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THE CARDIOVASCULAR EFFECTS OF TOBACCO PRODUCT-DERIVED
ALDEHYDES:
CONTRIBUTION OF TRANSIENT RECEPTOR POTENTIAL ANKYRIN-1

By

Lexiao Jin

M.S., Wenzhou Medical University, 2008

M.D., Wenzhou Medical University, 2003

A Dissertation

Submitted to the Faculty of the
School of Medicine of the University of Louisville
In Partial Fulfillment of the Requirements
for the Degree of

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Department of Pharmacology and Toxicology
University of Louisville
Louisville, KY

May, 2020

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Dissertation Approved on

03 - 02 - 2020

By the following Dissertation Committee

Daniel J. Conklin, Ph.D.

Aruni Bhatnagar, Ph.D.

Sanjay Srivastava, Ph.D.

John Pierce Wise, Ph.D.

Lu Cai, M.D., Ph.D.

Gary W. Hoyle, Ph.D.

DEDICATION

This dissertation is dedicated to my family who have
given me educational opportunities.

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First and foremost, I would like to thank my mentor, Dr. Conklin, for always believing in my scientific abilities, for his guidance, patience and support in my research. And he always pushes me to be my best and think critically. I would also like to thank my committee members, Dr. Bhatnagar, Dr. Srivastava, Dr. Hoyle, Dr. Wise, and Dr. Cai, for their comments and assistance in completing my dissertation research.

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ABSTRACT

THE CARDIOVASCULAR EFFECTS OF TOBACCO PRODUCT-DERIVED ALDEHYDES: ROLE OF TRPA1

Lexiao Jin

03-02-2020

Background

Cardiovascular diseases (CVD) are the leading cause of death in most countries and are a significant health and financial burden worldwide (Bhatnagar, 2004; Ramana et al., 2006). Smoking contributes to more than 480,000 premature deaths annually -- accounting for one of every five deaths in the United States each year (Centers for Disease Control and Prevention, 2018; U.S. Department of Health and Human Services, 2014). Numerous studies have shown that smoking increases the risk of factors including endothelial dysfunction, thrombosis, and atherosclerosis, that may lead to incidence of myocardial infarction, coronary artery disease, and stroke (Srivastava et al., 2011; U.S. Department of Health and Human Services, 2014). Electronic cigarettes (e-cigarettes) are a new kind of tobacco product, and some studies show that e-cigarettes also could induce CVD since both e-cigarettes and conventional tobacco products release many aldehydes. Extensive

epidemiological and experimental data show that aldehydes contribute to cardiovascular morbidity and mortality. However, whether these aldehydes contribute to the adverse effects of emerging tobacco-products such as e-cigarettes are unknown. The transient receptor potential ankyrin1 (TRPA1) channel is a receptor of unsaturated aldehydes that mediates tobacco smoke-induced lung injury in some animal models (Andre et al., 2008; Cheah, Burcham, Mann, & Henry, 2014; D. J. Conklin, 2016; D. J Conklin, Haberzettl, Jagatheesan, Kong, & Hoyle, 2017; Simon & Liedtke, 2008). Thus, our central hypothesis is that aldehydes derived from e-cigarettes or conventional tobaccos contribute to vascular toxicity through the activation of TRPA1 pathway.

Methods

Both in vivo and in vitro studies were performed using male and female C57BL/6J mice. For in vivo studies, mice were exposed to aldehyde gas (1-5 ppm, 6 h/day) for 4 days or 12 weeks (6 h/day, 5 days/week). Non-invasive blood pressure and heart rate were measured weekly for 12 weeks by tail cuff system. Measure real-time blood pressure and heart rate by telemetry system. After exposures, we collected aortas and examined vascular function ex vivo, specifically, testing for any changes in sensitivity and efficacy of the aorta in response to pharmacological agents: phenylephrine (PE; contractions), acetylcholine (ACh; relaxation) and sodium nitroprusside (SNP; relaxation). PE-induced isometric contractions (mN) were normalized by segment length and/or volume to compare aortic contractility. Relaxations were calculated as a

percentage reduction in PE-induced tension. Sensitivity of each agent was calculated as the effective concentration producing 50% response (EC₅₀).

In our *in vitro* studies, we examined the effects and underlying mechanisms of action (relaxation) of aldehydes in both isolated mouse superior mesenteric artery (SMA) and aorta. To test specific pathways, we used pharmacological inhibitors of nitric oxide synthase (NOS), guanylyl cyclase, TRPA1, and K⁺ channels as well as mechanical injury of the endothelium.

Results

In our *in vivo* studies, C57BL/6J (wild type, WT) mice were exposed to HEPA-filtered room air (control), formaldehyde (FA; 5 ppm) or acetaldehyde (AA; 5ppm) for 4 days. There were no differences in the relaxations of ACh and SNP or in the PE-induced contractility of between control and aldehyde-exposed groups. So, short-term exposure to FA or AA at levels present in tobacco product aerosols did not alter aortic function. Mice were exposed to crotonaldehyde (CR) or filtered room air for 12 weeks (1 ppm, 6h/day, 5 d/week). Non-invasive blood pressure was measured by tail cuff (once a week) for 12 weeks. Blood pressures (systolic, mean, diastolic) were significantly lower in the CR group than in the air group over the last several weeks, yet there was no difference in heart rate between these two groups. Blood pressure changes were accompanied by changes in aortic vasoreactivity *ex vivo*. Following 12-wk CR chronic exposure, the sensitivity of ACh-induced relaxation was significantly increased in the CR

group, however, there was no change in aortic response to PE. Similarly, aortic sensitivity to SNP relaxation was enhanced by CR compared with air control group. To dissect the mechanism of CR-induced vascular changes, we performed acute 4-day exposures (1 and 3 ppm). Similarly, CR exposure also altered aortic responses to ACh and slightly to SNP. Because a 4-day CR exposure was sufficient to alter aortic reactivity, we tested whether the transient receptor potential ankyrin 1 (TRPA1) channel was involved. In TRPA1-null mice exposed to CR (4-days, 1ppm), there were no changes in aortic reactivity to any agonist (PE, ACh, and SNP) compared with air group, indicating a distinct role of TRPA1 in CR-induced changes. Interestingly, there also were no differences in any aortic responses in CR- (1 ppm 4 days) versus air-exposed female WT mice.

For the *in vitro* studies, we explored the direct effects of FA, AA and CR in isolated murine superior mesenteric artery (SMA) and aorta. FA (30-1,200 mM) and AA (1-100 mM) strongly and reversibly relaxed agonist-induced contractions of SMA including phenylephrine (PE), thromboxane A₂ analog (U46,619) and high potassium (high K⁺, 60 mM) without toxicity. The sensitivity but not the efficacy of AA-, FA- and CR-induced relaxations was dependent on blood vessel (wherein SMA was more sensitive than aorta) and contractile agonist (PE; U46,619; high K⁺) yet independent of circadian cycle and sex. All three aldehyde-induced relaxations were significantly inhibited (at low concentrations) in the presence of: 1) mechanically-impaired endothelium; 2) N ω -Nitro-L-arginine methyl ester hydrochloride (L-NAME); and 3) guanylyl cyclase (GC) inhibitor (ODQ). Positive immunofluorescent staining for TRPA1 was co-localized in

endothelium of isolated SMA and the TRPA1-specific gene sequence was detected in SMA. The relaxations of FA and CR but not AA at low concentrations were all inhibited in the presence of TRPA1 antagonist (A967079).

Conclusion

These studies indicate that short-term exposure of mice to FA or AA have limited direct toxicity in aorta, however, CR exposure (acute and chronic) leads to altered vascular function that may lead to vascular disease. Moreover, we describe direct effects of FA, AA, and CR that are dependent on an EDRF/NO and VSMC GC/PKG pathway in SMA, with FA- and CR-induced relaxations also dependent on an endothelium-localized TRPA1 receptor. From our vitro study, we conclude that aldehydes play a key role in the regulation of blood flow in the gastrointestinal (GI) tract to augment digestion and nutrient absorption and induce postprandial hyperemia, and TRPA1 receptor plays an important role in the aldehyde-induced vasodilation in vasculature. Future studies are required to better understand how CR exposure modifies cardiovascular pathophysiology, and the specific roles of TRPA1 and sex play in contributing to burden of tobacco-related CVD including pathophysiological processes of atherosclerosis.

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CHAPTER I

INTRODUCTION

Background and Rationale

Conventional tobacco smoking and cardiovascular disease (CVD)

Cardiovascular diseases (CVDs) are the leading cause of death in most countries and it kills 1 million people per year; accounting for over 40% of all deaths (Levy D, 2000). More than 900 000 deaths for the reason of CVD in the United States in 2016 (Roth et al., 2018). It is a significant health and financial burden worldwide (Bhatnagar, 2004, 2006). In 2011, the United States spent \$316.6 billion directly and indirectly on CVD, more than the cost of other diagnostic group (Writing Group et al., 2019), and the total cost of CVD is increased exponentially over the past two decades (Heidenreich et al., 2011). Based on these data, looking for the reason and the way of prevention for the CVD showed to be urgent to humans.

Smoking has been used for thousands of years in the worldwide and has been studied for several decades. In 2017, the number of smoker exceeded 1 billion globally and the Centers for Diseases Control and Prevention (CDC) reported that 14% of U.S. adults were current smokers (Centers for Disease Control and Prevention, 2017). In 2018, 27.1% of high school students and 7.2%

middle school students were current tobacco users (Centers for Disease Control and Prevention, 2018). In 2017, the World Health Organization (WHO) reported that tobacco caused about 8 million global deaths each year (World Health Organization, 2019). In the United States, Smoking kills more than 480,000 deaths annually and accounted for one of every five deaths in the United States each year (Centers for Disease Control and Prevention, 2018; U.S. Department of Health and Human Services, 2014). Numerous studies showed that smoking increased the risk of thrombosis, atherosclerosis, etc. which narrowed the vascular lumen and caused myocardial infarction, coronary artery disease and stroke (Srivastava et al., 2011; U.S. Department of Health and Human Services, 2014). Cigarette smoking also is a cause of peripheral arterial disease (PAD), aortic aneurysm, and cerebrovascular disease. Over 90% of patients with symptomatic PAD have a chronic smoking history (Hughson, Mann, & Garrod, 1978; Powell et al., 1997). About 11% of Americans are affected by PAD, 11.08% developed to limb ischemia (Nehler et al., 2014). PAD affects 22.7% of those aged > 80 years, especially 59% in male African American are affected (Benjamin et al., 2019; Writing Group et al., 2019).

Electronic-cigarette smoking and CVD

Except for conventional tobacco smoking, there are more and more new forms of smoking, such as electronic cigarette, hookahs, JUUL, etc... Electronic cigarette is a new kind of tobacco product that was introduced to the commercial market in 2004. E-cigarette includes aerosol delivery devices and a battery, the

cartridge contains e-liquids that contain a mixture of glycerol and propylene glycol, flavors, and most commonly with variable concentrations of nicotine (T. Cheng, 2014; Hutzler et al., 2014) .

As e-cigarettes initially were exclaimed to be less toxic for the smoker's health, there has been a steady increase in e-cigarette users especially among high school students, wherein e-cigarette users increased from 1.5% in 2011 to 20.8% in 2018 (Cullen et al., 2018; Gentzke et al., 2019). More and more research shows the potential harm to e-cigarette users (Benowitz & Fraiman, 2017; Bhatnagar et al., 2019). In animal studies, Olfert *et al.* (2018) showed that mice were caused cardiovascular dysfunction such as impaired vascular function and increased arterial stiffness after had been exposed to e-cigarette aerosol for 8 months (Olfert et al., 2018). Another study showed that e-cigarette exposure induces cardiac dysfunction and atherosclerosis in apolipoprotein-E-null mice (Espinoza-Derout et al., 2019). Two human studies showed that sympathetic activity and oxidative stress significantly increased in e-cigarette users (Carnevale et al., 2016; Moheimani et al., 2017). Another one clinical study showed that e-cigarette aerosol could suppress the immune system and inflammatory response genes in nasal epithelial cells similar to cigarette smoke (Martin et al., 2016).

