–transduced SMCs compared with miR-Ctr–treated cells (Figure 4D). Cocaine treatment further increased H_2O_2 in miR-Ctr– and miR-30c-5p–transduced SMCs but had no significant effects on H_2O_2 in miR-Zip30c–transduced SMCs (Figure 4D). A similar pattern of results was seen for nitrotyrosine—the peroxynitrite modification of tyrosine residues to 3-nitrotyrosine (Figure 4E). Overexpression of miR-30c-5p enhanced nitrotyrosine levels, which were further increased by cocaine in a dose-dependent manner compared with miR-Ctr–treated cells (Figure 4E). MiR-Zip30c treatment, however, reduced nitrotyrosine levels to that below those seen in miR-Ctr–treated cells even in the presence of cocaine (Figure 4E).

To show that the miR-30c-5p–Me1 axis mediated cocaine-increased ROS, SMCs were transduced with lentiviral vectors encoding a miR-30c-5p–resistant version of Me1 lacking the 3′UTR (Me1-del 3′UTR) or an empty vector control. Treatment with miR-30c-5p or cocaine inhibited endogenous Me1 expression (Figure 5A). However, SMCs transduced with Me1-del 3′UTR showed elevated Me1 expression compared with cells treated with vector control. Neither miR-30c-5p nor cocaine significantly affected Me1 expression in Me1-del 3′UTR–transduced cells (Figure 5A). Moreover, Me1-del 3′UTR transduction abrogated the effects of miR-30c-5p or cocaine on increasing prostaglandin (Figure 5B) or nitrotyrosine (Figure 5C) levels. In contrast, the vector control–transduced cells showed both prostaglandin and nitrotyrosine elevation when treated with miR-30c-5p overexpression or cocaine (Figure 5C).

