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Postnatal Cardiovascular Consequences in the Offspring of Pregnant Rats Exposed to Smoking and Smoking Cessation Pharmacotherapies

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Abstract

Approximately 20% of pregnant women smoke despite intentions to quit. Smoking cessation drugs, such as nicotine replacement therapy (NRT) and bupropion, are recommended treatments. Adverse cardiovascular outcomes in offspring have raised concerns about NRT's safety during pregnancy. However, the effect of bupropion is unknown. Using a rat model, we determined whether NRT and bupropion interventions during pregnancy are safer than continued smoking on offspring's cardiovascular function. Male offspring of controls and dams exposed to cigarette smoke (1.6 packs/day, inhalation), nicotine (2 mg/kg/d subcutaneously), and bupropion (13 mg/kg twice daily orally) were assessed for fetoplacental weight, cardiac function, blood pressure, and vascular reactivity. Fetoplacental weights were decreased and spontaneous beating and intracellular calcium in neonatal cardiomyocytes were increased in smoking, nicotine, and bupropion offspring; however, these effects were more accentuated in smoking followed by nicotine and bupropion offspring. Increased heart rate and decreased cardiac output, stroke volume, and left ventricular percent posterior wall thickening were observed in smoking, nicotine, and bupropion offspring. The left ventricular mass was reduced in smoking and nicotine but not in bupropion offspring. Blood pressure was higher with decreased endothelium-dependent relaxation and exaggerated vascular contraction to angiotensin II in smoking and nicotine offspring, with more pronounced dysfunctions in smoking than nicotine offspring. Maternal bupropion did not impact offspring's blood pressure, endothelium-dependent relaxation, and vascular contraction. In conclusion, maternal nicotine intervention adversely affects offspring's cardiovascular outcomes, albeit less severely than continued smoking. However, bupropion causes cardiac derangement in offspring but does not adversely affect blood pressure and vascular function.

Keywords

nicotine, bupropion, offspring, cardiac function, vascular function

Introduction

Smoking during pregnancy is associated with a higher risk of poor pregnancy outcomes, including placental abruption, miscarriage, preterm birth, and fetal death.[1,2] In addition, maternal smoking is related to short- and long-term health risks for the fetus, such as fetal growth restriction and increased risks of obesity, diabetes, asthma, behavioral disorders, and cancer.[3-6] However, studies on offspring's cardiovascular outcomes have presented mixed results. Some studies show that maternal smoking increases infant, childhood, and adolescence blood pressure by 0.9 to 5.4 mm Hg.[7-12] Some studies found no effect,[13-15] and one study found an interaction between prenatal smoking exposure and gestational length (ie, preterm and full term leading to decreased and increased offspring blood pressure, respectively).[16] Pooled data in a meta-analysis estimated the effect to be 0.62 mm Hg.[17] These associations of maternal smoking with blood pressure in the offspring are limited to observational studies, and they may be confounded by several factors, such as nutrition, dose and time of exposure, lack of compliance, and genetic factors. In addition, smoking mothers are more likely to drink alcohol and abuse other recreational drugs, which may also confound the outcomes.[18] Thus,

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controlled animal studies utilizing pregnant animals subjected to cigarette smoking would help confirm a definitive link between maternal smoking and long-term cardiovascular function.

The broad detrimental effects of maternal smoking have led some public health scientists to believe that stopping smoking during pregnancy would lead to positive maternal and fetal outcomes. Accordingly, studies show that smoking cessation interventions during pregnancy have reduced the prevalence of low birthweight, preterm births, and infant morbidity and mortality.[19] Although smoking cessation counseling is recommended as the first-line intervention for pregnant smokers, many women continue to smoke.[20] For women who do not respond to counseling, clinical practice guidelines recommend that pharmacological interventions be considered for smoking cessation.[21] Nicotine replacement therapy (NRT) and bupropion are commonly advocated smoking cessation agents. Nicotine replacement therapy is the only Food and Drug Administration–approved aid for smoking cessation during pregnancy and is the first-line pharmacotherapy for pregnant women who cannot quit smoking by other means.[21] However, the use of NRT during pregnancy continues to be controversial since recent clinical studies with NRT in pregnant women have reported adverse pregnancy effects, including increased fetal heart rate and aortic pulsatility, decreased fetal movements, reduced umbilical artery blood velocity, intraventricular hemorrhage, neonatal convulsions, and congenital heart diseases.[22-26] In addition, animal studies have clearly demonstrated that maternal nicotine has toxic effects on the fetus, leading to low birth weight and cardiovascular dysfunctions, such as adult-onset hypertension, reduced endothelial relaxation, enhanced vascular smooth muscle contractility, and increased heart susceptibility to ischemia and reperfusion injury.[27-34] Although it is conceivable that substituting ''clean'' nicotine without the 4000 other toxins that are inhaled while smoking[35,36] is safer than tobacco smoking, it is important to perform controlled animal studies to directly compare maternal NRT versus smoking and determine the health benefits and relative safety of NRT over smoking on fetal cardiovascular outcomes. These studies are particularly important because the prescriptions for NRT to pregnant women are steadily increasing.[37]

Bupropion is an approved and highly prescribed antidepressant for pregnant women and is also a highly effective smoking cessation agent compared to NRT.[38-41] Bupropion does not deliver nicotine, like NRT, but it inhibits dopamine and norepinephrine reuptake and attenuates stimulant effects of nicotine on nicotinic acetylcholine receptors.[42,43] To date, there is limited clinical evidence available regarding the safety of bupropion use during pregnancy.[36,44,45] Despite this lack of data, studies have reported that 3% of pregnant smokers take bupropion during pregnancy.[46,47] Preclinical data obtained from ex vivo and in vitro studies indicate that bupropion has a better short-term fetal safety profile than nicotine.[48-50] However, the long-term effect of maternal bupropion treatment on offspring cardiovascular function is not known. In this study, using a pregnant rat model of cigarette smoke (via inhalation), NRT, and bupropion exposures at clinically relevant concentrations, we determined whether (1) maternal smoking during pregnancy affects fetal and adult cardiovascular function; (2) NRT intervention is relatively safer than continued smoking on fetal and adult life cardiovascular function; and (3) other therapies, such as bupropion, during gestation are safe for the developing fetus and its cardiovascular system.

Materials and Methods

Animals and Treatment

All experimental procedures were approved by the Animal Care and Use Committee at The University of Texas Medical Branch (UTMB) at Galveston and were in accordance with the guidelines published by the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). Timed-pregnant Sprague Dawley rats (gestational day [GD] 3) were purchased from Harlan (Houston, Texas) and housed in temperature-controlled rooms (23°C) with a 12:12 hour light/dark cycle and with unlimited access to food and water. The animals were randomly divided into control, cigarette smoking, nicotine, and bupropion groups ($n = 12$ in each group) and exposed to these treatments from GD 4 to until delivery to focus on the consequences of in utero exposure. The cigarette smoke exposure was performed at UTMB's inhalation core facility. Briefly, animals were exposed to mainstream cigarette smoke generated from 3R4F reference cigarettes (1.6 packs per day; Tobacco Health Research Institute, Lexington, Kentucky) using an automated cigarette generation system (Jaeger-Baumgartner cigarette smoking machine; CH Technologies, Westwood, New Jersey) for 6 hours a day. Chamber levels of total particulate matter (TPM) were monitored throughout the exposure using an Electro-Medical Measurement Systems (CH Technologies Westwood, New Jersey); mean TPM levels were determined gravimetrically from filters weighed before and after sampling. The control rats were put in an identical chamber but exposed to air for the same time period. This cigarette smoke exposure paradigm produced plasma cotinine levels of 198 ± 20 ng/mL in the dams, equivalent to that observed in moderate human smokers.[51,52] Nicotine was administered to pregnant rats through a subcutaneous osmotic minipump at 2 mg/kg/d, as described previously.[31,32] This dose of nicotine resulted in blood cotinine levels of 217 ± 38 ng/mL, which closely resembles humans using transdermal nicotine patch.[53,54] Control rats received saline from the osmotic minipump. Bupropion was gavaged orally (13 mg/kg/d) twice daily. This dose increased plasma levels of bupropion and its metabolite hydroxybupropion to 765 ± 168 ng/mL and 850 ± 146 ng/mL, respectively, which mimic average steady-state C_{max} levels in pregnant women.[55,56] Cotinine, bupropion, and hydroxybupropion levels in rats' plasma were analyzed by liquid chromatography–mass spectrometry (LC-MS), as described by us previously.[57] A subset of animals ($n = 6$ in

Table 1. Primer Sequences for L-Type Ca^{2+} Channels, Sarcoplasmic Ca^{2+} ATPase, Na^{+}/Ca^{2+} Exchanger, Ryanodine Receptors, Protein Kinase C Isoforms, and Transient Receptor Potential Channels.