E-cigarettes do not induce combustion as in conventional tobacco smoking. During heating of e-cigs, the humectants (VG, PG) are thermally decomposed and oxidized into short, toxic aldehydes including formaldehyde, acetaldehyde, and acrolein (D. J. Conklin et al., 2018; Ogunwale et al., 2017).

These aldehydes are well associated with endothelium dysfunction of cigarette smoking (Anderson, Majeste, Hanus, & Wang, 2016; G. E. Hill et al., 1998; S. E. Lee & Park, 2013; Lino-dos-Santos-Franco et al., 2011; Lino-dos-Santos-Franco et al., 2010; Park & Taniguchi, 2008). What's more, different brands of e-cigarettes contain varying amounts of nicotine along with multiple other elements in the smoke of the e-cigarette (Hahn et al., 2014). Numerous studies showed the cardiovascular effects of nicotine. During smoking, nicotine stimulates the release of catecholamines, thereby increases heart rate and blood pressure (Benowitz & Gourlay, 1997; Benowitz, Hansson, & Jacob, 2002). Nicotine could also increase platelet aggregation (Hom et al., 2016), induce vascular smooth muscle cell proliferation (Egleton, Brown, & Dasgupta, 2009), and increase atherosclerosis (Santanam et al., 2012).

We still do not know much about the risks of e-cigarettes to the smoker or second-hand smoker or the extent of harm. Many health care providers continue to urge people to be cautious in considering this as an alternative to cigarette smoking.

Aldehydes and CVD

Aldehydes are toxic components of automobiles exhaust and smoke, and have been detected in high concentrations in cigarette, cotton, wood, and coal smoke. Aldehydes are even present in drinking water. Cooking and frying further

would increase aldehydes concentration in foods. The metabolism of several pollutants and toxicants also generates aldehydes.

Extensive epidemiological and experimental data show that aldehydes contribute to cardiovascular mortality and morbidity. Lots of our previous studies showed the effects of aldehydes on lipid metabolism, atherosclerosis, platelet activation, thrombosis, myocardial function, and sensitivity to ischemia—suggesting that environmental aldehydes could increase CVD (D. J. Conklin, R. A. Prough, et al., 2011; B. G. Hill, Haberzettl, Ahmed, Srivastava, & Bhatnagar, 2008; Makia, Bojang, Falkner, Conklin, & Prough, 2011; O'Toole et al., 2014; Sithu et al., 2010; Srivastava et al., 2011; Srivastava et al., 2009). Some aldehydes, such as acrolein, acetaldehyde, and formaldehyde are considered the most significant cardiovascular and pulmonary toxins in tobacco smoke (Hausmann, 2012). Wheat *et al.* found acrolein inhalation exposure suppressed circulating endothelial progenitor cell (EPC) levels, indicating that EPC depletion might be a significant contributory factor linking pollutant exposure to CVD risk (Wheat et al., 2011). Other studies showed that e-cigarette aerosol including α,β -unsaturated aldehydes and saturated aldehydes led to inflammatory changes such as induction of cyclooxygenase-2 and cytokines, interacted with endothelium–leukocyte, increased lung vascular permeability, induced reactive oxygen species, DNA damage, apoptosis and programmed necrosis pathways in vascular endothelial cells (Anderson et al., 2016; S. E. Lee & Park, 2013; Lino-dos-Santos-Franco et al., 2011; Lino-dos-Santos-Franco et al., 2010). However, whether these aerosolized aldehydes contribute to the adverse effects of e-

cigarette that could induce endothelium dysfunction via its produced aldehydes is still unclear.

The metabolism of several industrial pollutants such as butadiene, vinyl chloride, benzene, and allylamine, generate aldehydes that may be the common metabolite responsible for the cardiotoxicity of these pollutants. For example, acrolein and crotonaldehyde are also formed during the metabolism of 1,3-butadiene (Nauhaus, Fennell, Asgharian, Bond, & Sumner, 1996; Richardson et al., 1998), which is used in the rubber industry. Butadiene is also a reactive constituent of cigarette smoke (Himmelstein, Acquavella, Recio, Medinsky, & Bond, 1997). Occupational exposure to butadiene is associated with a significant risk of death from arteriosclerotic heart disease (Matanoski, Santos-Burgoa, & Schwartz, 1990). Moreover, butadiene exposure has been shown to increase atherosclerosis in cockerels (Penn & Snyder, 1996a, 1996b) and generation of hemangiosarcomas in the heart (Solleveld, Miller, Banas, & Boorman, 1988). Aldehydes are metabolized in cardiac myocytes, smooth muscle cells and endothelial cells by glutathione-S-transferases (GSTP) and aldehyde dehydrogenase (ALDH) (D. J. Conklin et al., 2004; D. J. Conklin, Haberzettl, et al., 2015b; D. J. Conklin, Haberzettl, Prough, & Bhatnagar, 2009b). Formaldehyde is metabolized to formate, and acetaldehyde is metabolized to acetate. Those two metabolites are extremely high in the urine (D. J. Conklin et al., 2018; D. J. Conklin, S. Schick, et al., 2019). Our preliminary data showed that formate had no relaxation in precontracted aorta, formate and acetate had some

relaxation in superior mesenteric artery (SMA), but significantly lower than formaldehyde and acetaldehyde (Jin, Jagatheesan, et al., 2019).

Toxic metabolites of aldehydes and other environmental pollutants may actually be contributing to cardiovascular effects in vivo. Since many environmental pollutants are metabolized to aldehydes, research into the mechanisms of how aldehydes induce CVD could help study the mechanisms of these environmental pollutants as parent compounds. Rewrite. However, there was still a long way to study how aldehydes impair cardiovascular function or enhance cardiovascular diseases.

Endothelium Dysfunction and CVD

Endothelium is important in the regulation of vascular tone through the production of a variety of mediators such as NO, ROS, prostanoids, and endothelin. It acts as an active signal transducer instead of as a barrier (Vita & Loscalzo, 2002). Bunches of studies showed that smoking could directly damage to endothelial cells and affect endothelial function (Barua, Ambrose, Srivastava, DeVoe, & Eales-Reynolds, 2003; Messner & Bernhard, 2014; Raij, DeMaster, & Jaimes, 2001). Alteration in endothelial function causes the initiation of prothrombotic, proinflammatory and proliferative states and induces the development of morphological atherosclerotic changes. Endothelial dysfunction is the first step in vascular disease, since it leads to vascular inflammation, cell proliferation, and thrombosis, seems to be the most notable indicator of the

progression of cardiovascular disease (CVD), leading to high morbidity and mortality from CVD (Daiber et al., 2017; Huveneers, Daemen, & Hordijk, 2015; Lerman & Zeiher, 2005; Schachinger, Britten, & Zeiher, 2000; Yannoutsos, Levy, Safar, Slama, & Blacher, 2014). Evaluation of endothelial function is crucial to our study about the vascular effects of aldehydes.

Endothelial dysfunction refers to the impairment of endothelium-dependent vasodilatation, which implies abnormalities in endothelial integrity and homeostasis and usually occurs at an early stage of hypertension (Deanfield, Halcox, & Rabelink, 2007). The imbalance between the dilator and constrictor forces in the vasculature leads to injurious changes in the endothelium, these changes are considered as an evidence of dysfunction and as a sign of unhealthy vasculature (Kolluru, Bir, & Kevil, 2012).

Endothelial cells synthesize and secrete a broad of anti-atherosclerotic substances, the principal of which is nitric oxide (NO) that is generated from the metabolism of L-arginine by endothelial NO synthase (eNOS), constitutively expressed in endothelial cells (Luscher, 1990). Endothelial dysfunction is defined as a reduced NO bioavailability. Endothelial progenitor cells (EPCs) have multiple different cell types that play roles in the regeneration of the endothelial lining of blood vessels. They are released from the bone marrow to the sites of endothelial damaged blood vessels and promote an angiogenesis, which play an important role in the proper endothelial function (Asahara et al., 1999; Pelosi, Castelli, & Testa, 2014). The levels of endothelial progenitor cells might be a surrogate biologic marker for vascular function and cumulative cardiovascular

risk (Vasa et al., 2001). An increase in EPC levels was also shown to correlate with the occurrence of ischemic stroke (Liao et al., 2017).

Transient receptor potential ankyrin1 (TRPA1) and CVD

Despite this high disease burden, we still know little about the mechanisms by which smoking causes CVD. The transient receptor potential family is a large family of cation selective ion channels that are extensively expressed in the sensory nerves, endothelium and vascular smooth muscle cells. Transient receptor potential ankyrin1 (TRPA1) is a promiscuous sensory receptor that is activated by a variety of environmental and endogenous noxious compounds, including α,β -unsaturated aldehydes such as acrolein (Bautista et al., 2006; Cheah et al., 2014; D. J. Conklin, 2016; D. J Conklin et al., 2017), cinnamaldehyde (Bandell et al., 2004; Bodkin & Brain, 2011; Pozsgai et al., 2010), and 4-hydroxynonenal (4HNE) (Trevisani et al., 2007). TRPA1 mediates pain-reception/transmission (nociceptors). Recently, the distribution of TRPA1 receptor has been expanded to include non-neuronal locations, such as the heart, endothelium, and urothelium (Bautista et al., 2006; Earley, Gonzales, & Crnich, 2009a; Kolluru et al., 2012; Pelosi et al., 2014), it is also present in vasculature (Earley et al., 2009a; Sinharoy et al., 2017).

Activation of peripheral sensory fiber TRPA1 leads to pain signaling and likely triggers the opening of the cation channel, allowing calcium entry, neuronal activation, and release of vasoactive peptides, substance P (SubP) and

calcitonin gene-related peptide (cGRP) (Macpherson et al., 2007; Trevisan et al., 2016), which may contribute to increased vasodilation. These peptides increase local tissue inflammation, blood flow, vascular permeability, edema and leukocyte binding and extravasation (Andrade, Meotti, & Calixto, 2012). Thus, activation of the TRPA1 channel by acrolein is linked with inflammation, which could contribute to acute tobacco smoking- or acrolein-induced injury in the airways and the cardiovascular system (Bautista et al., 2006; Earley et al., 2009a; Inoue et al., 2006). Our previous study showed that pre- and post-exposure TRPA1 antagonism significantly protected against acrolein-induced mortality showed a complex role of TRPA1 in cardiopulmonary injury (D. J Conklin et al., 2017). The airway-located TRPA1 triggers an irritant response (e.g., coughing and “respiratory braking”) that alters the rate and depth of breathing to reduce exposure, what’s more, the morbidity and mortality significantly increased in the TRPA1-null mice might be for the reason that these TRPA1-null were deficient in this “respiratory braking” (D. J Conklin et al., 2017). Cinnamaldehyde-induced dose-dependent blood vessel relaxation, blood pressure, heart rate and local blood flow was significantly less potent in TRPA1-null compared with wide type arteries (Pozsgai et al., 2010). However, the relationship between TRPA1 and e-cigarettes aerosol induced endothelial dysfunction and CVD risk is still unclear.