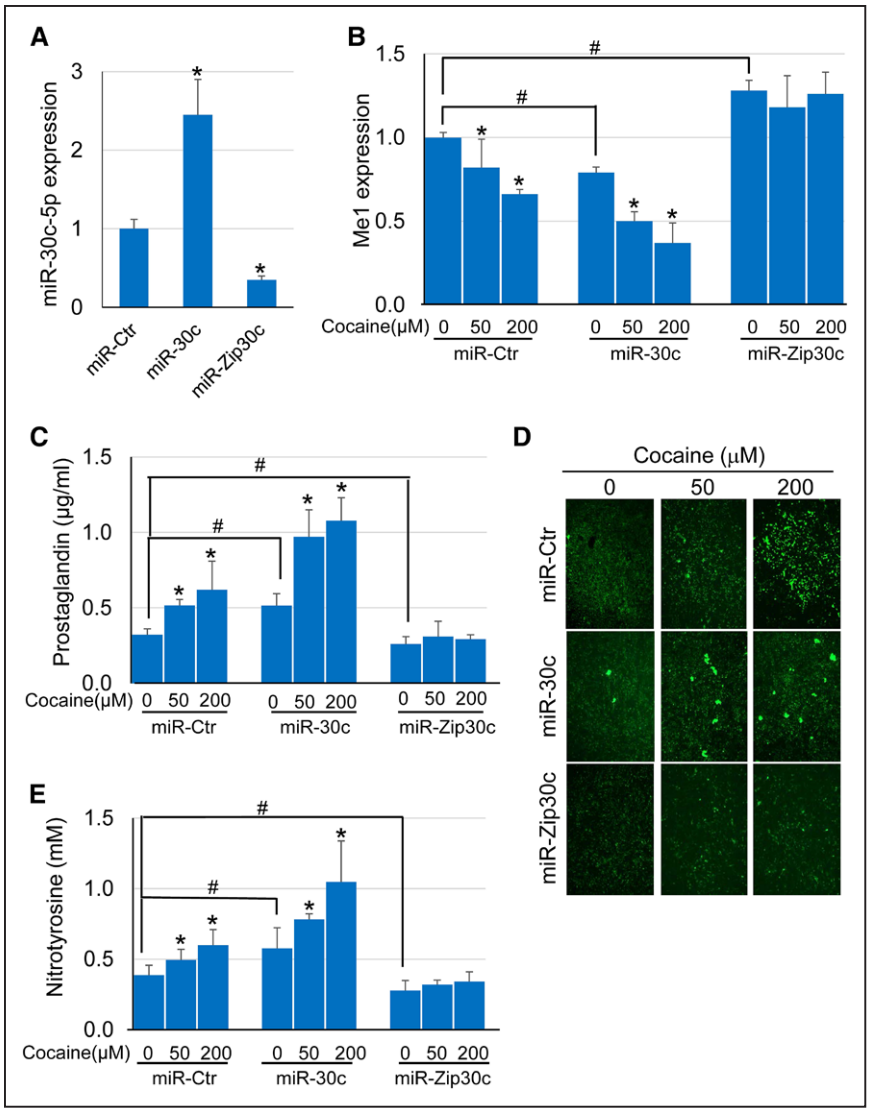

Figure 4. Perturbing the miR-30c-5p–Me1 (malic enzyme 1) axis affects cocaine-induced reactive oxygen species (ROS) elevation in mouse aortic smooth muscle cells (SMCs). SMCs were stably transduced with lentiviral vectors expressing miR-30c-5p, miR-Zip30c, or miR-scrambled control (miR-Ctr). **A**, miR-30c-5p expression was increased in the miR-30c-5p–transduced cells and decreased in the miR-Zip30c–transduced cells (*P<0.05 vs miR-Ctr). **B**, Cocaine dose dependently decreased Me1 expression, an effect that was exacerbated by overexpression of miR-30c-5p. MiR-Zip30c transduction led to increased Me1 expression, which was unaltered after cocaine treatment. **C**, Prostaglandin levels were elevated in miR-30c-5p transduced cells and were further elevated by cocaine in a dose-dependent manner. MiR-Zip30c not only decreased the basal level of prostaglandin but also abrogated the effect of cocaine on increasing prostaglandin. **D** and **E**, Overexpression of miR-30c-5p exacerbated the effect of cocaine on increasing H_2O_2 (detected by DCF [2',7'-dichlorofluorescein] staining) and nitrotyrosine levels. Conversely, silencing miR-30c-5p eliminated cocaine-induced increases in H_2O_2 and nitrotyrosine levels (*P<0.05 vs respective miR-transduced SMC line alone; #P<0.05 vs miR-Ctr–transduced SMCs alone).

Perturbing the miR-30c-5p–Me1–ROS Pathway Suppresses Cocaine-Induced Increases in BP and Aortic Stiffness

To validate the cocaine–↑miR-30c-5p–↓Me1–↑ROS axis in vivo, mice were injected with lentiviral vectors encoding miR-Zip30c or no gene (vector control) driven by an SMC-specific promoter, Sm22a, before cocaine exposure. Additional mice were pretreated with NAC that acts a scavenger of free oxygen radicals.[27] Consistent with the in vitro results, cocaine treatment for 10 days, which decreased Me1 expression (Figure S1A in the online-only Data Supplement) and elevated O_2^- levels in the aortas (Figure S1B and S1C), led to a significant increase in both systolic (Figure 6A) and diastolic (Figure 6B) BP compared with saline-treated mice. However, these cocaine-induced changes in Me1 expression and O_2^- levels were significantly suppressed by pretreatment with miR-Zip30c (Figure S1A through S1C), which reduced miR-30c-5p expression in the aortas (Figure S1D). Reduced BP levels were also seen from day 3 to day 10 in miR-Zip30c–pretreated animals following cocaine exposure (Figure 6A and 6B). In contrast, pretreatment with vector control had no effects on cocaine-induced Me1 expression, O_2^- levels (Figure S1A through S1C), and BP changes (Figure 6A and 6B). Pretreatment with NAC also significantly inhibited the increases in O_2^- and BP after cocaine exposure, without affecting the cocaine-induced reduction of Me1 (Figure S1A through S1C). Similarly, pretreatment with vector control did not affect cocaine-induced increases in aortic stiffness measured by PWV, whereas pretreatment with miR-Zip30c or NAC dampened the cocaine-induced PWV increases by ≈20% (Figure 6C).