Gene Name	Forward (5′→3′)	Reverse (5′→3′)
Cacna1c	GATTCGATGTGAAGGCACTGAGAGC	ATGGCCTTGATGATGGAGTTCAGGA
Cacna1d	AATCAGGAGGCTTTGATGTCAAAGCC	AGGGGGACCATGGCTTTTATAATGGAG
Cacna1s	CCATCATCTTGCTCACCATCTTCGC	GAGAAGACGATGAGGAAGAAGTACTCCAG
Cacna1f	TGAGCTACCTTGGGTGTACTTTGTGAG	GCTTCTCCCGAAGCTTCTGAAAGTC
Ryr1	CGAGGACGAGGTCCAGTTTTTGCGGACG	ACACAACTTGAGCTGCTCCTTGAGCA
Ryr2	GATGTCAAATCAGCACGAATGGGATCC	TCGTGCTGCGATCTGGATAAGTTCA
Ryr3	AATGCTTGCCAACACCGTTGAAAATGG	CTGAAGGAGTGCCGCAGGAGAATTGCA
Pkc α	GATTTACCTGAAGGCAGAGGTCACAGAT	GGGTCAGGAATAAGTTTCAGCTTCACGTA
Pkc δ	GCCTTTCTGTGCCGTGAAGATGA	ATAGATGTGGGCGTCGAATGTTGACT
Pkc ε	GCGGAAACACCCTTATCTAACCCAAC	GGAACATGAGGTCTCCACCGTTTACATA
Trpc1	ATATACTGCAGCTGCTTTTGGACTACGG	GTGATGATCGTTTTGGCCGATGGTTAAG
Trpc2	ATAGCCATGGAGAACCAGATTCCAGTC	ACTTTCCTCCTCTCCTTGTT
Trpc3	GGCCAAAGTAAACCCTGCTTTTACCAC	GACAACAGAAGTCACTTCAGAGAGTCCAAATA
Trpc4	GAGTGGATGATATTACCGTGGGTCCT	CACCAGTCGTGGATGTAATCCTGAAGT
Trpc5	CCAAGCTGAAGGTGGCAATCAAATATCAC	AGTGTTTTCTTCGCCATCCAGGGAA
Trpc6	TTTAGAACTCAGCAATGAGCTGGCAGT	TCCAGGAGACCCACAACAAAATCCTT
Trpc7	GCATCAAACTTGCCATTAAGTACGAAGTCAA	GATAGACTGTTGCCGTAAGCCTGAGAG
Atp2a1	ATGACCATGGCCTTGTCTGTGTTG	AGATGGAACCGAGAAGCCAGATGTT
Atp2a2	TTGCTGGAACTTGTGATCGAGCAGT	GGCTGTAATCGTTTCTTCACCTTCTTCG
Atp2a3	TGTACGTAGGCCTGGCTACAGTG	TGGATTGTCTTCAGAGCACTTCAGGAAG
Slc8A1	CATTCTAGGCGAACACACCAAGCTG	GGCCAGGTTCGTCTTCTTAATGAGTTTG
Slc8A2	GTTTTTAGAGGCAGTTACAGTGAGCGC	AAGTGCATCACGTAGTCAAAGCAGGAT
Slc8A3	GGGAGCTGGAGTTTAAGAATGATGAAACG	CAAGGGCAATGAAGAAATTCTCTTGCCT

Abbreviations: *Atp*, ATPases; *Pkc*, protein kinase C; *Ryr*, ryanodine receptors; *Slc*, Na^{+}/Ca^{2+} exchangers; *Trpc*: transient receptor potential channels.

each group) were killed on GD 20 using carbon dioxide inhalation; their placentas were isolated and blotted to remove fluids and blood, and the junctional and labyrinth zones were separated and weighed. Fetal weights were also measured. Other dams in all groups were allowed to deliver at term. The number of pups in the control and treatment litters were adjusted to 8 pups per dam to ensure equal nutrient access for all offspring (pups with weights at each extreme were removed from the study). The ratio of male to female pups remained equivalent after culling, when possible. Heart/cardiomyocytes were isolated from 1-day-old pups to determine spontaneous beating frequency and intracellular calcium ($[Ca^{2+}]_i$) and for RNA analysis. The pups were weaned at 21 days of age, males and females were housed separately, and only males were used for the study. Animals were reared up to 6 months of age, and cardiac function and blood pressure were determined using ultrasound and telemetry, respectively. Then, the animals were killed and mesenteric arteries were isolated for vascular reactivity studies. Although each treatment group had its own control (ie, air inhalation, subcutaneous saline, and oral saline), the studied outcomes did not significantly differ between these control groups, hence combined control data are presented for easy comparison across and between treatment groups.

Isolation of Neonatal Cardiomyocytes and Spontaneous Beating Frequency

Neonatal ventricular myocytes were isolated and cultured as described previously.[58,59] Briefly, 1-day-old pups were euthanized, and their hearts were immediately excised. The ventricles were separated, transferred into ice-cold phosphate-buffered saline (Sigma, St Louis, Missouri), and quickly digested with 0.04% collagenase II (Fisher, Newark, Delaware) and 0.05% pancreatin (Sigma) at 37°C. The dispersed cells were suspended in DMEM-M19 (4:1) with 10% fetal bovine serum (Gibco, Grand Island, New York) and plated on a dish for 90 minutes at 37°C to allow attachment of fibroblasts and endothelial cells. Then, the unattached cardiomyocytes in the supernatants were collected, cultured on glass cover slips for 4 to 7 days, placed on a stage-mounted microscope, and observed for several minutes to calculate the spontaneous cell beats per minute, as described previously.[60]

Measurement of $[Ca^{2+}]_i$ Concentration

The measurements of $[Ca^{2+}]_i$ were performed with Fura-2 methodology.[61] Briefly, neonatal cardiomyocytes cultured on glass bottom dishes were loaded with 5-μM Fura-2 AM (Life Technology, Grand Island, New York) in HEPES-buffered salt solution (HBSS; 152 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4) for 1 hour in an incubator humidified with 95% air/5% CO_2, washed 3 times with HBSS, and then incubated in that medium for 30 minutes for dye esterification. The cells that show beating property (which are characteristic of cardiomyocytes) were focused for $[Ca^{2+}]_i$ measurement. Fluorescence quantitation was performed with the Nikon Eclipse TS100 inverted microscope equipped with a 20× S Fluor objective (Nikon, Japan),

Fura-2 filter set (Chroma, Bellows Falls, Vermont), Basler scA640-74fm CCD camera (Basler AG, Germany), and shutter wheel changer (Lambda LS 10-B; Sutter Instruments, Novato, California) controlled by a computer using InCytIm2 imaging software (University of Cincinnati, Ohio). Changes in Fura-2 fluorescence intensities emitted at 2 excitation wavelengths (340 and 380 nm) were acquired, and $[Ca^{2+}]_i$ was determined from Fura-2 ratio images using InCytIm2 imaging software (Version 5.0). During each scanning session, 6 individual cardiomyocytes were randomly selected and continuously imaged for 3 minutes to monitor basal $[Ca^{2+}]_i$ levels.

Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from neonatal heart using TRIzol reagent (Invitrogen, Grand Island, New York) and purified by RNeasy cleanup kit (QIAGEN Inc., Valencia, California). Total RNA quality was determined using an ND-1000 model Nanodrop (Thermo Fisher Scientific, Newark, Delaware). One microgram of total RNA was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, California), and real-time reverse transcription PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) with gene-specific primers for L-type Ca^{2+} channels (*Cacna1c*, *Cacna1d*, *Cacna1s*, and *Cacna1f*), sarcoplasmic reticulum Ca^{2+} ATPases (*Atp2a1*, *Atp2a2*, and *Atp2a3*), Na^+/Ca^{2+} exchangers (*Slc8A1*, *Slc8A1*, and *Slc8A3*), ryanodine receptors (*Ryr1*, *2*, and *3*), protein kinase C isoforms (*Pkc* α, δ, and ε), and transient receptor potential channels (TRPC; *Trpc1*, *Trpc2*, *Trpc3*, *Trpc4*, *Trpc5*, *Trpc6*, and *Trpc7*). Primers were designed based on the Ensembl Rat genome version Rnor_6.0 using Primer3 and purchased from Integrated DNA Technologies (Coralville, Iowa; Table 1). Results were calculated using the $2^{-\Delta\Delta CT}$ method and expressed as fold changes in the treatment group versus control. All reactions were performed in duplicate, and β-actin was used as an internal control.

Heart Functional Analysis Using Cardiac Ultrasound

At 6 months of age, offspring underwent echocardiography evaluation as reported previously.[62] Animals were anesthetized with inhaled continuous isoflurane via facemask. After using a chemical depilatory agent (Nair; Church & Dwight Co., Princeton, New Jersey) to remove chest wall hair, transthoracic M-mode, B-mode, and pulsed Doppler echocardiography were performed using an MS250 probe with a frequency of 13 to 24 MHz and 30-μm resolution to capture 240 frames per second (Vevo 2100; VisualSonics, Toronto, Ontario, Canada). Long-axis measurements at the minor chord dimension were taken in a standard long-axis view. Short-axis measurements were taken just below the level of the midpapillary muscle. Vevo 2100 software was used to calculate end-diastolic and end-systolic interventricular septal thickness, left ventricular percent posterior wall thickness, heart rate, cardiac output, stroke volume, left ventricular mass, and left ventricular ejection fraction and fractional shortening for each animal.

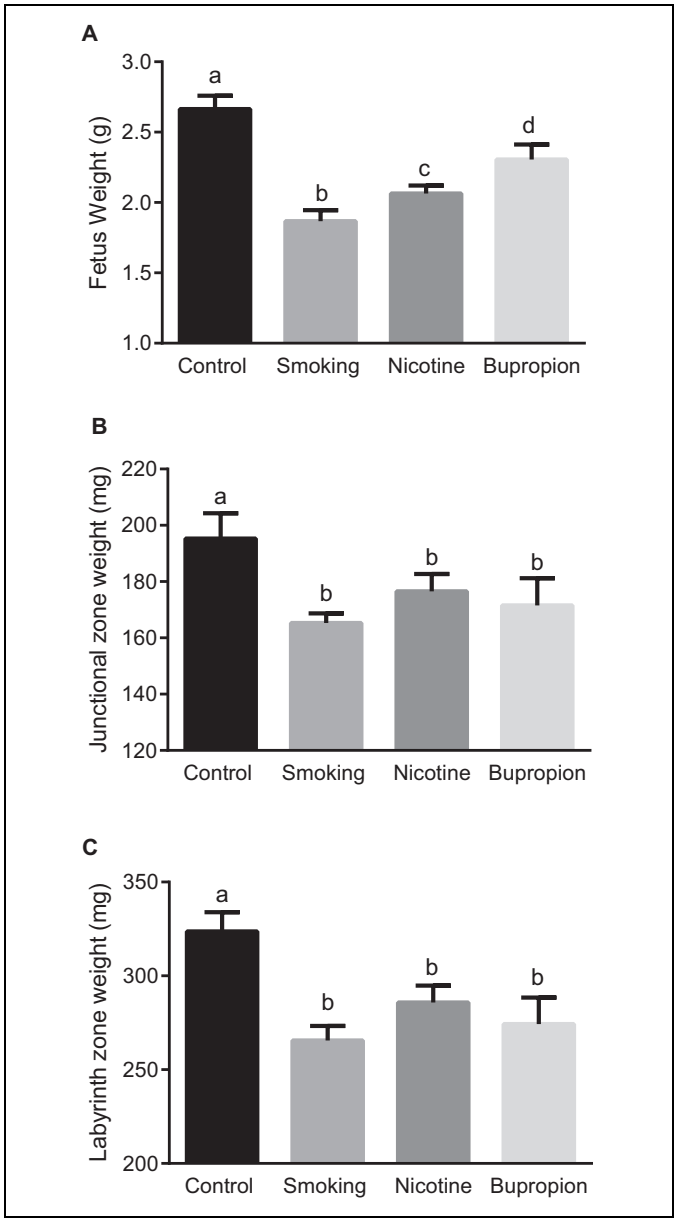

Figure 1. Fetal and placental weights were reduced in pregnant rats exposed to cigarette smoke, nicotine, and bupropion. The animals were killed on gestational day (GD) 20, and (A) fetal, (B) placental junctional zone, and (C) placental labyrinth zone weights were measured. Data are expressed as mean ± standard error of the mean (SEM) of 6 rats in each group. Bars with different alphabet superscripts differ significantly ($P < .05$).

Blood Pressure Measurement by Radiotelemetry

Mean arterial pressure in conscious free-moving rats was determined using the telemetry system as previously described.[63] Briefly, rats were anesthetized with 2.5% isoflurane, and a flexible catheter attached to a radio transmitter (TA11PA-C10; Data Sciences, Minneapolis, Minnesota) was inserted into

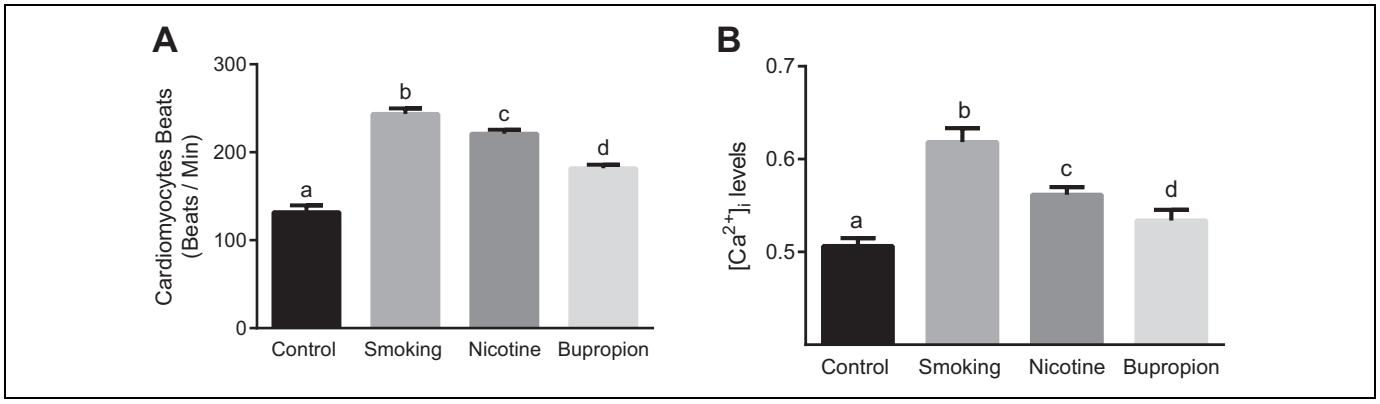

Figure 2. Maternal cigarette smoke, nicotine, and bupropion exposure increased spontaneous beating and $[Ca^{2+}]_i$ in neonatal cardiomyocytes. Cardiomyocytes isolated from day-old pups were examined for (A) spontaneous beating frequency and (B) $[Ca^{2+}]$i levels. Cultured cardiomyocytes were observed for several minutes under microscope to calculate spontaneous beats per minute. Fura-2-loaded cardiomyocytes were examined for $[Ca^{2+}]_i$ levels that is expressed as F340/380 ratio. All data are expressed as mean $\pm$ standard error of the mean (SEM); n = 6 in each group. Bars with different alphabet superscripts differ significantly ($P < .05$).

the left femoral artery. After surgery, rats were housed in individual cages and allowed to recover for a week. Blood pressure measurements obtained with a 10-second sampling period were averaged and recorded every 10 minutes, 24 hours a day, using the software (Dataquest 4.0; Data Sciences, St. Paul, Minnesota) provided by the manufacturer. Averaged 24-hour blood pressure was calculated for each animal.