In summary, voluntary and involuntary exposures to cigarette smoke (and perhaps e-cigarette aerosols) are major causes of CVD such as CHD, stroke, aortic aneurysm, and PAD. Smoking releases aldehydes, and numerous studies show that aldehydes can also induce CVD. The risk is seen both as an increased

risk of endothelial dysfunction and acute thrombosis of narrowed vessels and as an increased of atherosclerosis in the blood vessels involved. The cardiovascular risks attributable to cigarette smoking increase with the number of cigarettes smoked and the duration of smoking. The mechanisms of the smoking induce endothelial dysfunction are still unclear. TRP family play important roles in the development of CVD, especially TRPA1 receptor. It is important to clarify this mechanism in order to decrease the impact that cigarette products exposure has on the incidence of endothelial dysfunction and CVDs. Once this mechanism has been better clarified, and the toxicity of cigarette smoking or e-cigarette smoking has been identified, we can begin to develop health guidelines and pharmaceutical interventions to reduce the frequency of CVD both nationally and globally. A decreased rate of cigarette smoking and a decreased incidence of CVDs would have a significant impact on the cost of medical treatment and the worldwide financial burden and, more importantly, on the global quality of life.

B. Specific aims in this study

The primary goal of the proposed research is to determine whether the aldehydes derived from tobacco products (electronic cigarettes, conventional tobacco) may be harmful to the vasculature, and, if so, what is the underlying mechanism. Over-exposure to aldehydes is known to produce cardiovascular toxicity. However, whether these aldehydes contribute to the adverse effects of tobacco-products such as electronic cigarettes are unknown. The transient receptor potential ankyrin1 (TRPA1) channel is a receptor of aldehydes and

mediates tobacco smoke-induced lung injury in some animal models. Thus, our central hypothesis is that tobacco product-derived aldehydes contribute to vascular toxicity through activation of the TRPA1 pathway. To test this hypothesis, we explored the following aims:

Aim 1. Determine the direct vascular effects of tobacco-product derived aldehydes in vitro.

We explored the possible pathways of vascular effects of aldehydes, and found that the relaxation of formaldehyde (FA), acetaldehyde (AA) and crotonaldehyde (CR) were all endothelium dependent, nitric oxide dependent, and cGMP dependent. We also showed that the relaxation of FA and CR was TRPA1 dependent. Furthermore, we tested the vascular effects of aldehydes metabolites. Formate (main metabolite of FA; up to 1 mM) nor methanol (a stabilizer in formalin, up to 1 mM) induced a weak relaxation in SMA indicating that FA-induced relaxation was not a result of formate or methanol. Ethanol and acetate could dilate blood vessels but much lower than AA and were all nitric oxide dependent. Because smoking is often inhaled with nicotine, we tested if nicotine modified aldehyde effects, but nicotine [1 μ M] had no effect on subsequent FA, AA and CR-induced vasorelaxation.

Aim 2. Determine the vascular effects (endothelium dysfunction) of tobacco-product derived aldehydes exposures in vivo.

We hypothesize that the of electronic cigarettes or conventional tobacco could cause cardiovascular injury through its aldehydes. Mice were exposed to

FA (5ppm) or AA (5ppm) for 4 days, there was no significant change in aorta function after FA or AA exposure. Mice were exposed to CR or air for 12 weeks (1 ppm, 6h/day, 5 d/week). Blood pressure was significantly lower in the CR group than in the air group for the last several weeks, but there was no difference in the changes of heart rate between these two groups. Hemodynamic changes were accompanied by changes in aortic vasoreactivity ex vivo. Following 12-wk CR chronic exposure, there was no change in aortic response to phenylephrine (PE), however, the sensitivity of ACh-induced relaxation was significantly increased in the CR group. Similarly, aortic sensitivity to SNP relaxation was enhanced by CR. To dissect the mechanism of CR-induced vascular changes, we performed acute 4-day exposures (1 and 3 ppm). Similarly, CR exposure also altered aortic responses to ACh and somewhat to SNP.

Aim 3. Are vascular effects of aldehydes mediated by TRPA1?

We hypothesize that the aldehydes derived from tobacco products induce vascular injury might through the TRPA1 pathway. In our vivo study, since a 4-day CR exposure was sufficient to alter aortic reactivity, we tested whether the transient receptor potential ankyrin 1 (TRPA1) channel was involved. In TRPA1-null mice exposed to CR (4-days, 1ppm), there were no changes in aortic reactivity to any agonist (PE, ACh, and SNP) compared with air group, indicating a distinct role of TRPA1. In our vitro study, since histology staining showed there were a lot of TRPA1 proteins in endothelium in superior mesenteric artery (SMA). We tested whether the relaxation of FA, AA or CR via TRPA1 pathway. TRPA1 antagonist (A967079) has significantly inhibited the relaxation of FA and CR at

low concentrations in SMA. The relaxation of CR significantly decreased in TRPA1-null mice. We concluded that the relaxation of FA and CR are TRPA1 dependent.

We also have tested the sex dependence in the cardiovascular effects of aldehydes. In our vivo study, we tested a 4-day CR exposure to female mice, Interestingly, there were also no differences in any aortic responses in CR versus air-exposed female mice. For the in vitro study, there was no difference in the relaxation of FA and AA between male and female mice. The mechanism of female-dependent protection in mice is not clear.

CHAPTER II

A SIMPLE METHOD FOR NORMALIZATION OF AORTA CONTRACTILITY

Vascular contractile function changes in proliferative vascular diseases, e.g., atherosclerosis, and is documented using isolated blood vessels, yet, many laboratories differ in their approach to quantification. Some use raw values (e.g., mg, mN); others use a 'percentage of control agonist' approach; and, others normalize by blood vessel characteristic, e.g., length, mass, etc. A lack of uniformity limits direct comparison of contractility outcomes. To address this limitation, we developed a simple 2-step normalization method: 1) measure blood vessel segment length (mm) and volume (mm^3); and, 2) normalize isometric contraction (mN) by segment length and/or volume. Normalization reduced variability, and thus, normalized aortic contractions but not raw values were statistically different between normal chow (NC) and high fat diet-fed (HFD) mice supporting the practical utility and general applicability of normalization. It is recommended that aortic contractions be normalized to segment length and/or volume to reduce variability, enhance efficiency and to foster universal comparisons across myography platforms, laboratories and experimental settings.

1. Introduction

Cardiovascular disease is the leading cause of death worldwide.

Atherosclerosis is the primary form of cardiovascular disease that increases the risk of myocardial infarction, stroke and peripheral arterial disease. An underlying mechanism of atherosclerosis involves a 'phenotypic switch' in vascular smooth muscle cells (VSMC) that transcriptionally moves VSMC from a contractile to a proliferative or "synthetic phenotype" (Li et al., 2013; Z. Zhang et al., 2012). Thus, (D. J. Conklin, Haberzettl, et al., 2009b) phenotypic change reflective of the underlying local cellular and biochemical alterations such as in atherosclerosis. Thus, laboratories around the world measure vascular contractile function *in vitro* wherein an isolated blood vessel, e.g., aorta, is contracted with an agonist, e.g., phenylephrine (PE), endothelin-1, U46,619 – a thromboxane A₂ analog, etc... and data reported (D. J. Conklin & Boor, 1998; D. J. Conklin, Boyce, Trent, & Boor, 2001b; D. J. Conklin, Haberzettl, et al., 2009b). The widespread use of *in vitro* vascular contractility assays is revealed in PubMed search using the terms "vascular" and "dysfunction," which netted 214,358 hits, indicating a sizeable research investment.

Although raw isometric data reflect vessel contractility, raw values (e.g., mg or mN tension)(Reynolds et al., 2012) cannot be compared across different laboratories due to obvious differences in the size of the blood vessel segment (e.g., length, diameter), species, animal size, age, diet, and disease state as well as the specific blood vessel or blood vessel location (e.g., thoracic vs abdominal aorta). Additionally, different platforms or myography systems (e.g., DMT) are used

to assess isometric force production. So, even if two laboratories use the same system, the reporting of data can make it difficult to compare contractility due to dissimilar units (mg vs mN) or a different data normalization procedure, e.g., % of control agonist such as % contraction of high potassium solution)(Kim, Avila, & Massett, 2016; Schepelmann et al., 2016) or differential normalization, e.g., mg tension/cross-sectional area; mg tension/mg wet weight; mN/mm length (D. J. Conklin & Boor, 1998; D. J. Conklin et al., 2004; D. J. Conklin, Haberzettl, et al., 2009b). To address this obvious limitation and to foster more across-study and laboratory comparisons, aortic contractions were normalized by several easily measured parameters.

Several criteria need to be met to establish a universal normalization method. For example, the method needs to be accessible, and thus, it should not require costly equipment that may exclude users. It should be simple enough that all users can implement the method. Moreover, the method should be cost effective in that the time spent performing should be worth the effort. Because current laboratories already use some form of normalization (e.g., as “% of control agonist”) (Kim et al., 2016), a new approach must be cost effective and enhance data quality over an existing practice. Finally, the method should foster data comparisons for both cross-laboratory fertilization and greater biological understanding. To this end, we offer a simple and cost-effective method for normalization of vascular contractility that requires minimal equipment and effort on the user's behalf, yet refines the measurement of aortic contraction to a degree wherein even modest changes in contractility (e.g., due to a treatment, aging, diet,

etc...) are more readily discerned. Universally-normalized contractility data can be shared and compared by laboratories around the globe. Adoption of this approach may improve assessment of the biological actions of drugs, for example, and thus, minimize the overall use of resources.

2. Materials and Methods

Rodents:

Male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or raised in house. Mice were fed normal chow (NC; 13% kcal fat, Rodent Diet 5010, LabDiet; St. Louis, MO) or high fat (HFD; 42% kcal fat, TD.88137, Harlan Teklad, Madison, WI) diet from 8 to 20 weeks of age. Male Sprague-Dawley rats (wild type and low-density lipoprotein receptor deficient; LDLR-null) were obtained from SAGE (Sigma-Aldrich) and fed normal chow diet up to 1 yr of age (Sithu et al., 2017). Rodents were treated according to APS's Guiding Principles in the Care and Use of Animals and all protocols were approved by University of Louisville IACUC. All rodents were anesthetized with sodium pentobarbital (i.p., 150 mg/kg) and exsanguinated via right ventricle blood draw.

Equipment:

Digital handheld microscope (zORB, Carson, New York); scientific ruler (1 mm increments; Fine Science Tools); 10% neutral buffered formalin solution (NBF; Sigma); Falcon® 12-well, clear, flat bottom plate (Corning Costar); ImageJ (NIH free imaging software); razor blades; dental wax (Z Pink); general surgical tools

(scissors, forceps); microfuge tubes; organ bath myography system; and, microbalance.

Aorta Isolation and Organ Bath Systems:

Aortas were isolated via ventral thoracotomy and placed into cold phosphate buffered solution (PSS) containing (in mM): NaCl, 130; KCl, 4.7; MgSO₄·7H₂O, 1.17; KH₂PO₄, 1.18; NaHCO₃, 14.9; CaCl₂, 2.0; glucose, 5.0; pH 7.4. Distal thoracic aorta (located 0.8-1 cm from aortic arch apex) segments were cut from each aorta (2-4 mm), cleaned of perivascular adipose tissue, and mounted in one of two systems: 1) horizontal pin (DMT, Ann Arbor, MI); or, 2) vertical wire system. In the horizontal pin system, a short, aortic segment (≈2 mm) was mounted on stainless steel pins in a 5-ml heated (37 °C) organ bath. One pin was connected to an isometric strain gauge transducer, and the other was attached to a micrometer. Transducer signals were fed directly into a PC with LabChart software (v. 7 or 8; ADI). In the vertical wire system, an aortic segment (3-5 mm) was placed on wire hooks (100 μm dia. tungsten wire) with a hook connected to an isometric strain gauge transducer (Kent Scientific, Litchfield, CT) and the other attached to a fixed support glass rod in a 15-ml heated (37 °C) organ bath. Transducer signals were fed into an 8-channel PowerLab A/D converter and recorded on a PC using LabChart software (v. 4.3.2; ADI). Prior to each use, each system was calibrated using standardized weights. In both systems, after 10 min without tension, aortic rings were equilibrated to ≈9.81 mN (≈1 g) loading tension over 30 min before agonist stimulation (D. J. Conklin, Haberzettl, Prough, & Bhatnagar, 2009a).