Discussion

Current evidence suggests that cocaine impacts the cardiovascular system directly and indirectly through complex mechanisms. For example, cocaine acts directly on cardiomyocytes to block the voltage-gated sodium and potassium channels in the sinoatrial node and myocardium, leading to impaired contractility, arrhythmia, and decreased left ventricular function.[28–30] Cocaine is also known to potentiate the effect of norepinephrine on cardiovascular function by blocking norepinephrine transporters from transporting norepinephrine back to the presynaptic neurons.[1–3] Moreover, Schindler et

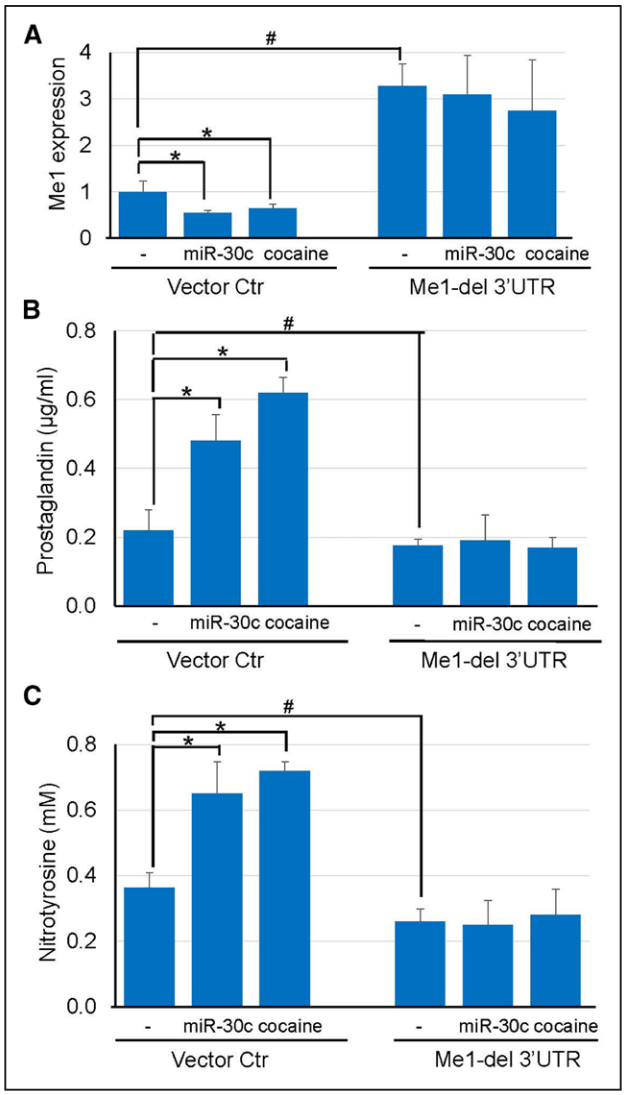

Figure 5. Expression of a microRNA (miRNA)-resistant form of Me1 (malic enzyme 1) abrogates cocaine-induced reactive oxygen species (ROS) elevation in mouse aortic smooth muscle cells (SMCs). **A**, Transduction of SMCs with lentiviral vectors expressing Me1 lacking the 3′UTR (Me1-del 3′UTR; untranslated region) overcame the inhibitory effect of exogenous miR-30c-5p or cocaine treatment on Me1 expression. **B** and **C**, Unlike vector control (Ctr)–transduced cells, Me1-del 3′UTR–transduced SMCs showed decreased levels of prostaglandin and nitrotyrosine, and that were unchanged by miR-30c-5p overexpression or cocaine treatment (*P<0.05 vs vector Ctr alone; #P<0.05 vs Me1-del 3′UTR alone).