Vascular Reactivity Studies

Rats were killed, and mesenteric arcade was removed and placed directly into ice-cold Krebs buffer (in mM: NaCl, 119; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.17; $NaHCO_3$, 25; KH_2PO_4, 1.18; EDTA, 0.026; and D-glucose, 5.5; pH 7.4). Under a dissecting microscope, the third-order mesenteric resistance arteries (100-200 μm diameter) were dissected. Mesenteric vessels were cut into 1.5-mm segments, and two 25-μm wires were threaded though the lumen of the vessel segment. One wire was attached to a stationary support driven by a micrometer, whereas the other was attached to an isometric force transducer (Multi Myograph, Model 610M; Danish Myo Technologies, Aarhus, Denmark). The myograph organ bath (6 mL) was filled with Krebs buffer maintained at 37°C and aerated with 95% O_2/5% CO_2. The vessels were then washed and incubated for 30 minutes under zero force. Then, the rings were normalized to an internal diameter of 0.9 of $L_{13.3kPa}$ using a normalization software package (Myodata; Danish Myotechnology, Aarhus, Denmark). The rings were then assessed for vascular function. The presence of intact endothelium in the vascular preparations was confirmed by observing the relaxation response to acetylcholine (10^{-6} M; Sigma) in rings precontracted with phenylephrine (10^{-6} M; Sigma), as described previously.[64] Endothelium-dependent relaxation responses to cumulative concentrations of acetylcholine (10^{-9}-10^{-5} M) in endothelium-intact arterial rings and endothelium-independent relaxation responses to sodium nitroprusside (10^{-9}-10^{-6} M; Sigma) in rings precontracted with phenylephrine (10^{-6} M) were determined. In endothelium-denuded arterial rings, vascular contractile responses to cumulative additions of angiotensin II (10^{-13} to 3×10^{-8} M; Sigma) were determined. Since angiotensin II causes tachyphylaxis, only 1 dose response per arterial ring was measured.

Statistical Analysis

Contractile responses to angiotensin II were calculated as percentages of its maximal contraction. Relaxant responses to acetylcholine were calculated as inhibition percentages of the phenylephrine-induced contraction. Sigmoidal functions were modeled to individual dose–response curves (GraphPad Prism, San Diego, California). Pharmacological concentration–response curves were defined by the negative log concentration that produced half of the maximum effect (pD_2) and by the maximum response (E_{max}). Data from multiple offspring of the same dam were averaged and presented as the datum for 1 dam, with the "n" representing the number of dams. Statistical comparisons were made by analysis of variance followed by the Bonferroni post hoc test. All values are expressed as means $\pm$ standard error (SE). Differences are considered statistically significant at $P < .05$.

Results

Maternal Smoking and Smoking Cessation Agents Decrease Fetal and Placental Weights

Maternal smoking, nicotine, and bupropion treatments caused significant decreases in body weight of near-term (20-day) fetuses compared with controls; however, the effect was more pronounced in smoking (−17%), followed by nicotine (−9%) and then bupropion (−5%; Figure 1A; $P < .05$; n = 6 in each group).

Placental weights (junctional and labyrinth zones) were significantly lower in smoking, nicotine, and bupropion groups compared with controls (Figure 1B and C; $P < .05$; n = 6 in

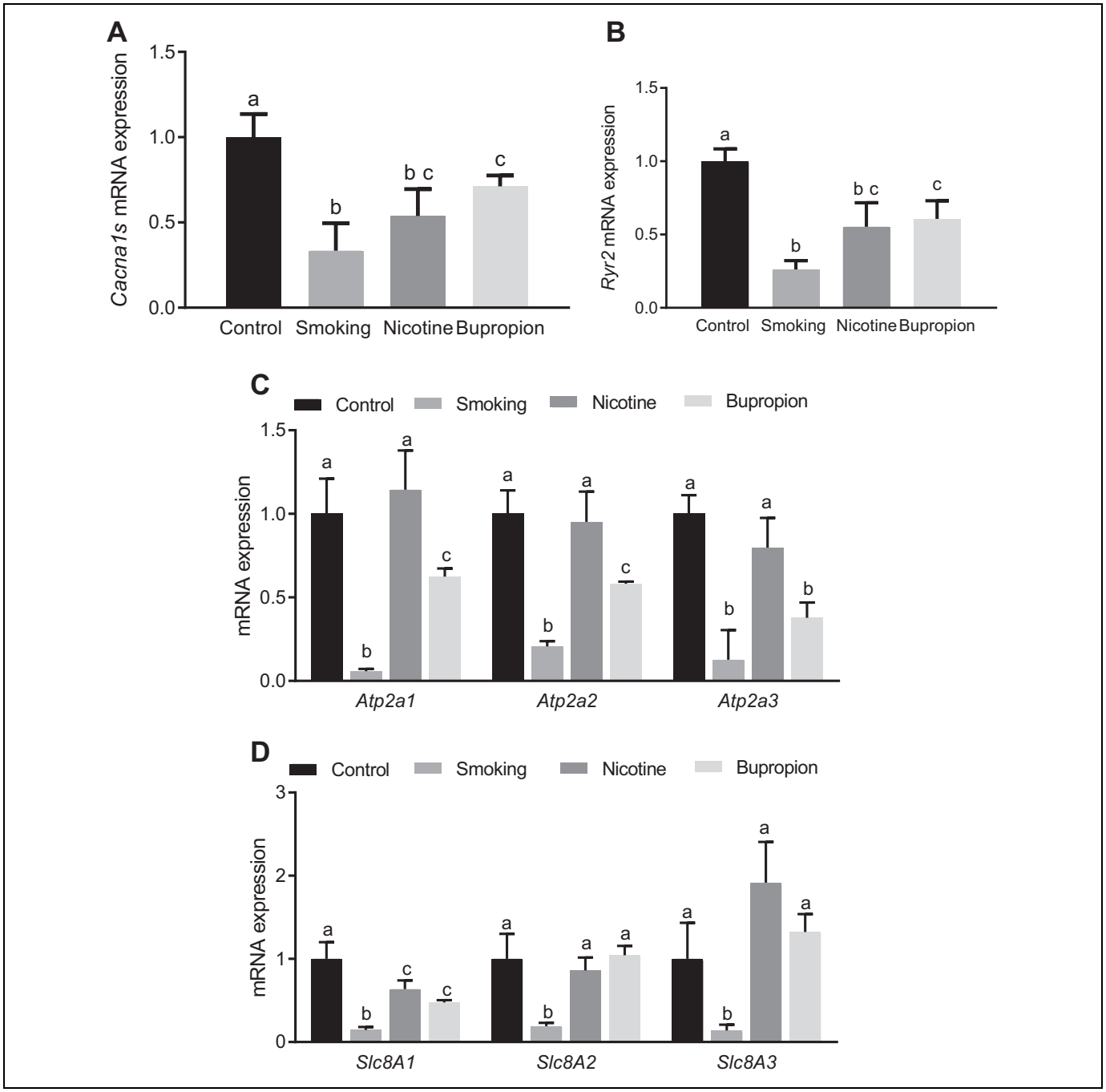

Figure 3. Maternal cigarette smoking, nicotine, and bupropion exposure decreased L-type calcium channel and ryanodine receptor messenger RNA (mRNA) expression in neonatal hearts. Real-time reverse transcriptase polymerase chain reaction (PCR) was used to assess (A) *Cacna1s* (calcium channel, voltage-dependent, L-type, alpha-1S subunit), (B) *Ryr2* (ryanodine receptor), (C) Ca^{2+} ATPases (*Atp2a1*, *Atp2a2*, and *Atp2a3*), and (D) Na^{+}/Ca^{2+} exchangers (*Slc8A1*, *Slc8A1*, and *Slc8A3*) mRNA expression. Quantitation of fold change in *Cacna1s*, *Ryr2*, *Atp2a1*, *Atp2a2* *Slc8A1*, *Slc8A1*, *and Slc8A3* was normalized relative to β-actin levels. Values are given as means ± standard error of the mean (sem) of 6 rats in each group. Bars with different alphabet superscripts differ significantly ($P < .05$).

each group). No significant difference in weights was found between smoking-, nicotine-, and bupropion-treated placentas (Figure 1B and C; n = 6 in each group).