Statistics.

Values are expressed as mean $\pm$ SD or $\pm$ SE (where appropriate). Comparisons between 2 groups (e.g., horizontal vs vertical system; User 1 vs User 2; etc...) were done with a paired or unpaired *t*-test as appropriate. $P < 0.05$ was considered significant.

3. Results and Discussion

Acquisition of Aortic Segment Parameters:

Normalization of contractions was done using a simple, sequential, multi-step process that is described pictorially in **Fig. 1**, and described here as a general protocol: 1) immediately formalin-fix a blood vessel segment; 2) obtain wet weight (to 0.1 mg; dab extra fluid on tissue paper); 3) image segment lengthwise with scientific ruler (**Fig. 1a**); 4) cut (with razor blade) and image (with handheld digital microscope) a thin (0.5 mm) blood vessel cross-section (x-sec) with a ruler in the image (**Fig. 1b**); 5) using ImageJ, quantify segment length (mm) and x-sec area (mm^2) (**Fig. 1c**); and, 6) calculate segment volume (mm^3) as the product of length (mm) and x-sec area (mm^2), and the normalize isometric contractions by segment length (mN/mm), x-sec area (mm^2), and volume (mN/mm^3).

Figure 1. Images of method for aortic parameter data acquisition. **a)** Digital images of blood vessel segment length (mm) acquired with digital mini-microscope camera. **b)** Digital image of blood vessel cross-section over-laid with digital outlines of outer and inner circumferences (NIH ImageJ). The outer area (OA) minus the inner area (IA) is the segment cross-sectional area (mm²). **c)** Calculation of blood vessel segment volume (volume = cross-section area * length = mm³). (Permission with S. Karger AG, Basel.)

c $\text{Volume} = \text{length} \times \text{wall area (OA - IA)} = \text{mm}^3$

Aortic Function:

Aortic vascular contractile function was assessed using both a horizontal and a vertical isometric tension organ bath system as reported previously (D. J. Conklin, Haberzettl, et al., 2009a). To illustrate the utility of the normalization protocol, the distal thoracic aorta of a mouse was sectioned into 2 obvious, unequal lengths. A shorter piece (1.5-2.5 mm) was mounted in the horizontal pin system and a longer piece (3-4 mm) was mounted in the vertical system. Both segments were equilibrated similarly (≈ 9.81 mN tension). As intended in this 'proof of principle' example, the absolute (raw) tension of aortic contractions (mN) induced either by cumulative concentrations of PE (1 nM-10 μ M; **Fig. 2a**) or by high potassium solution (100 mM; **Fig. 2b**) was significantly different between the two due to the intentional difference in blood vessel segment size ($\approx 2\times$).

Figure 2. Normalization of vascular contractility data. Contraction data (Raw, mN) and data normalized by aorta cross-sectional area (mN/mm^2), length (mN/mm), and volume (mN/mm^3) were plotted. To generate tension data, two aortic segments were exposed to contractile agents: **a**) phenylephrine (PE, 1 nM-10 μM); and, **b**) high potassium (100 mM) buffer; in a horizontal pin (DMT; 1, filled symbols), or a vertical wire (2, open symbols) organ bath system. After experimentation, length and cross-sectional area of the blood vessel segments were measured and the segment volume calculated from these values (volume = cross-section area * length; see **Fig. 1**). (Permission with S. Karger AG, Basel.)

Normalization of Aortic Contraction:

After acquisition of aortic parameters, cumulative PE and high potassium-induced contractions were normalized to x-sec area, length, and volume (**Fig. 2**). Normalization of raw tension by segment volume (mN/mm^3) resulted in the most similar PE-induced tension curves (see curves of “Volume 1” and “Volume 2”; **Fig. 2a**). Whereas normalization of raw values to either x-sec area or length were beneficial in reducing the difference between “Raw 1” and “Raw 2” values, but neither was superior to normalization by volume. Notably, in a separate study, a group used a standardized mouse aorta segment length (3 mm) to normalize contractility and arrived at values similar to ours, thus, supporting the idea that normalized contractions even to one dimension (e.g., length; mN/mm) likely is better than no normalization (**Fig. 2a**) (Silva et al., 2016). Normalization efficacy was independent of the contractile agonist because a similar effect was observed when high potassium-induced maximal tension was normalized to x-sec area, length and volume (**Fig. 2b**). As for PE, normalization to volume appeared to perform better than using either length or area alone.

In a second, real-world example, contractility was measured in aortas isolated from C57BL/6 mice fed either a normal chow (13% kcal fat) or high fat diet (42% kcal fat) for 12 weeks. As all aorta segments had similar lengths, x-sec areas, and thus, volumes (**Table 1**), direct comparison of raw contractility values revealed that 12 weeks of HFD appeared to decrease PE-induced contractions relative to aortas of NC-fed mice *yet there were no statistically significant differences* (**Fig. 3a**). To assess whether normalization would unmask differences in contractility, raw

values were normalized by area (**Fig. 3b**), length (**Fig. 3c**) and volume (**Fig. 3d**). When normalized to aortic segment length or volume, contractions were statistically different between NC and HFD mice (**Fig. 3c, d**). Regression analyses revealed that aortic length (mm; **Fig. 3e**) and volume (mm³; **Fig. 3f**) were correlated with contractility (mN) more strongly in aorta of NC- than in HFD-fed mice. Moreover, volume appeared to perform better as a denominator than did length in both NC- and HFD-fed mice (**Fig. 3e, f**). Overall, these data indicate that normalization of aortic contractility enhanced detection of statistically significant changes induced by HFD (**Fig. 3d**). Another real world example was performed using aortas isolated from 1-year old Sprague-Dawley rats that were wild type or deficient of LDLR (LDLR-KO). As all aorta segments had similar lengths, x-sec areas, and thus, volumes (**Table 2**), direct comparison of raw contractility values revealed that genetic deletion of LDLR increased PE-induced contractions relative to aortas of WT rats (**Fig. 4a**). To assess whether normalization would alter the difference in contractility based on raw values, values were normalized by length (**Fig. 4b**), x-sec area (**Fig. 4c**) and volume (**Fig. 4d**). Normalization of raw contractility data to aortic segment length, area or volume only enhanced the statistically significant differences between WT and LDLR-KO rats (**Fig. 4b, c, d**). Overall, these data indicate that normalization of aortic contractility improves detection of statistically significant changes induced by genetic deletion of LDLR in rats.

Table 1. Thoracic aorta segment parameters from C57BL/6 mice fed normal chow (n=9) or high-fat diet (n=10) for 12 weeks.

Parameter	Diet	
	Normal chow	High-fat diet
Wall area, mm ²	0.246±0.024	0.225±0.020
Length, mm	3.96±0.56	4.37±0.41
Volume, mm ³	0.976±0.178	0.990±0.171

Values = means ± SE; *n* = 9,10 mice, respectively.

Figure 3. Normalization of vascular contractility of aortas from C57BL/6 mice fed normal chow (NC; n=9 mice) or high fat diet (HFD; n=10 mice) for 12 weeks. Phenylephrine (PE)-induced raw contraction data (**a**, mN) and raw data normalized by aorta length (**b**, mN/mm), x-sec area (**c**, mN/mm²), and volume (**d**, mN/mm³). Values = mean $\pm$ SE. n = 9 or 10 aortas (1 aorta per mouse). *, P<0.05 between NC and HFD. (Permission with S. Karger AG, Basel.)

Table 2. Normalization of high potassium-induced (100mM) isometric contractions in thoracic aortas isolated from male mice fed either normal chow (NC) or high fat diet (HFD) for 12 weeks.

Group	mN	mN/mm	mN/mm ²	mN/mm ³
NC	8.26±1.03	2.08±3.32	33.16±3.32	8.37±0.71
HFD	5.54±0.61	1.26±0.13	24.61±2.60	5.66±0.57
<i>p</i> value	0.0330	0.0047	0.0562	0.0079

Value = means ± SE; *n* = 9,10 mice, respectively; *p* values shown are results of unpaired *t* tests between NC and HFD groups.

Table 3. Thoracic aorta segment parameters from wild-type (WT) and LDLR-null (LDLR-KO) 1-year-old Sprague-Dawley rats.

Parameter	Strain	
	WT	LDLR-KO
Wall area, mm ²	1.251±0.213	1.168±0.224
Length, mm	3.07±0.49	2.74±0.48
Volume, mm ³	3.90±1.055	3.269±1.138

Values = means ± SE. *n* = 5,6 rats, respectively.

Table 4. Normalization of PE-induced (10 μ M) isometric contractions in thoracic aortas isolated from wild-type (WT) and LDLR-null (LDLR-KO) 1-year-old Sprague-Dawley rats.

Group	mN	mN/mm	mN/mm ²	mN/mm ³
NC	15.10 $\pm$ 2.55	4.85 $\pm$ 0.66	12.09 $\pm$ 1.59	3.98 $\pm$ 0.52
HFD	22.29 $\pm$ 2.34	8.36 $\pm$ 1.09	19.57 $\pm$ 2.35	7.50 $\pm$ 1.27
<i>p</i> value	0.0680	0.0283	0.0327	0.0413

Value = means $\pm$ SE; *n* = 5,6 rats, respectively; *p* values shown are results of unpaired *t* tests between WT and LDLR-KO groups.

Figure 4. Normalization of vascular contractility of aortas from Sprague-Dawley wild type (WT; n=5 rats) and LDLR-KO rats (n=6 rats) fed normal chow for 1 year. Phenylephrine (PE)-induced raw contraction data (**a**, mN) and raw data normalized by aorta length (**b**, mN/mm), x-sec area (**c**, mN/mm²), and volume (**d**, mN/mm³). Values = mean $\pm$ SE. n = 5 blood vessels. *, P<0.05 between WT and LDLR-KO rats. (Permission with S. Karger AG, Basel.)

Method Validation:

The 'proof of principle' tests (i.e., platforms; NC vs HFD; WT vs LDLR-KO) support the general applicability of our normalization approach, so we also performed four validation tests. These were: 1) two different 'users' quantified the same ring images using ImageJ; 2) two different users quantified the same image multiple times (e.g., 10 times); 3) two different users imaged and then quantified multiple sections from a single aorta; and, 4) normalization was performed using segment volume calculated by 2 different methods: a) our image analysis approach; and, b) volume estimated from segment weight (in mg) and density ($\text{mg}/\mu\text{l}$; mg/mm^3).

Reproducibility.

Image analysis for length and x-sec area proved highly reproducible (**Fig. 5**). It appeared that users vary in their first attempt to quantify length, but users rapidly adjusted and subsequently made consistent measurements of the same image. These data support the robust and cost-effective nature of our approach. Images were made with an inexpensive microscope camera (<\$50) and were analyzed with free imaging software (NIH ImageJ). The approach was independent of the user because measurements made by 2 users of both area (**Fig. 6a**) and length (**Fig. 6b**) using the same 5 blood vessel segments were similar. Although noticeable divergence in volumes between users was present (**Fig. 6c**), both variability and directionality of values were preserved -- indicating both robustness and consistency. Our approach also compared favorably to using blood vessel volume estimated as the quotient of blood vessel mass and reported aortic density, i.e., 0.86 mg/L (Weind, Ellis, & Boughner, 2002) (**Fig. 6d**). We think that there is inherently more difficulty in accurately measuring the mass of a wet blood vessel than our image-based approach. First, it should be recognized that a short, cleaned segment of mouse aorta weighs approximately 0.1-0.2 mg, and a high-quality balance capable of such a measurement is costly. Second, much smaller blood vessels, e.g., mesenteric, may be difficult to weigh accurately because of lumen fluid retention that is difficult to remove without rapidly “drying out” the blood vessel segment (Murphy, 1980). Thus, our low-cost approach is more practical and avoids these pitfalls.