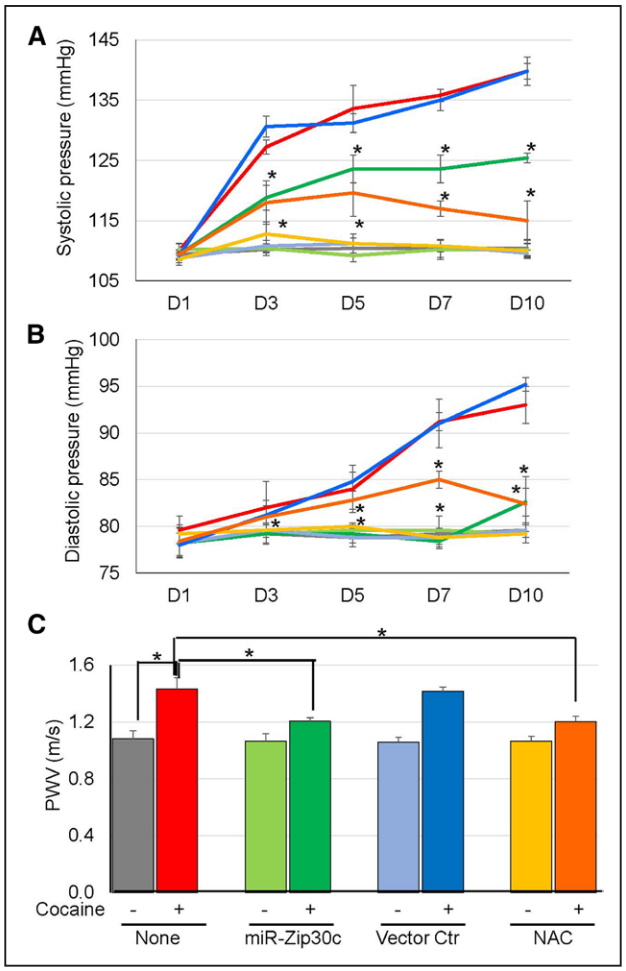

Figure 6. Smooth muscle cell (SMC)–specific knockdown of miR-30c-5p suppresses cocaine-induced increases in blood pressure (BP) and aortic stiffness. Mice (n=5) were injected with cocaine or saline each day for 10 consecutive days. In parallel experiments, mice were injected with lentiviral vectors expressing miR-Zip30c or no gene (vector control [Ctr]) underdriven by a Sm22a promoter, or antioxidant NAC, before cocaine exposure. **A** and **B**, Cocaine exposure elevated systolic and diastolic BP levels compared with saline- or vector Ctr–treated mice, whereas miR-Zip30c or N-acetyl cysteine (NAC) pretreatment partially reduced the cocaine-induced BP elevation from day 3 to day 10 (*P<0.05 vs cocaine treatment alone at each time point). **C**, Similar to BP results, pretreatment of miR-Zip30c or NAC significantly decreased cocaine-induced increases in pulse wave velocity (PWV) levels (*P<0.05 vs cocaine treatment alone at D12). Line/bar colors, red: cocaine; gray: saline; light green: miR-Zip30c; green: miR-Zip30c+cocaine; light blue: vector Ctr; blue: vector Ctr+cocaine; yellow: NAC; and orange: NAC+cocaine.

al[31] reported that cocaine directly elevates BP and heart rate at doses that do not potentiate norepinephrine, and administration of various doses of cocaine or CM could not enhance norepinephrine's pressor effect in anesthetized animals, implicating that additional mechanisms other than blockade of norepinephrine reuptake may mediate the cardiovascular effects of cocaine. In this study, we examined the molecular bases by which cocaine affects the cardiovascular system using a mouse model that recapitulates the cocaine-induced increases in ROS and BP as seen in humans. Although miRNAs play important roles in cardiomyocyte dysfunction–mediated myocardial infarction,[32,33] Endothelial cell (EC) and SMC senescence and apoptosis associated with biological aging,[34–36] and cocaine-seeking behaviors in the central nervous system,[37] no reports have defined the involvement of miRNAs in cocaine-driven CVD. Our results revealed a novel role for miR-30c-5p in regulating cocaine-induced increases in BP and aortic stiffness by elevating ROS production through directly suppressing Me1 in aortic SMCs. We also found that inhibiting norepinephrine transporters or adding exogenous norepinephrine had no effect on cocaine-induced upregulation of miR-30c-5p expression in SMCs (Figure S2), supporting the hypothesis that norepinephrine-independent

mechanisms may be operating to mediate the effect of cocaine on the cardiovascular system.

The putative miRNA predicted to target Me1 expression,[23,24] miR-30c-5p has not been previously linked to CVD. In cocaine- or CM-treated mice that exhibit increased BP and aortic stiffness, we first reported a significant upregulation of miR-30c-5p in the aortas. Moreover, Me1 was confirmed to be a direct target of miR-30c-5p, which recognized specific miRNA-binding sites in the Me1 3′UTR. Unlike miR-30c-5p, several lines of evidence suggest an association of Me1 with CVD. A meta-analysis of genome-wide gene expression studies in the spontaneously hypertensive and Lyon hypertensive rats found differential Me1 expression between onset and progressive stages of hypertension.[38] A locus on chromosome 6q12-16 encompassing the Me1 gene was linked to autosomal dominant dilated cardiomyopathy in a French family with 9 affected individuals.[39] Our study provides additional evidence linking Me1 to CVD, because perturbing Me1 expression in SMCs through miR-30c-5p regulation affects cocaine-induced BP and aortic stiffness.