Compared to controls, maternal body weight at term (GD 20) was significantly decreased by smoking (−16.34%) but not by nicotine (−6.41%) and bupropion (−6.08%) treatments (Supplementary Figure 1). There was no significant difference in the food intake between the control and smoking-, nicotine-, and bupropion-treated groups over the course of gestation (Supplementary Figure 2). No significant differences were noted in mean litter sizes between control (12.4 ± 2.7), smoking (11.7 ± 1.2),

nicotine (13.1 ± 1.8), and bupropion (12.4 ± 2.3) dams (n = 6 in each group).

Increased Spontaneous Beating and $[Ca^{2+}]_i$ in Cardiomyocytes

To address the impact of smoking and smoking cessation agents on neonatal cardiomyocyte function, single cells were observed over several minutes to quantify beating frequency and $[Ca^{2+}]_i$ levels. Spontaneous beating frequency (Figure 2A) and basal $[Ca^{2+}]_i$ levels (Figure 2B) were significantly increased in smoking, nicotine, and bupropion offspring compared to controls ($P < .05$; n = 6 in each group). The magnitude of increase in beating frequency and basal $[Ca^{2+}]_i$ levels was more pronounced in cardiomyocytes of the smoking group than the nicotine group. The beating frequency and basal $[Ca^{2+}]_i$ levels in the nicotine group were significantly higher compared to the bupropion group (Figure 2A and B; $P < .05$; n = 6 in each group).

Impaired Expression of Cardiac Signaling Genes in Heart

To determine whether increased spontaneous beating of isolated fetal cardiomyocytes correlated with alteration of excitation–contraction signaling molecules, messenger RNA levels of L-type calcium channels, ryanodine receptors, protein kinase C, and transient receptor potential channels were determined using quantitative real-time PCR. The expression of L-type calcium channel *Cacna1s* (Figure 3A) and ryanodine receptor *Ryr2* (Figure 3B) showed significant downregulation in the smoking-, nicotine-, and bupropion-treated groups compared to controls ($P < .05$; n = 6 in each group). The expression of L-type calcium channel *Cacna1c* was not different between the control and treatment groups (data not shown), and other L-type calcium channels (*Cacna1d* and *Cacna1f*) and ryanodine receptors (*Ryr1* and *Ryr3*) were below detectable limits. Moreover, Ca^{2+} ATPases (*Atp2a1*, *Atp2a2*, and *Atp2a3*) were significantly downregulated in the smoking and bupropion groups ($P < .05$; n = 6 in each group) but not in the nicotine group compared to controls (Figure 3C). The Na^+/Ca^{2+} exchangers (*Slc8A1*, *Slc8A2*, *and Slc8A3*) were downregulated in the smoking group compared with controls, and only *Slc8A1* was downregulated in the nicotine and bupropion groups compared with controls ($P < .05$; n = 6 in each group; Figure 3D).

Expression of protein kinase C-α, -δ, and -ε was significantly higher in the smoking- and nicotine-exposed groups compared to controls (Figure 4; $P < .05$; n = 6 in each group). Only protein kinase C-δ was significantly higher in the bupropion group compared to controls (Figure 4; $P < .05$; n = 6 in each group). Overall, the expression of protein kinase C was less affected by bupropion treatment compared to smoking and nicotine exposures.

Transient receptor potential channels were differentially expressed depending on exposure. In smoking offspring, *Trpc2* was downregulated, whereas *Trpc3* was upregulated. In the nicotine offspring, *Trpc3* was upregulated. In the bupropion offspring, *Trpc3* was downregulated and *Trpc7* was upregulated (Figure 5; $P < .05$; n = 6 in each group).

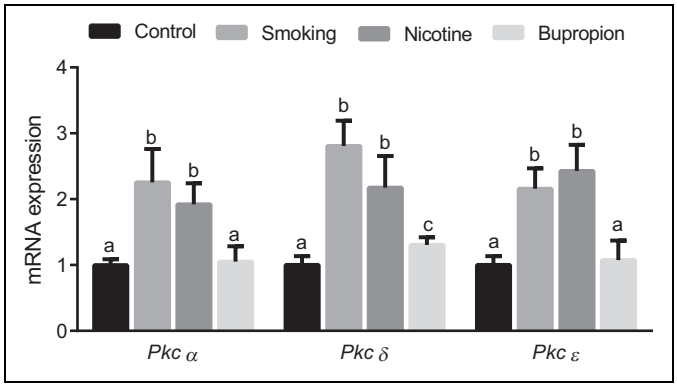

Figure 4. Maternal cigarette smoke, nicotine, and bupropion exposure increased protein kinases C isoforms messenger RNA (mRNA) expression in neonatal hearts. Real-time reverse transcriptase polymerase chain reaction (PCR) was used to assess protein kinase C-α, -δ, and -ε mRNA expression. Quantitation of fold change in protein kinase C was normalized relative to β-actin levels. Values are given as means ± standard error of the mean (sem) of 6 rats in each group. Bars with different alphabet superscripts differ significantly ($P < .05$).

Cardiac Dysfunction in Adult Offspring

We next examined the cardiac function in 6-month-old adult offspring using ultrasound. At 6 months of age, the offspring's body weights were not significantly different between the control and treatment groups (control: 506.5 ± 8.07, smoking: 495.5 ± 12.10, nicotine: 498.5 ± 4.15, and bupropion: 508.833 ± 5.98). Heart rate was significantly higher in smoking, nicotine, and bupropion offspring compared to controls; however, the magnitude of increase was greater in smoking than nicotine and bupropion offspring (Figure 6A; $P < .05$; n = 6 in each group). No significant difference was found in heart rate between nicotine and bupropion offspring (Figure 6A). Cardiac output, stroke volume, and left ventricular percent posterior wall thickening were significantly decreased in smoking, nicotine, and bupropion offspring compared to controls (Figure 6B-D; $P < .05$; n = 6 in each group). Compared to controls, the left ventricular mass was significantly reduced in smoking and nicotine but not in bupropion offspring (Figure 6E; $P < .05$; n = 6 in each group). There were no significant differences in interventricular septal thickness, ejection fraction, and fractional shortening between groups (data not shown).

Elevated Blood Pressure in the Adult Offspring

Blood pressure was significantly higher in the 6-month-old smoking (124 ± 2.22 mm Hg) and nicotine (120 ± 1.13 mm Hg) offspring compared to controls (115 ± 1.2 mm Hg); however, the magnitude of increase was significantly higher in the smoking (+9 mm Hg) compared to nicotine (+5 mm Hg) offspring (Figure 7A; $P < .05$; n = 6 in each