We also tested whether a single blood vessel segment would yield different x-sec areas along its length and whether this was dependent on user evaluation. Although variation in x-sec area was apparent, it appeared less dependent on user and more on image quality and segment location (**Fig. 7**). These data further emphasize that specific user-based analysis of an image is a less important determinant of x-sec area than is the image quality and blood vessel location (e.g., thoracic aorta tapers proximal to distal). Of course, quality control of image analysis needs to be established in each laboratory adopting this approach. We purposefully used a low cost, highly portable 'hobby' or classroom-style digital camera/microscope to emphasize that even rudimentary optics are sufficient to perform blood vessel analysis yet practice is required.

Resiliency to Prolonged Formalin Fixation.

Formalin fixation for 1 week (**Fig. 8a**), 1 month (**Fig. 8b**) and up to 2 months (**Fig. 8c**) had little effect on quantified aortic segment volume, so it is feasible to formalin fix a blood vessel and still obtain reliable and robust measurements for normalization long after contractility experiments were performed. Additionally, fixed blood vessel segments can be processed, embedded, sectioned and stained for more refined morphometry of the blood vessel wall (D. J. Conklin, Kong, & Committee, 2015).

Figure 5. Reproducibility of aorta parameter data acquisition: intra-assay variation.

The same images of a blood vessel lengthwise and in cross-section (ring) were repeatedly examined for length and cross-sectional area (x-sec area), respectively, as described above using ImageJ a total of 10 times by two different observers. Blood vessel volume (mm^3) was calculated by multiplying length (mm) $\times$ x-sec area (mm^2). Values = mean $\pm$ SD. (Permission with S. Karger AG, Basel.)

Figure 6. Reproducibility of aorta parameter data acquisition: inter-assay variation.

a) Aorta cross sectional area (mm^2) measured in five separate aortas independently by two users (User 1; User 2). **b)** Aorta segment length (mm) measured independently by two different users in five separate aortas. **c)** Volume (mm^3) calculated five times by two users. **d)** Aortic volume calculated from image analysis compared with volume derived from segment weight and aorta density (0.86 mg/L). Values = mean $\pm$ SD. (Permission with S. Karger AG, Basel.)

Figure 7. Intra-aortic variation in cross-sectional area. A single thoracic aorta segment was cut into five cross-sections (“donut”) and each cross-section was imaged and the area measured by two independent users (User 1; User 2). Values = mean $\pm$ SD. (Permission with S. Karger AG, Basel.)

Figure 8. Resiliency of aorta parameter data acquisition over time. Comparison of aorta volumes measured after formalin fixation of the blood vessel. **A)** Aorta volume measured 1 week after formalin fixation. **B)** Aorta volume measured 1 month after formalin fixation. **C)** Aorta volume measured 2 months after formalin fixation. Values = mean $\pm$ SD. (Permission with S. Karger AG, Basel.)

Major Advantages and Limitations:

The current study describes a novel method for normalization of vascular contractility *in vitro*, and highlights 4 major advantages: 1) the method is simple and low cost, and thus, accessible world-wide; 2) the method is validated and is reproducible across different platforms and users; 3) the method is resilient to the chronic storage of blood vessels in formalin; and, 4) if adopted, normalized vascular contraction data with a 'universal unit' (either mN/mm or mN/mm³) will allow for more across study and across laboratory comparisons, and thus, more efficient use of research resources.

There are several caveats to this method. This method does not define the optimal state of contractility or the mechanisms of change in contractility. It is expected that laboratory conditions likely will vary in many ways that meaningfully contribute to real differences in contractility (e.g., buffer composition, loading tension, temperature, inhibitors, denuding, etc...), but "all things being equal," this approach provides a common denominator, which currently is not used by most laboratories. As indicated above, the method reduces variability in raw values by correcting for differences that arise as a function of blood vessel segment size. Blood vessel segments differ in size simply because two different laboratories use different platforms. An obvious limitation is that we only tested the protocol using aortas from mice and rats, however, the method will likely work in other blood vessels because there is an inherent reliance of vascular contraction on smooth muscle that makes up the bulk of the media in aorta and other arteries as well. In any case, we predict that normalization (with segment length and/or volume) is

superior to using only raw contraction values. In our real-world example, we detect significant changes in aortic contractility with HFD feeding (vs NC) *only when we normalized contractions* (see **Fig. 3**). This outcome has value because importantly it reduces the overall cost of a study by reducing the number of animals needed in a study. Finally, our approach is validated to be robust, consistent, reproducible and resilient. It is recommended that isometric vascular contractions be normalized to either blood vessel segment length (mN/mm) or volume (mN/mm³) to foster both improved quantitative assessment of contractility changes and across study comparisons.

CHAPTER III

FORMALDEHYDE INDUCES MESENTERIC ARTERY RELAXATION VIA A SENSITIVE TRANSIENT RECEPTOR POTENTIAL ANKYRIN-1 (TRPA1) AND ENDOTHELIUM-DEPENDENT MECHANISM

Introduction Formaldehyde (FA), the smallest aldehyde, is generated endogenously, and is widespread in the environment in foods, beverages and as a gas phase product of incomplete combustion. **Methods and Results** The main metabolite of FA, formate, was increased significantly in murine urine ($\approx 3\times$) after overnight feeding. Because feeding increases mesenteric blood flow, we explored the direct effects of FA in isolated murine superior mesenteric artery (SMA). Over the concentration range of 30-1,200 μM , FA strongly and reversibly relaxed contractions of SMA induced by three different agonists: phenylephrine (PE), thromboxane A_2 analog (U46,619) and high potassium (high K^+ , 60 mM K^+). Formate (to 1.5 mM) did not induce relaxation. FA ($>1,500 \mu\text{M}$) irreversibly depressed vascular function in SMA indicating vasotoxicity. The sensitivity (EC_{50}) but not the efficacy (% relaxation) of FA-induced relaxations was dependent on blood vessel type (SMA $\ll$ aorta) and contractile agonist (PE, $EC_{50}=52\pm 14 \mu\text{M}$; U46,619, $EC_{50}=514\pm 129 \mu\text{M}$; 60K, $EC_{50}=1,093\pm 87 \mu\text{M}$). The most sensitive

component of FA vasorelaxation was within physiological levels (30-150 μ M) and was inhibited significantly by: 1) mechanically-impaired endothelium; 2) N ω -Nitro-L-arginine methyl ester hydrochloride (L-NAME); 3) transient receptor potential ankyrin-1 (TRPA1) antagonist (A967079); 4) guanylyl cyclase (GC) inhibitor (ODQ); and, 5) K⁺ channel inhibitor (BaCl₂). A similar mechanism of SMA vasorelaxation was stimulated by the TRPA1 agonist cinnamaldehyde. Positive TRPA1 immunofluorescent staining and gene-specific sequence were present in SMA but not in aorta. **Conclusion** These data indicate FA, but not formate, robustly relaxes SMA via a sensitive TRPA1- and endothelium-dependent mechanism that is absent in aorta. Thus, as FA levels increase with feeding, FA likely contributes to the physiological reflex of post-prandial hyperemia via SMA vasodilatation.

1. Introduction

Formaldehyde (FA) is the smallest aldehyde and the concentration of FA in the blood ranges from 0.0127 to 2.28 μ g/mL (1.9 to 5.4 mg/L; or 63-179 μ M)(Kleinnijenhuis, Staal, Duistermaat, Engel, & Woutersen, 2013; Y. S. Wang et al., 2011). FA is in food and beverages (likely the largest quantitative source of exogenous FA) and is metabolically-derived (D.J. Conklin, P. Haberzettl, J. Lee, & S. Srivastava, 2011). Exogenous FA is a volatile indoor and outdoor pollutant emitting from building materials (e.g., plywood, glues, carpet, drapery), and present in cosmetics, vehicle exhaust, photochemical smog and tobacco-derived aerosols (D. J. Conklin, P. Haberzettl, J. Lee, & S. Srivastava, 2011; Ogunwale et al., 2017). Endogenous FA is generated by breakdown of L-methionine, histamine,

methanol and methylamine, and may contribute to biological methylation of folic acid (Yu & Zuo, 1996). Thus, blood levels of FA are in constant flux due to rapid changes in exposure, absorption, distribution, metabolism (to formate and CO₂) and excretion.

Although direct effects of FA on cardiovascular targets are described, the role of FA in cardiovascular physiology is not well explored. For example, intra-arterial or intravenous FA reduces heart rate (bradycardia), decreases arterial pressure (hypotension) and causes arrhythmias in animals (Strubelt, Brasch, Pentz, & Younes, 1990; Tani & Horiguchi, 1990; Tani, Kogi, & Horiguchi, 1986; Tani, Satoh, & Horiguchi, 1978), but these are considered toxicological effects due to high doses administered directly into the blood. Because FA is toxic in cultured endothelial cells (inhibition of mitosis, increased apoptosis), vascular smooth muscle cells and cardiomyocytes (D. J. Conklin, Langford, & Boor, 1998), it is, thus, speculated that endogenously-generated FA induces endothelium dysfunction by which it accelerates diabetic complications such as atherosclerosis (Deng & Yu, 1999). However, FA induces a concentration-dependent relaxation in isolated blood vessels (D. J. Conklin et al., 2004; Tani, 1981; Q. Zhang et al., 2018) yet the relevance of this effect in vascular physiology is unclear.

Despite myriad sources of FA and its provoked cardiovascular effects, few studies address the vascular effects of physiological levels of FA. Formate is a stable metabolite of FA generated from a 2-step enzymatic process involving glutathione-dependent formaldehyde dehydrogenase (FALDH2) hemi-thioacetal formation and aldehyde dehydrogenase 2 (ALDH2) mediated oxidation (Conklin et

al., 2006). In the current study, we measured urine levels of formate, the primary metabolite of FA, to estimate changes in endogenous FA levels. As murine urine levels of formate increased ≈ 3 times overnight (with feeding) compared with levels after daytime fast, we think feeding increased endogenous FA levels (directly or indirectly). As such, the gastrointestinal vasculature represents a likely target of changes in FA levels following feeding when reflexively blood flow increases in the gut (Takagi, Naruse, & Shionoya, 1988). Thus, to address potential FA-induced vascular effects, isolated superior mesenteric artery (SMA) and aorta were exposed to FA across a physiologic to toxic range (30-1,500 μM). Although an FA-induced vasorelaxation was present in both blood vessels, the most sensitive relaxation was present in SMA indicating that physiologic levels of FA likely stimulate dilation in SMA before aorta. Thus, we identified the mechanisms of the FA-induced relaxation (30-150 μM) as dependent on: a functional endothelium, TRPA1 activation, NO formation, guanylyl cyclase activation, and potassium channels. Formate, though, had no effect in blood vessels up to 1,500 μM . These data collectively support the idea that feeding increases FA levels, and that FA (and not formate) likely contributes to increased gastrointestinal blood flow of feeding (i.e., postprandial hyperemia) through a sensitive, robust and reversible TRPA1- and endothelium-dependent mechanism.

2. Material and Methods

Chemicals and Solutions.

Reagent grade chemicals were purchased from Sigma-Aldrich or other commercial sources as indicated: A967079 (AdooQ); acetylcholine chloride (ACh); 4-aminopyridine (4-AP); barium chloride (BaCl₂); cinnamaldehyde (%); formalin (formaldehyde, 37% and methanol, 10-15%); formate; 1h-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ); N^ω-nitro-L-arginine methyl ester hydrochloride (L-NAME); L-phenylephrine hydrochloride (PE); sodium nitroprusside (SNP); tetraethylammonium chloride (TEA); and, U46,619 (thromboxane A₂ analog). Krebs physiological salt solution (PSS) for SMA was (in mM): NaCl, 119; KCl, 4.7; CaCl₂, 2.5; MgCl₂, 1.2; KH₂PO₄, 1.2; NaHCO₃, 24; glucose, 7.0; pH 7.4. Krebs PSS for aorta was (in mM): NaCl, 118; KCl, 4.7; CaCl₂, 2.5; KH₂PO₄, 1.2; MgSO₄, 1.2; NaHCO₃, 12.5; and, glucose, 5.5; pH 7.4. High K⁺ PSS (60K; 60 mM) substituted equimolar K⁺ for Na⁺.