Growing evidence supports the importance of ROS in CVD pathogenesis. A significant link between cocaine exposure and increased oxidative stress was found in mitochondrial dysfunction of cardiomyocytes that leads to cardiomyopathy, arrhythmias, and heart failure.[40–42] Elevated ROS have been observed in spontaneous hypertensive rats,[13] animals with renovascular hypertension,[14] salt-sensitive hypertension,[15] and obesity-induced hypertension.[16] Human hypertension also displays signs of increased ROS.[12,17,43] Moreover, ROS affects cell proliferation, apoptosis, senescence, and lipid oxidation, processes key to vascular aging.[44] On the basis of our data, the increased BP and arterial stiffness seen by repeated administration of cocaine or CM could also be directly linked to ROS elevation, because treatment with antioxidant NAC markedly inhibited these cocaine-induced cardiovascular phenotypes. Although miR-Zip30c transduction almost completely abolished the effect of cocaine on increasing ROS in SMC cultures, the in vivo tail vein injection of miR-Zip30c led to a significant suppression but not complete reversal of cocaine-increased BP and aortic stiffness in mice. This is not unexpected because it is far more difficult to achieve high levels of miR-30c-5p silencing in vivo than it is in cultured cells. Moreover, it is possible that additional, as of yet unidentified, biological pathways are functioning in vivo to mediate the effect of cocaine on BP and PWV.

SMCs, the major cellular component of the medial aorta wall that controls blood flow by contracting or relaxing in response to external stimuli, were the focus of this study. The precise mechanism(s) of how ROS elevation in SMCs leads to hypertension or aortic stiffness are, however, incompletely understood. ROS-dependent aortic stiffness may be associated with ERK1/2 phosphorylation[45] or osteopontin[46]-mediated osteogenic differentiation of vascular SMCs that leads to aortic medial calcification. ROS overproduction may also increase angiotensin II activity leading to increased SMC proliferation, migration, and Ca^{2+} release, events associated with hypertension development.[47,48] Other than aorta wall, impairment of the endothelial layer of the aorta may participate in the cocaine-induced hypertension and aortic stiffness. For example, cocaine-mediated ROS elevation leads to loss of the tight junction protein Zo-1 and increases EC monolayer permeability in human pulmonary artery EC.[49] EC release of NO is also affected by ROS,[50] and the formation of ONOO− by reactions between O_2^- and NO not only reduces the bioavailability of NO but also induces SMC-mediated vasoconstriction.[51] With this in mind, cocaine may cause dysfunction of both SMCs and ECs that act synergistically to mediate the effect of cocaine on inducing hypertension and aortic stiffness. Moreover, structural changes of the aortas associate with their pathogenic phenotypes. Consistent with previous findings,[52] we observed thickening of the intima–media of aortas from cocaine-treated mice (Figure S3A and S3B), indicating their contribution to peripheral resistance control in the pathogenesis of hypertension. The thickened and stiffer (increased PWV) aortas in cocaine-treated mice may suggest a dampened contractibility of SMCs because of cocaine exposure. Although both peripheral resistance and cardiac output contribute to the development of hypertension, we did not detect increased heart rate in cocaine-treated mice (data not shown). This could be associated with the cocaine dosage used in our animal model, because previous studies showed that lower doses of cocaine produced an immediate increase in heart rate, whereas heart rate increases were delayed to be observed with higher cocaine doses.[31]

In summary, using an animal model combined with ex vivo aortic tissue and primary SMC culture, we demonstrate, for the first time, a critical role of the ↑miR-30c-5p–↓Me1–↑ROS pathway in mediating the cocaine-induced increases in BP and aortic stiffness. Future studies are needed to corroborate these findings in vascular cells and aortic tissues obtained from cocaine abusers and patients with CVD.

Perspectives

The results of this study suggest that modulating the miR-30c-5p–Me1 pathway may have therapeutic benefits for the attenuation of cocaine-induced hypertension and aortic stiffness. More broadly, perturbing the miR-30c-5p–Me1 axis may control excessive ROS levels known to contribute to a wide range of cardiovascular complications.

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Disclosures

None.

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Novelty and Significance

What Is New?

- The first report of a novel miR-30c-5p–Me1 (malic enzyme 1)–reactive oxygen species pathway that mediates cocaine-induced increases in blood pressure and aortic stiffness.

What Is Relevant?

- Understanding the miR-30c-5p–Me1–reactive oxygen species pathway could facilitate the identification of new molecular targets for intervention to combat cocaine abuse-related hypertension and aortic stiffness.

Summary

The miR-30c-5p–Me1 pathway plays a critical role in mediating the effect of cocaine on inducing hypertension and aortic stiffness.