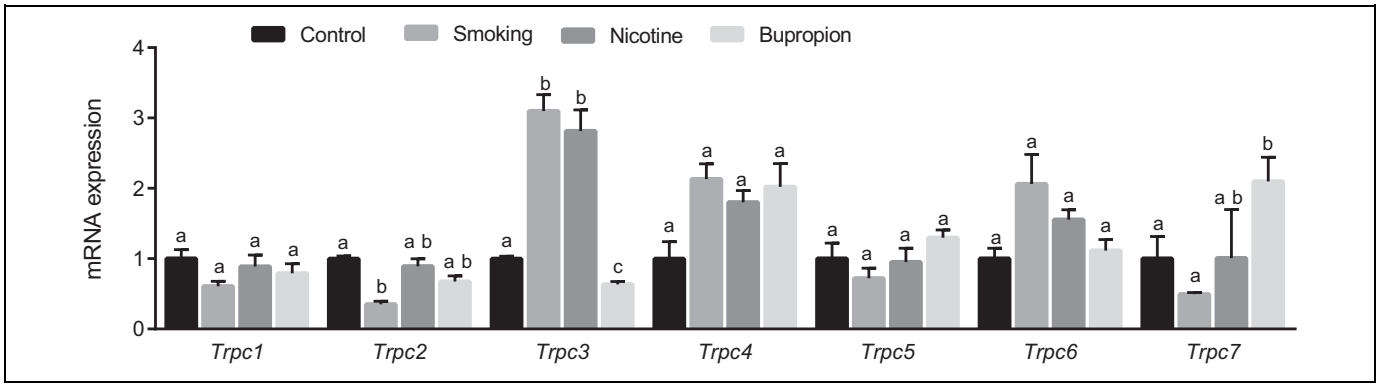

Figure 5. Maternal cigarette smoke, nicotine, and bupropion exposure dysregulates messenger RNA (mRNA) of transient receptor potential channels (*Trpc1*, *Trpc2*, *Trpc3*, *Trpc4*, *Trpc6*, and *Trpc7*) in the neonatal heart. Quantitation of fold change in *Trpc* was normalized relative to β-actin levels. Values are given as means ± standard error of the mean (sem) of 6 rats in each group. Bars with different alphabet superscripts differ significantly ($P < .05$).

group). The arterial pressure in the bupropion offspring (114 ± 2.1 mm Hg) was comparable to that in the controls (115 ± 1.2 mm Hg; Figure 7A; $P < .05$; n = 6 in each group). Heart rate recorded from conscious free-moving rats was significantly higher in smoking, nicotine, and bupropion offspring compared to controls; however, the magnitude of increase was greater in smoking than nicotine and bupropion offspring (Figure 7B; $P < .05$; n = 6 in each group). No significant difference was found in heart rate between nicotine and bupropion offspring (Figure 7B).

Decreased Endothelium-Dependent Relaxation

Endothelium-dependent acetylcholine-induced relaxation in mesenteric arterial rings was significantly reduced in smoking- ($pD_2 = 6.13 \pm 0.09$) and nicotine-treated groups ($pD_2 = 6.33 \pm 0.12$) compared to their controls ($pD_2 = 7.03 \pm 0.12$; Figure 8A; $P < .05$; n = 6 in each group). Besides, the maximal vascular relaxation to acetylcholine was significantly decreased in smoking (E_{max}: 78.71% ± 3.81%) and nicotine exposure (E_{max}: 71.35% ± 6.55%) compared to controls (E_{max}: 98.68% ± 0.73%; Figure 8A; $P < .05$; n = 6 in each group). On the other hand, acetylcholine-induced relaxation in the bupropion offspring was not significantly altered ($pD_2 = 7.27 \pm 0.11$; E_{max}: 94.28 ± 1.62%) compared to controls (Figure 8A; n = 6 in each group). Endothelium-independent vascular relaxation to sodium nitroprusside in mesenteric arteries was not significantly different between groups (Figure 8B; n = 6 in each group).

Exaggerated Contractile Response to Angiotensin II

Angiotensin II induced a dose-dependent increase in contractile responses in endothelium-denuded mesenteric arterial rings in all 4 groups. However, angiotensin II-mediated dose-dependent contractions were exaggerated, with a leftward shift in smoking ($pD_2 = 10.62 \pm 0.12$) and nicotine ($pD_2 = 10.12 \pm 0.15$) offspring compared to controls ($pD_2 = 9.51 \pm 0.11$; Figure 8C; $P < .05$; n = 6 in each group). The pD2 for angiotensin II was significantly higher in arteries of smoking than nicotine offspring. Angiotensin II-induced contractile responses were comparable between bupropion offspring ($pD_2 = 9.47 \pm 0.10$) and controls (Figure 8C; n = 6 in each group).

Discussion

For the first time, this study has demonstrated that cigarette smoking during pregnancy in rats leads to cardiac abnormalities and hypertension in the adult offspring, with an associated decrease in endothelium-dependent relaxation and exaggerated vascular contractile responses to angiotensin II. Maternal nicotine administration at concentrations used for NRT also induced similar cardiac and vascular abnormities in the offspring, albeit at a lesser magnitude than maternal smoking. However, bupropion administration caused minimal fetal and adult cardiac abnormalities but no adverse changes in vascular function and blood pressure. These results indicate that the use of NRT has significant cardiovascular risk, whereas bupropion has less risk for the development of adult-onset cardiovascular dysfunctions than nicotine; however, translation of these findings to human warrants further studies.

In our study, we observed that maternal smoking, nicotine, and bupropion exposure during pregnancy induced fetal growth restriction, which is in accordance with previous human and animal studies.[28,33,65-68] In addition, the present study revealed the magnitude of fetal growth restriction is different between maternal exposures, with a more pronounced effect in the smoking group, followed by the nicotine and then bupropion groups. These adverse effects on fetal growth could be from indirect action of these maternal exposures on the placenta. This theory is supported by the fact that maternal smoking causes lower placental weights and changes essential features of placental function, such as progesterone production,[69] estrogen metabolism,[70] and amino acid transport.[71] In addition, nicotine is shown to have a direct vasoconstrictive effect on pregnant uterine arteries[33] and decrease uterine blood flow to the fetoplacental unit.[72] These changes may conceivably have a