Animals.

Wild type C57BL/6J mice from in house breeding pairs or purchased (The Jackson Laboratory, Bar Harbor, ME) (12-20 weeks old; 25-35g) were used in these studies. Mice were treated according to American Physiological Society *Guiding Principles in the Care and Use of Animals*, and all protocols were approved by University of Louisville Institutional Animal Care and Use Committee. Mice were housed under pathogen-free conditions in the University of Louisville vivarium under controlled temperature and 12h light:12h dark cycle. Mice were provided a standard chow diet (Rodent Diet 5010, 4.5% fat by weight, LabDiet; St. Louis, MO).

Urine Collection and Formate Measurement.

Urine samples were collected, centrifuged (2,500 RPM, 10 min, 4 °C), decanted, and stored at -80 °C until analysis (D. J. Conklin et al., 2017). In brief, each mouse was briefly exposed to D-glucose/saccharin solution (w/v; 3.0%/0.125%; Sigma) immediately prior to a 6h fast after which each was placed singly in a metabolic cage (Harvard Apparatus) with glucose/saccharin solution in drinking water without food to collect urine (in 4°C water-jacketed organ baths) for 3h. In total, mice were fasted for 9-h. After fasted urine collection, mice were provided food along with glucose/saccharin solution for an overnight urine collection (O/N). Urine samples were collected, centrifuged, decanted, and stored at -80 °C (D. J. Conklin et al., 2017).

The primary urinary metabolite of FA is formate and it was measured using GC-MS as described previously (Kage, Kudo, Ikeda, & Ikeda, 2004; Lamarre et al., 2014) with minor modifications. Briefly, urine (50 µL) was mixed with sodium phosphate (20 µL 0.5M, pH 8.0) containing internal standard (¹³C-formate, 2.3 mM). Samples were incubated with PFBBr (130 µL, 0.1M) for 15 min at 60°C, extracted with hexane (330 µL), and analyzed by GC-MS in electron ionization (EI) mode. Six-point calibration curve was used to calculate the concentration of formate. Results were corrected for the natural abundance of ¹³C isotope. Formate level was normalized to urinary creatinine (mg) to account for urine dilution.

Isolated Superior Mesenteric Artery (SMA) and Aorta.

Mice were anesthetized with sodium pentobarbital (0.1 ml, 150 mg/kg, i.p.), and SMA and aorta were removed via mid-ventral thoracotomy. Thoracic aorta rings (3-4 mm) were hung on stainless steel hooks in 15-ml water-jacketed organ

baths and SMA rings (2 mm) were hung on tungsten wire (75 μm dia.) in 5-ml heated organ baths (MultiWire Myograph System 620M, DMT, Denmark) in PSS bubbled with 95% O₂: 5% CO₂ at 37 °C. After 10 min without tension, aorta rings were equilibrated to $\approx 1\text{g}$ loading tension over 30 min and SMA rings were equilibrated to $\approx 0.25\text{g}$ loading tension over 1h. All rings were stimulated with high K⁺ to test for viability, washed 3 times with PSS over 30 min, and re-equilibrated to appropriate resting tension.

FA-Induced Relaxation in Isolated Superior Mesenteric Artery (SMA) and Aorta:

Formaldehyde (30-1,500 μM) was added to organ baths containing either aorta or SMAs pre-contracted with 1 of 3 contractile agonists: phenylephrine (PE, 10 μM), U46,619 (0.1 μM), or high K⁺ (60 mM). The efficacy of FA-induced relaxation was calculated as the % reduction in agonist-induced contraction. The sensitivity of FA-induced relaxation was calculated as the effective concentration producing 50% response (EC₅₀), i.e., cumulative concentration responses were normalized to 100% with interpolation of EC₅₀. To assess whether time of day affected SMA response to FA, mice were euthanized both in day and in night cycle. Sensitivity to a known TRPA1 agonist, cinnamaldehyde (1-100 μM), were conducted, and because cinnamaldehyde is immiscible in aqueous solution, dilutions were vigorously vortexed immediately prior to addition to organ baths.

Effects of Formate (FA metabolite) and Methanol: FA is oxidized to formate (primary urinary metabolite), and thus, we tested whether formate could induce vasorelaxation or vascular toxicity (50 to 1,500 μM) in PE pre-contracted aorta and

SMA. Because formalin (a source of FA) contains 10-15% methanol (as a stabilizer), we also tested whether formalin or methanol alone (to 0.001%, equivalent to amount present in 1,200 μ M formalin) contributed, in part, to FA-induced relaxation.

Role of Endothelium, Nitric Oxide Synthase (NOS), and cGMP:

To evaluate the role of NOS, L-NAME (0.1 mM) was added prior (15 min) to addition of PE (10 μ M), U46,619 (0.1 μ M) or High K^+ (60 mM). Pre-contracted SMA rings were then relaxed with cumulative concentrations of FA (50, 150, and 300 μ M). The role of the endothelium was assessed as vasorelaxation to increasing FA concentrations (50, 150, 300, and 600 μ M) in aorta and in SMA with intact and injured endothelium. The endothelium was mechanically-injured by air perfusion, and effective impairment was confirmed by near complete abolition (>95%) of ACh-induced dilation of PE pre-contracted blood vessels. To assess whether cGMP was involved in FA-induced vasorelaxation, isolated blood vessels were pre-incubated with ODQ (3 μ M) to inhibit guanylyl cyclase (GC) and subsequent formation of cGMP (Jiang, Wan, Wang, & Wu, 2015).

Role of TRPA1 Channel:

The role of the TRPA1 channel was assessed by addition of TRPA1 antagonist (A967079, 1 μ M; 10 min; Cat. #: A12353, AdooQ, Irvine, CA) or DMSO (vehicle) in PE-precontracted aorta and SMA followed by cumulative addition of FA (30-300 μ M).

Role of K^+ Channels:

The role of K⁺-channels in FA-induced responses were interrogated using K_{IR} channel inhibitor (BaCl₂, 1 mM) and the non-specific Kv channel inhibitor (tetraethylammonium, TEA, 10 mM).

Role of nicotine:

Because most cigarette and e-cigarette contains nicotine, we tested whether nicotine would affect FA-induced relaxation in PE-precontracted SMA.

Histology and TRPA1 Immunofluorescence.

Thin sections (4 µm) of formalin-fixed, paraffin-embedded SMA and dorsal root ganglia (DRG; positive control) of WT mice were stained with H&E and TRPA1 antibody (1:200; Alomone Labs, Israel; Cat. #: ACC-037)(N. Green, Weech, & Walters, 2005). Images of SMA sections were made using a digital Spot camera mounted on an Olympus microscope. Immunofluorescence microscopy was performed with rabbit polyclonal antibody against TRPA1 without or with a blocking peptide of the TRPA1 antibody and Alexa Fluor 647 goat anti-rabbit secondary antibody (1:400 dilution; Invitrogen; Cat. #: 21244). To label endothelial cells, sections were co-stained with isolectin GS-IB4 Alexa Fluor 594 conjugate (1:200 dilution; Invitrogen; Cat. #: I21413). Briefly, the slides were deparaffinized, and heat-mediated antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) for 20 min at 95 °C. The slides were cooled to room temperature and then incubated with blocking buffer (10% goat serum, 1% BSA, 0.025% triton X-100 in 1X TBS) for 2h at RT and then incubated with either TRPA antibody in blocking buffer without and with blocking peptide against TRPA1 (the TRPA1 antibody and the

blocking peptide at 1:1 ratio; $\mu\text{g}/\mu\text{g}$) was mixed and pre-incubated for 1h at RT before adding on top of the tissue sections) in blocking buffer overnight at 4° C. The slides were incubated with anti-rabbit Alexa Fluor 647 secondary antibody in the dark for 1h at RT. The slides were washed 3 X 5 min with 1X TBST after each step. Tissue sections incubated with secondary antibody only served as a negative control. Slides were covered with DAPI containing Slow Fade[®] Gold anti-fade reagent (Invitrogen; Cat. #: S36938) and fluorescence was visualized on a Nikon eclipse Ti fluorescence Microscope using NIS-Elements (Nikon; Japan) at 200X. A DAPI filter was used for nuclear staining and a Cy5 Red filter for TRPA1 staining.

TRPA1 qRT-PCR.

Total RNA was isolated from approximately 10 mg of SMA (8 arteries pooled) and DRG (positive control) from WT mice with Trizol (Invitrogen) and RNeasy MiniKit (QIAGEN) according to manufacturer's instructions. RNase-free DNase (QIAGEN; Cat. # 79254) was used to remove any contaminating DNA. RNA was eluted from RNeasy mini-columns into 30 μL of RNase-free water and the concentration measured using NANODROP 2000C (Thermo Scientific). Total cDNA was synthesized using iScript cDNA synthesis Kit (BIO-RAD; Cat. # 170-8891) according to manufacturer's instructions. Briefly, 15 μL denatured RNA (250 ng), 4 μL 5X iScript reaction mix and 1 μL of iScript reverse transcriptase were mixed (total volume, 20 μL) and cDNA synthesis was carried out in a Bio-Rad mycycler Thermocycler using the following conditions: 42 °C for 60 min and 94 °C for 5 min. The resultant cDNA was target amplified using TRPA1 specific primers. (Forward 5'-GAT GCC TTC AGC ACC CCA TTG- 3'; Reverse 5'-CAC

TTT GCG TAA GTA CCA GAG TGG- 3'). Real-time quantitative PCR was done using a murine specific TaqMan gene expression assays. Briefly, the following were added to each well of a 384-well plate sequentially: 5.0 μ L of 2X TaqMan Fast Universal PCR Master Mix (Cat.no. 4440040), 2 μ L undiluted cDNA from target specific PCR and 3 μ L of murine primers for *mTrpa1*, (Mm01227447_m1, Cat. # 4351372) or *β -actin* (Mm00607939_m1, Applied Biosystems, Cat. # 4331182) for TaqMan assays (total = 10 μ L final volume), and then subjected to qRT-PCR using standard protocols on an Applied Biosystems 7900 HT Real-Time PCR system. For each RNA sample, the cDNAs were run in duplicate for each assay set in the same plate along with β -actin. The expression level of TRPA1 in both SMA and DRG are expressed as ct values.

DNA Sequencing and Agarose Gel Electrophoresis.

PCR products from TRPA1 TaqMan assay were run on 1.5% agarose gel and stained with ethidium bromide to visualize the expected 101 bp band in WT SMA and DRG. Also, the PCR product from Target specific TRPA1 amplification (309 bp) of SMA and DRG were sequenced on Illumina NextSeq 500 by the Center for Genetic and Molecular Medicine (CGEMM University of Louisville) sequencing service core.

Statistical Analyses.

Data are expressed as means $\pm$ SE. When comparing two groups, a *t*-test was used (paired or unpaired as appropriate), and statistical significance was assumed where $P < 0.05$ (SigmaPlot, ver. 11).

3. Results

Formate: the primary FA metabolite is increased with feeding.

Formate is the primary metabolite of FA, and formate in the urine is a consequence of endogenous FA metabolism, so we measured urinary formate by GC-MS. We found that feeding significantly increased the urinary levels of formate indicating a major source of endogenous FA is either directly from food or related to feeding, e.g., metabolism of food constituents, intermediary metabolism, etc... In male C57BL/6J mice, a daytime fast (9 h) resulted in a basal level of urinary formate that was tripled with overnight feeding (**Table 5**). A similar pattern occurred in female C57BL/6J mice (data not shown). Thus, we infer that feeding increased FA levels that subsequently increased formate excretion in urine.

FA-induced relaxation.

FA (from 30 μ M to 1.2 mM) induced a robust, repeatable and concentration- and agonist-dependent relaxation in isolated SMA and aorta of both male and female mice (**Fig. 9 A-D; Table 6**). Regardless of agonist (PE, U46,619, high K^+), FA relaxed agonist-induced tension by >90% in SMA and aorta (**Table 7**), yet when added to an uncontracted vessel, FA did not affect basal tone (myogenic tone; data not shown). FA-induced relaxation up to 1.2 mM (SMA) and 1.5 mM (aorta) did not alter subsequent agonist-induced contractions or ACh-induced (endothelium-dependent) relaxation indicating no overt vasotoxicity. Irreversible toxicity was, however, detected following exposure to high levels of FA (>1.2 mM in SMA; >1.5 mM in aorta) with vasotoxicity defined as near loss of contractility

even after multiple bath changes (e.g., 3 washouts, <10% tension production of first agonist-induced contraction; data not shown).

FA: formate and methanol.

Because FA is metabolized to formate, it is possible that vascular effects of FA (and its precursors) are the result of FA metabolism to formate. To address this possibility, formate was cumulatively added up to 2.1 mM in PE pre-contracted SMA. Formate induced a weak relaxation indicating that FA-induced relaxation was not a result of formate production (**Table 7**). Formalin, a mixture of FA (37%) and methanol (10-15% as stabilizer), induces TRPA1-dependent vasodilation and pain (McNamara et al., 2007), yet it is unknown if this is due to either FA or methanol (or both). Because FA also is a product of methanol metabolism, we tested whether methanol alone (at levels comparable with those present in formalin) had any effect in PE pre-contracted SMA. Methanol also induced a weak, modestly concentration-dependent relaxation in SMA. Thus, neither formate nor methanol appears responsible for the efficacy of FA- or formalin-induced relaxations, respectively. These data also implicate real time metabolism of FA (to formate) as a relevant vaso-regulatory step because it converts an active agent (FA) into an inactive metabolite (formate). Thus, alterations in FA metabolism in the vascular wall may be a targetable process to promote or diminish feeding-induced hyperemia.

Role of endothelium, nitric oxide synthase (NOS), and GC in FA-induced vasorelaxation.

To investigate the mechanism of the most sensitive component of the FA-induced relaxation, we used PE-precontracted SMA (PE<U46,619<60K). Because the SMA has a robust ACh-induced, endothelium-dependent relaxation that was impaired significantly by air perfusion (**Fig. 10**), we tested for the role of endothelium in FA-induced relaxation. Air perfusion significantly shifted FA-induced relaxation rightward in SMA (**Fig. 11 A, B**). Similarly, the sensitivity (EC_{50}) but not the efficacy of FA-induced vasorelaxation in PE-pre-contracted SMA was affected by L-NAME (100 μ M) (**Fig. 11 C, D**). However, L-NAME had no effect on sensitivity or efficacy of FA-induced relaxation in either U46,619 (514 ± 129 vs 569 ± 98 μ M) or High- K^+ ($1,093 \pm 87$ vs 984 ± 92 μ M) pre-contracted SMA (**Fig. 10 C, D**). The rightward shift due to L-NAME indicated a role of NO (e.g., eNOS) as part of the most sensitive component of the FA-induced relaxation in PE-precontracted SMA. Because NO stimulates guanylyl cyclase (GC) to generate cGMP in smooth muscle cells, we tested ODC, an irreversible inhibitor of GC, and ODC also rightward shifted the FA-induced relaxation (**Fig. 11 E, F**). The endothelium contributes to overall contractility in SMA (**Fig. 10 B, E**). Collectively, these data show that the most sensitive component of the FA-induced relaxation is dependent on a functional endothelium, NOS and an NO-mediated activation of GC.

Role of TRPA1 channel.

Because formalin-induced pain is associated with activation of TRPA1 channel as well as an increase in blood flow (McNamara et al., 2007), we hypothesized that the TRPA1 channel may contribute to the FA-induced, endothelium-dependent relaxation in SMA. In support, the TRPA1 antagonist

A967079 (1 μ M) significantly inhibited the most sensitive portion of the FA-induced relaxation (30 μ M) in PE-contracted SMA (**Fig. 12 A, B**). In paraffin-embedded, thin cut sections of mouse SMA, TRPA1 (green) was co-localized with isolectin (red) in endothelium (**Fig. 12 C**). More modest yet specific TRPA1 staining was present in SMA media and adventitia as well. Positive and selective TRPA1 staining was confirmed by inclusion of a blocking peptide and by using dorsal root ganglion (DRG; **Fig. 12 D**) as a positive control. A PCR product specific to TRPA1 exons 23-25 mRNA (**Fig. 12 Ei**) matched both the predicted fragment size (101 bp; **Fig. 12 Eii**) and by gene sequence (**Fig. 12 Eiii**) -- confirming TRPA1 presence in SMA. These image and molecular data support that TRPA1 contributed to the FA-induced, endothelium-dependent relaxation in SMA.

Cinnamaldehyde is a known TRPA1 agonist, and, unsurprisingly, it induced a concentration- and TRPA1-dependent relaxation in SMA (**Fig. 13 A**). This relaxation, like FA, this sensitive cinnamaldehyde-induced relaxation in SMA was dependent on a functional endothelium (**Fig. 13 B**), TRPA1 (**Fig. 13 C**), NOS (**Fig. 13 D**) and GC (**Fig. 13 E**).

Role of K⁺-channels.

In SMA pre-contracted with High K⁺ (60mM), FA induced an insensitive (rightward-shifted) relaxation (compared with both PE and U46,619). From these data, we surmised that alteration of smooth muscle membrane potential, likely due to K⁺ channel activation, contributed to FA-induced vasorelaxation in PE-contracted SMA. In confirmation, BaCl₂ (1 mM), which blocks inward rectifier K⁺ channels, significantly inhibited FA-induced relaxation in PE-contracted SMA (**Fig.**

14 A, B) and also in aorta (**Fig. 14 C**). However, we observed no effect of the non-selective K⁺ channel inhibitor, TEA (tetraethylammonium, 10 mM), on FA-induced relaxation in SMA (**Tables 7, 8**). Collectively, these results indicate that the activation of K_{IR} channels but not of other K⁺ channels was involved in FA-induced vasorelaxation in PE-precontracted SMA. The endothelium also contributed to FA-induced relaxation in aorta (**Fig. 14 D**)

Nicotine and FA:

In tobacco users, FA may be present in blood simultaneously with nicotine, so we also tested whether nicotine altered FA-induced relaxation in SMA. Nicotine alone (1 µM) had no effect in PE-contracted SMA, and also had no effect on subsequent FA-induced vasorelaxation.

Fig. 9. Formaldehyde (FA) induced relaxation in superior mesenteric artery (SMA). **A)** Representative traces of concentration-dependent relaxation induced by cumulative addition of FA (50-1,200 μ M) in isolated SMA contracted by one of three different contractile agonists: phenylephrine (PE); thromboxane A₂ analog (U46,619); or, high potassium (60K). **B)** Summary data of the efficacy (% relaxation) of FA-induced relaxation in isolated pre-contracted SMA. Summary data of the efficacy (% relaxation; **C)** and of the sensitivity (**D)** of FA-induced relaxation in isolated PE-precontracted SMA from male and female mice isolated either during day time or night time. Values are means $\pm$ SE of 3-10 preparations.

Table 5. Levels of urinary formate following either a daytime fast or overnight feeding in male C57BL/6J mice.

Urine Metabolite	Condition	
	Daytime Fast (9h, no food, 3h water)	Overnight Feeding (16h, food & water)
Formate (μg/ml)	0.72±0.03	2.73±0.95*
Creatinine (mg/dl)	6.88±0.32	7.23±0.66
Formate (μg/mg creatinine)	10.54±0.74	36.82±10.30*

Values = means ± SE. N = 3-5 per group. *, P<0.05 vs fasting.

Table 6. The effective concentration inducing 50% relaxation (EC_{50} ; in μM) of formaldehyde-induced vasorelaxation in isolated murine superior mesenteric artery (SMA; male and female; day and night) and thoracic aorta precontracted with different agonists.

Blood Vessel (sex, time of day)	EC_{50} by precontraction agonist		
	PE	U46,619	High K^+
SMA (male, day)	53 $\pm$ 3	514 $\pm$ 129*	1,093 $\pm$ 87*
SMA (male, night)	43 $\pm$ 2	--	--
SMA (female, day)	77 $\pm$ 9	--	--
Aorta (male, day)	261 $\pm$ 22*	--	--

Values = means $\pm$ SE. Abbr.: PE, phenylephrine, [10 μM]; U46,619, thromboxane A_2 analog, [0.1 μM]; High K^+ , high potassium solution, [60 mM K^+]; --, did not perform experiment. N=3-10 per group. *, $P < 0.05$ vs EC_{50} of "SMA (male, day)" PE agonist (i.e., baseline control).

Table 7. Efficacy of formaldehyde and associated compounds in relaxation of PE-precontracted superior mesenteric artery (SMA) and aorta without and with selective treatments (inhibitors).

Compounds	[mM]		Treatment	% Relaxation	
	SMA	Aorta		SMA	Aorta
Formaldehyde	0.3	0.6	-	-94.3±1.3	-99.6±1.2
Formate	2.1	-	-	-25.7±6.7	-
Methanol	2.1	-	-	-34.1±11.7	-
Formaldehyde	0.3	0.6	ED	-90.4±2.3	-94.0±2.6
Formaldehyde	0.6	-	+LNAME	-96.7±2.2	-
Formaldehyde	0.3	0.6	+A967079	-96.4±1.1	-100.7±1.2
Formaldehyde	0.6	-	+ODQ	-99.7±0.5	-
Formaldehyde	0.6	0.6	+BaCl ₂	-99.5±0.5	-81.7±3.7
Formaldehyde	0.2	0.6	+TEA	-90±1.8	-94.4±1.3

Values = means ± SE. Abbr.: PE, phenylephrine, 10 µM; ED, endothelial dysfunction induced by mechanical air perfusion; LNAME, N^ω-nitro-L-arginine methyl ester hydrochloride, 100 µM; A967079, TRPA1 antagonist, 3 µM; ODQ, 1h-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, BaCl₂, barium chloride, 1 mM; TEA, tetraethylammonium. N=3-6 per group.

Fig. 10. Regulators of agonist-induced tension in superior mesenteric artery (SMA). **A)** Phenylephrine (PE), U46,619- and 60K-induced tension (mN) in SMA in the absence and presence of LNAME. **B)** FA-induced relaxations (%) in PE, U46,619- and 60K-precontracted SMA in the absence and presence of LNAME. **C)** FA-induced relaxations (converted to 100 %) in PE, U46,619- and 60K-precontracted SMA in the absence and presence of LNAME. **D)** Representative tracings of PE-induced tension and ACh-induced relaxation in SMA with intact and mechanically-impaired endothelium (ED). **E)** Summary data of PE-induced tension in SMA in the absence and presence of LNAME, BaCl₂, ED, and ODQ. Values are means $\pm$ SE of 3-4 preparations. *, $P < 0.05$ vs Control.