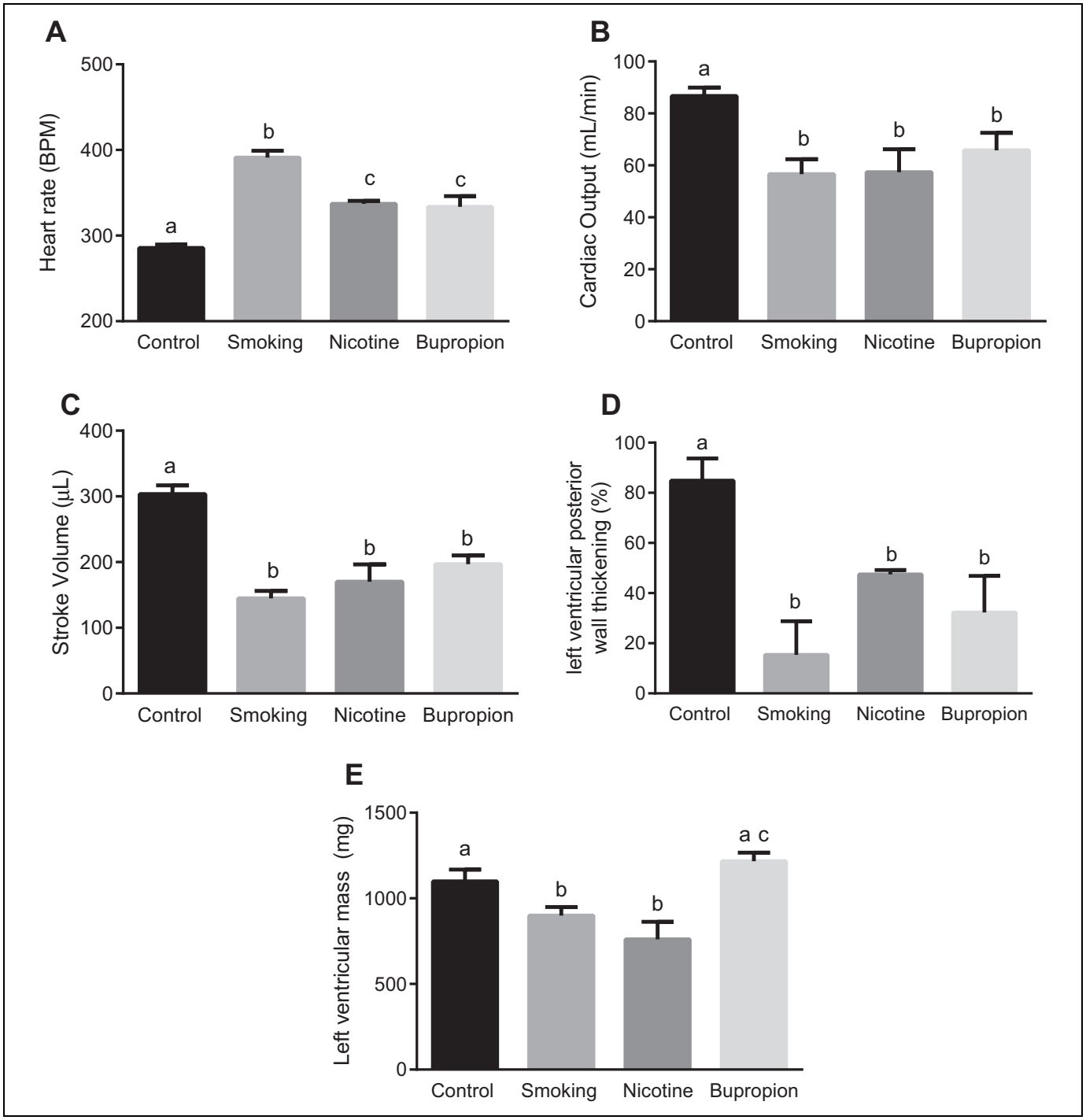

Figure 6. Cardiac morphology and functions were altered in offspring of dams exposed to cigarette smoke, nicotine, and bupropion. Cardiac morphology was analyzed in 6-month-old offspring by echocardiography to assess (A) heart rate, (B) cardiac output, (C) stroke volume, (D) left ventricular percent posterior wall thickening, and (E) left ventricular mass. Values are given as means $\pm$ standard error of the mean (sem) of 6 rats in each group. Bars with different alphabet superscripts differ significantly ($P < .05$).

detrimental effect contributing for fetal growth restriction in smoking and nicotine offspring. On the other hand, these agents can directly cause fetal damage. Indeed, studies have shown that nicotine readily crosses the placenta, and maternal cigarette smoking produces higher nicotine concentrations in fetal circulation than that experienced by the mother.[73,74] Bupropion is also known to cross placenta.[75] Thus, it is possible that fetal growth restriction may have an indirect effect on placental function and a direct effect on fetal organogenesis.

It is generally accepted that smoking and smoking cessation drugs mediate their therapeutic effects via specific interaction with a neurotransmitter signaling event in the central nervous

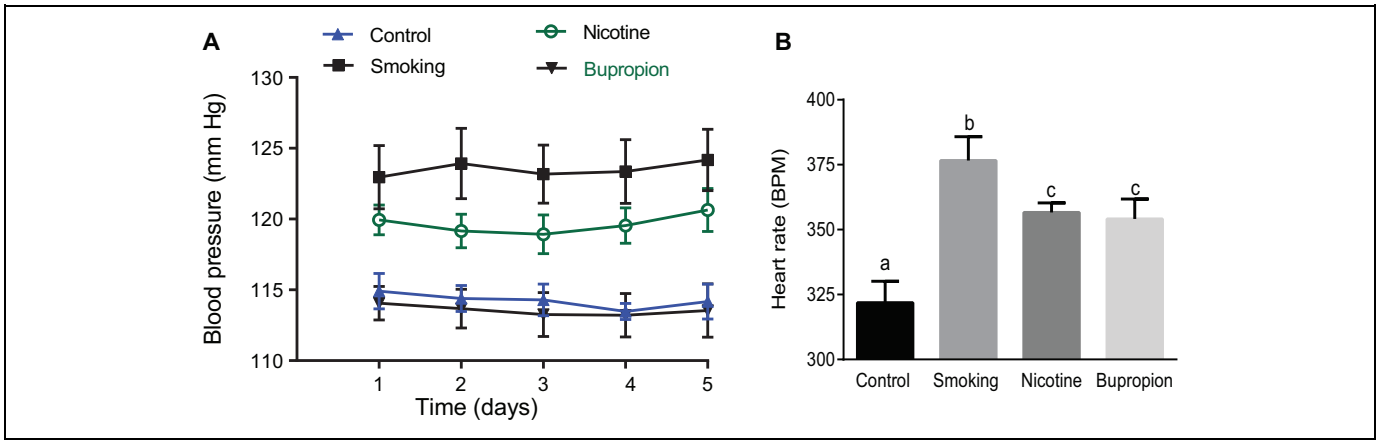

Figure 7. Maternal smoke and nicotine but not bupropion exposure increased blood pressure in adult offspring. Heart rate was significantly higher in smoking, nicotine, and bupropion offspring compared to controls. (A) Blood pressure and (B) heart rate were measured 24 hours per day by telemetry at 6 months of age for 5 days. Values are given as means ± standard error of the mean (sem) of 6 rats in each group. Bars with different alphabet superscripts differ significantly ($P < .05$).

system.[42,43,76-78] However, most of these agents also interact with ion channels, which make these agents candidates for cardiotoxicity during embryonic development. In the present study, the spontaneous beating of isolated neonatal cardiomyocytes was increased following smoking, nicotine, and bupropion treatments, which is consistent with the increased fetal heart rate observed in smoking mothers,[79] maternal nicotine treatments in rats, and chewing nicotine gums in humans.[80,81] In addition, bupropion is known to induce fetal arrhythmias.[82] The increased beating observed ex vivo in cardiomyocytes indicates that this effect is independent of endogenous factors and possibly involves some inherent alterations in cardiomyocyte function. An increase in intracellular Ca^{2+} via the L-type Ca^{2+} channels, sarcoplasmic reticulum Ca^{2+} ATPase, and the ryanodine receptors is an important cellular process for excitation–contraction coupling.[83] Intriguingly, the expressions of *Cacna1s* and *Ryr2* in neonatal hearts were lower following exposure to smoking, nicotine, and bupropion, yet the spontaneous beating of the cardiomyocytes and $[Ca^{2+}]_i$ levels were higher. It is unclear whether the decreases in *Cacna1s* and *Ryr2* expressions are an attempt to compensate for increased beating frequency and $[Ca^{2+}]_i$ levels. Thus, other mechanisms, possibly the increased expression of protein kinases C and TRPCs and decreased expression of Ca^{2+} ATPases and Na^+/Ca^{2+} exchangers observed in the smoking-, nicotine-, and bupropion-exposed fetuses, may play a role in contributing to increased spontaneous beating and $[Ca^{2+}]_i$ in cardiomyocytes.[84-87] The increased expression of TRPCs (which are known to increase Ca^{2+} influx through activation of receptor-activated and store-operated mechanisms)[86,87] may play a role in increasing $[Ca^{2+}]_i$ in cardiomyocytes of smoking, nicotine, and bupropion offspring. Indeed, studies have shown that nicotine increases $[Ca^{2+}]_i$ through upregulation and activation of *Trpc3*.[88] It is interesting to note that the minimal upregulation of protein kinase C in bupropion-exposed neonatal hearts correlates well with the marginal increase in beating frequency. Also, the decreased expression of Ca^{2+} ATPase and Na^{2+}/Ca^{2+} exchanger may contribute to the decrease in reuptake of Ca^{2+} into the sarcoplasmic reticulum or removal by the sarcolemma leading to increase in $[Ca^{2+}]_i$. Further studies that delineate the exact signaling mechanism, including measurement of intracellular Ca^{2+} transients with selective inhibitors,[89] may help us understand the underlying changes that contribute to increased beating in cardiomyocytes and $[Ca^{2+}]_i$ of smoking, nicotine, and bupropion groups.

It is now well established that a variety of insults, when experienced in the prenatal period, can have long-term influences on the health of the individual. In the present study, the increased beating frequency of neonatal cardiomyocytes was maintained through adult life as evidenced by the increased heart rates in smoking, nicotine, and bupropion offspring. Whether altered control of cardiac sympathetic and parasympathetic activity or other mechanisms contribute to increased heart rate is not clear.[80] Prenatal nicotine is shown to alter the tone of cholinergic neurons through a programmed disruption, suggesting a possible deficiency in cholinergic activity in smoking/nicotine offspring.[90] The decreased cardiac output in the smoking, nicotine, and bupropion offspring may presumably be due to decreased stroke volume. In addition, this study identified changes in heart morphology, including decreased left ventricular percent posterior wall thickness in smoking, nicotine, and bupropion offspring. This decrease in ventricular wall thickness, together with the finding of decreased left ventricular mass, suggests that maternal smoking and nicotine may compromise myocardial development and function. Consistent with this notion, previous studies show that maternal smoking and nicotine decreased cardiomyocyte numbers in the fetal heart,[91] reduced the size of embryonic organs—including heart,[91,92] and increased risks for congenital heart defects.[93] Also studies have shown that maternal nicotine exposure leads to elevated left ventricle myocardial infarct size and decreased post-ischemic recovery following ischemia in the offspring.[34] It is also possible that the impaired cardiac function may be secondary to altered respiratory, metabolic, and hemodynamic

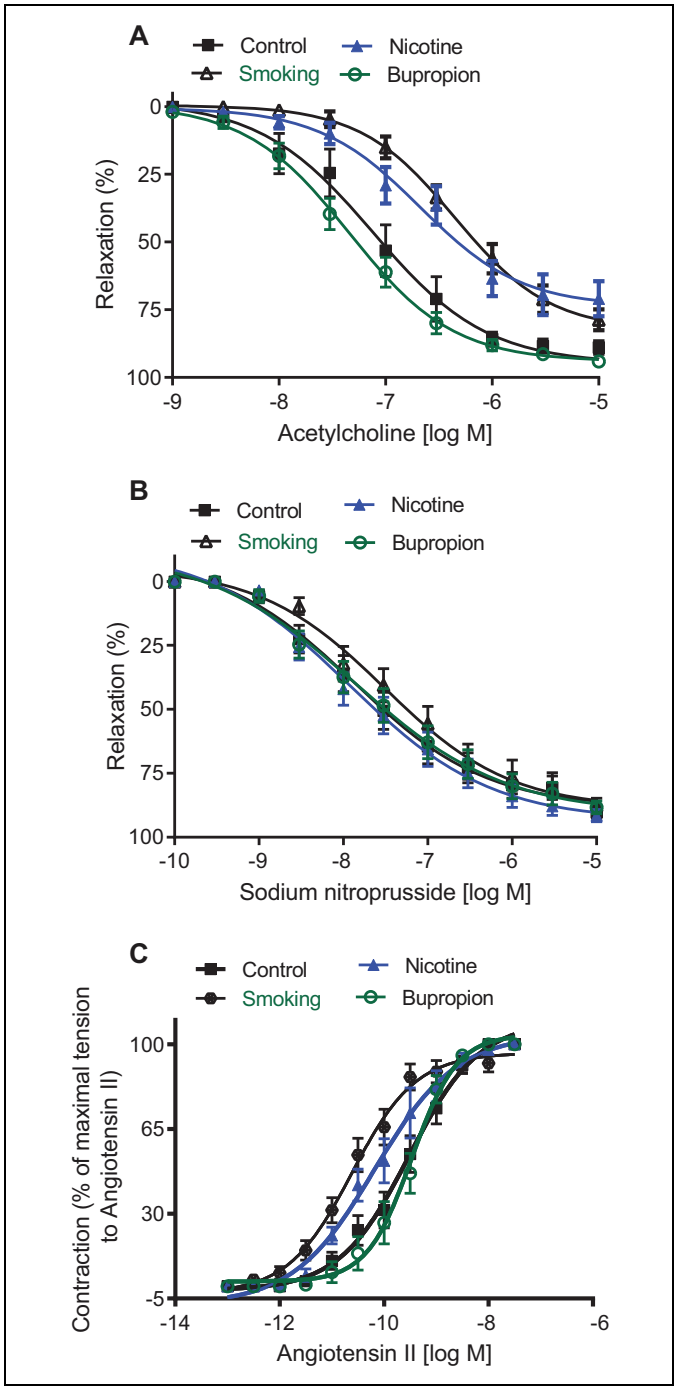

Figure 8. Maternal smoke and nicotine but not bupropion exposure altered vascular function in adult offspring. Mesenteric arterial rings were isolated from 6-month-old rats to assess (A) endothelium-dependent relaxation and (B) endothelium-independent relaxation. Endothelium-intact mesenteric arterial rings were precontracted with phenylephrine (10^{-6} M) and examined for relaxation to cumulative additions of acetylcholine or sodium nitroprusside and (C) vascular contractile responses. Endothelium-denuded mesenteric arterial rings were contracted to cumulative additions of angiotensin II. Values are given as means $\pm$ standard error of the mean (Sem) of 6 rats in each group.

functions.[30] The exact causes of impaired cardiac parameters are unknown, and further investigation at an ultrastructural, biochemical, and genetic basis may prove to be revealing.

The adult offspring of both smoke- and nicotine-exposed dams exhibited increased blood pressure. This result is consistent with the reports in children of smoking mothers[7-12,94] and offspring of nicotine-exposed pregnant rats.[27,95-97] However, this study provides new data that the magnitude of hypertension is different between smoking and nicotine offspring, with smoking offspring having a more pronounced effect (mean increase of 9 mm Hg in smoking vs 5 mm Hg in nicotine offspring). This outcome suggests that the smoking offspring are more susceptible and hypertension is more severe than in nicotine offspring. Interestingly, bupropion offspring had no blood pressure alterations, despite the fact that bupropion treatment has been reported to cause increased blood pressure in non-pregnant humans.[98]

To dissect the contribution of the vasculature to the altered blood pressure changes, we examined alterations in vascular function in the offspring. Responses to acetylcholine, an endothelium-dependent vasodilator, to sodium nitroprusside, an endothelium-independent vasodilator, and to angiotensin II, a vasoconstrictor, were investigated. Mesenteric vasorelaxation to acetylcholine was reduced in smoking and nicotine offspring compared to controls, but no differences could be detected to sodium nitroprusside. These observations suggest that it is not the smooth muscle vasodilating capability that is reduced in smoking and nicotine offspring but some function related to the endothelium. This result is in accordance with previous reports of altered endothelial function in adult offspring of maternal nicotine-exposed dams.[28,33] In the present study, angiotensin II-induced contractile responses were significantly increased in smoking and nicotine offspring compared to controls. Because smoke and nicotine exposure caused increases in angiotensin II-induced contractions in the absence of functional endothelium, we suggest the enhanced arterial sensitivity to angiotensin II primarily occurred in the vascular smooth muscle cells. It is possible that the vascular effects could be primary effects or secondary effects related to differences in blood pressure. Other studies in prenatally nicotine-exposed rats have also reported exaggerated vasomotor responses to angiotensin II in other vascular beds, such as the aorta.[28] The magnitude of vascular dysfunction is different between smoking and nicotine offspring, with smoking offspring having more pronounced impairment in endothelium-dependent relaxation and greater exaggeration in vasoconstriction to angiotensin II, which relates to a proportional increase in blood pressure. Although smoke and nicotine exposure in pregnant rats achieved comparable increases in plasma cotinine levels similar to those in moderate human smokers[51,52] and in humans who use transdermal nicotine patch,[54] the exaggerated adverse cardiovascular effect in the smoking offspring may be attributed to additional toxic components of cigarette smoking.[35,36] The finding that endothelium-dependent relaxation

response and angiotensin II-induced contractile response are preserved in the bupropion offspring suggests that the vascular function is not affected by developmental exposure to bupropion.

By controlling for external confounders present in human epidemiological studies, this study provides strong evidence of adverse consequences of maternal smoking on the cardiovascular health of the offspring, implicating the need to develop strategies that facilitate smoking cessation for pregnant women. The magnitude of outcomes in the fetus (fetal weight and beating frequency of cardiomyocytes) and adults (cardiac dysfunction, blood pressure, and vascular relaxation and contraction) is more severe in the smoking than in the nicotine offspring, yet the consequences are significant enough to raise potential safety concerns for the use of NRT during pregnancy. There was fetal growth restriction and cardiac derangement; however, the lack of adverse effects of developmental exposure to bupropion on adult life vascular function and blood pressure control is novel and intriguing. Similar findings that maternal bupropion does not cause significant metabolic and reproductive dysfunction in the offspring have been reported.[68] Although animal studies are reassuring, further clinical studies are required to evaluate the safety and efficacy of bupropion for smoking cessation during pregnancy.

Authors' Note

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

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Declaration of Conflicting Interests

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Supplemental Material

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