Fig. 11. Role of the endothelium and nitric oxide (NO) in the most sensitive component of formaldehyde-induced relaxation of phenylephrine (PE) pre-contracted superior mesenteric artery (SMA). **A)** Representative traces of FA-stimulated relaxation of PE-induced contraction without and with endothelium impairment due to 5 min of air perfusion. **B)** Summary data of FA-induced relaxation in isolated SMA pre-contracted with PE in the presence and absence of functional endothelium. **C)** Representative traces of FA-stimulated relaxation of PE-induced contraction in the presence and absence of LNAME. **D)** Summary data of FA-induced relaxation in isolated SMA pre-contracted with PE in the absence and presence of LNAME. **E)** Representative traces of FA-stimulated relaxation of PE-induced contraction in the presence and absence of the guanylyl cyclase (GC) antagonist, ODQ (3 μ M), added to bath after PE-induced contraction plateaued and prior to cumulative addition of FA. **F)** Summary data of FA-induced relaxation in isolated SMA pre-contracted with PE in the absence and presence of ODQ. Values are means $\pm$ SE of 3-4 preparations. *, $P < 0.05$ vs Control.

Fig. 12. Role of the TRPA1 channel in formaldehyde (FA)-induced relaxation in phenylephrine (PE) pre-contracted superior mesenteric artery (SMA). **A)** Representative traces of FA-stimulated relaxation of PE pre-contracted SMA in the absence (Control) and presence of the TRPA1 antagonist, A967079 (3 μ M), which was added after PE-induced contraction plateaued and prior to cumulative addition of FA in wild type (WT) SMA. **B)** Summary data of FA-induced relaxation in isolated SMA pre-contracted with PE in the absence and presence of TRPA1 antagonist. Immunofluorescence localization of TRPA1 in SMA (**C**) and in dorsal root ganglion (DRG; **D**). Formalin-fixed and paraffin-embedded sections of murine organs were stained with H&E (**Ci, Di**); TRPA1 only (green; **Cii**) or DAPI only (blue, nuclear stain; **Dii**); TRPA1 antibody (green), isolectin (red, endothelium) and DAPI (**Ciii**) or TRPA1 antibody (red) and DAPI (**Diii**); and, TRPA1 antibody, TRPA1 blocking peptide, isolectin and DAPI (**Civ**) or TRPA1 antibody, TRPA1 blocking peptide, and DAPI (**Div**). L, lumen of SMA. All images were at 200x magnification (scale bar = 100 μ m). **Ei)** Schematic depicting mouse *mTrpa1* gene exons (E) 23 to 25 (with introns, I) and their length (in base pairs, bp). TRPA1 mRNA with the spliced exons 23 to 25 makes a 309 bp product as indicated by TRPA1 specific primers used. **Eii)** Agarose gel electrophoresis of PCR amplified product from 309 bp cDNA by qRT-PCR (101 bp product from SMA or DRG of WT mouse using TaqMan Assay for TRPA1; ct values indicated). **Eiii)** DNA sequence of 309 bp PCR product identified as TRPA1. Sequence of DRG PCR product (309 bp) was used as validation (data not shown). Values are means $\pm$ SE of 3-4 preparations. *, $P < 0.05$ vs Control.

Fig. 13. Role of the endothelium and nitric oxide (NO) in the most sensitive component of cinnamaldehyde-induced (CA) relaxation of phenylephrine (PE) pre-contracted superior mesenteric artery (SMA). **A)** Representative traces of concentration-dependent CA-stimulated relaxation of PE-induced contraction. **B)** Summary data of CA-induced relaxation in isolated SMA pre-contracted with PE in the presence and absence of functional endothelium. **C)** Summary data of CA-induced relaxation in isolated SMA pre-contracted with PE in the absence and presence of TRPA1 antagonist. **D)** Summary data of CA-induced relaxation in isolated SMA pre-contracted with PE in the absence and presence of L-NAME. **E)** Summary data of CA-stimulated relaxation of PE-induced contraction in the presence and absence of the guanylyl cyclase (GC) antagonist, ODQ (3 μ M), added to bath after PE-induced contraction plateaued and prior to cumulative addition of CA. Values are means $\pm$ SE of 3-4 preparations. *, $P < 0.05$ vs Control.

Fig. 14. Role of potassium channel (K_{IR}) in formaldehyde (FA)-induced relaxation in phenylephrine (PE) pre-contracted blood vessels. **A)** Representative and **B)** summary curves of FA-induced relaxation in superior mesenteric artery (SMA) in the absence and presence of $BaCl_2$. Summary curves of FA-induced relaxation in aorta in the absence and presence of **C)** $BaCl_2$ or **D)** endothelium damage (ED). $BaCl_2$ (1 mM) was added to PSS after stable PE-induced contraction and prior to cumulative addition of FA. ED was induced by 5 min of air perfusion. Values are means $\pm$ SE of 3-4 preparations. *, $P < 0.05$ vs Control.

Table 8. The sensitivity of formaldehyde-induced vasorelaxation measured as the effective concentration inducing 50% relaxation (EC_{50} ; in μM) in isolated murine superior mesenteric artery (SMA; male) pre-contracted with PE in the absence and presence of functional endothelium and selective inhibitors of mediators of vasorelaxation.

SMA (+treatment)	EC_{50} (Control, PE+vehicle)	EC_{50} (ED or PE+inhibitor)
+ED	61 $\pm$ 8	142 $\pm$ 14*
+LNAME	52 $\pm$ 8	207 $\pm$ 10*
+A967079	52 $\pm$ 6	95 $\pm$ 18*
+ODQ	76 $\pm$ 15	182 $\pm$ 16*
+BaCl ₂	63 $\pm$ 10	248 $\pm$ 56*
+TEA	75 $\pm$ 3	93 $\pm$ 15

Values = means $\pm$ SE. Abbr.: PE, phenylephrine, 10 μM ; ED, endothelial dysfunction induced by mechanical air perfusion; LNAME, N^o-nitro-L-arginine methyl ester hydrochloride, 100 μM ; A967079, TRPA1 antagonist, 3 μM ; ODQ, 1h-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, BaCl₂, barium chloride, 1 mM; TEA, tetraethylammonium. N=3-6 per group. *, P<0.05 vs Control (PE+vehicle).

4. Discussion

To our knowledge, this is the first study to propose that FA may contribute to postprandial hyperemia via a TRPA1- an endothelium-mediated SMA vasodilation. Because rodents feed at night and sleep (fast) during the day, we measured and observed a circadian difference in urinary formate levels indicating endogenous FA level likely increases with feeding. As far as we know, this is a novel observation. Furthermore, we show that SMA is sensitive to FA significantly more so than aorta, and that FA not its metabolite, formate, stimulates a robust and reversible vasorelaxation. The SMA is a major arterial blood supply to the GI tract and it dilates postprandially in response to many stimuli e.g., nutrients, vago-vagal reflex, NO, substance P, etc... (Chou & Coatney, 1994; Lucchini, Saumet, Mei, & Garnier, 1996). Although FA is oft considered a toxicant, its natural abundance in food, water and beverages (Committee on, 1981; D. J. Conklin, P. Haberzettl, et al., 2011) and as an abundant metabolic product make it a plausible stimulus of GI blood flow. As this is the first suggestion of this physiological role, more research will be needed to establish FA as a *bona fide* contributor to postprandial hyperemia *in vivo*.

We show that there are 3 distinct pathways of FA-induced vasorelaxation in SMA (**Fig. 15**) and while this is fascinating in its own right, we focused on the mechanism of the most sensitive, and thus, most likely physiological pathway. FA-induced vasorelaxation requires a functional endothelium, TRPA1 channels, NOS (presumably eNOS), guanylyl cyclase (GC/cGMP) and K_{IR} channels. Likely, the TRPA1 and NOS components are in endothelial cells, whereas the latter two

components are likely present in vascular smooth muscle cells (VSMC). From these collective data, we propose a model featuring a stepwise sequence of events that is quite typical of agonists that elicit EDRF/NO such as ACh (see model in **Fig. 15**). That FA activates a TRPA1 channel likely present on the SMA endothelium is paralleled by FA action in rat aorta (Q. Zhang et al., 2018) and by systemically administered propofol – a general anesthetic that induces TRPA1-dependent hypotension (Sinha, Sinharoy, Bratz, & Damron, 2015). Similarly, TRPA1 is a known target of formalin (solution of FA and methanol) that increases blood flow and pain (McNamara et al., 2007). With regard to formalin, we show that it is likely the FA in formalin and not the methanol, which can be metabolized to FA, that elicits vasorelaxation.

TRPA1 is a well-known target of unsaturated aldehydes, such as acrolein and crotonaldehyde (Andre et al., 2008; Bessac et al., 2008; D. J. Conklin, 2016) and cinnamaldehyde (Pozsgai et al., 2010), but its activation by FA is somewhat surprising given the proposed mechanism of unsaturated aldehyde-mediated activation via conjugation with TRPA1 N-terminal free cysteines (Macpherson et al., 2007). In addition, several unsaturated aldehydes including acrolein, cinnamaldehyde and 4HNE induce sensitive and robust vasodilation in mesenteric arteries (Awe, Adeagbo, D'Souza, Bhatnagar, & Conklin, 2006; Romero et al., 1997) and other blood vessels (Yanaga et al., 2006) and smooth muscle (Cheah et al., 2014) indicating perhaps a shared (universal) aldehyde-TRPA1 pathway.

In any case, TRPA1 is a cation (calcium) channel and a promiscuous receptor concentrated in sensory fibers (unmyelinated C-fibers) that mediate pain

reception/transmission (nociceptors), and it is also present in vasculature (Earley et al., 2009a; Sinharoy et al., 2017). Activation of peripheral sensory fiber TRPA1 leads to pain signaling and release of vasoactive peptides, substance P (SubP) and cGRP (Trevisan et al., 2016), which may contribute to increased vasodilation. We confirm localization of TRPA1 in mouse DRG and show specific staining in the endothelium of murine SMA but not in aorta. Functionally, the specific TRPA1 antagonist, A967079 ($IC_{50} = 289$ nM), significantly blocks *the most sensitive component* of the FA-induced relaxation and shifts the EC_{50} to the right quantitatively similar to that of endothelium disruption but less so than LNAME treatment -- further supporting a role of endothelial cell TRPA1. Above 150 μ M FA -- likely an *unphysiological* level of FA -- the TRPA1 antagonist did not affect FA relaxation reflecting a non-TRPA1 pathway(s), yet perhaps via TRPV1 or TRPV4 channels (Ma et al., 2008). We cannot rule out the possibility that additional components in the vascular wall (perivascular nerves, SubP, CGRP, etc...) also may contribute to overall FA-induced relaxation (Nilius, Appendino, & Owsianik, 2012).

FA-induced vasorelaxation is well-documented for over 4 decades, yet the complex mechanisms of this phenomenon are only now becoming clearer. In contrast to the idea that FA induces endothelial dysfunction (Yu & Deng, 1998), our data indicate that endothelial dysfunction itself likely would block only the physiological (sensitive) component of FA-induced vasorelaxation. We did not observe evidence of FA-induced endothelial dysfunction even after exposing SMA to 1,000 μ M FA. Moreover, in isolated human CABG blood vessels with endothelial

dysfunction, FA still induces relaxation but in a far less sensitive manner (EC_{50} , $315 \pm 0 \mu M$) (D. J. Conklin et al., 2004) than that present in healthy mice ($\approx 65 \mu M$; this study).

It is clear that the vascular effects of FA *ex vivo* are not a result of FA metabolism into formate (primary metabolite). For example, formate addition (up to 2.1 mM) fails to evoke a strong vascular response, and thus, oxidative metabolism of FA regulates FA levels and subsequently any FA-evoked physiological vascular responses. Similarly, formate fails to recapitulate formalin-induced vasodepressor response in rats *in vivo* (Strubelt et al., 1990). Because FA metabolism involves a reduced glutathione (GSH)- and FALDH-dependent (aka ADH3) hemi-thioacetal formation (Kaiser, Holmquist, Vallee, & Jornvall, 1991), this process may be more affected by changes in oxidant load and GSH depletion in the vascular wall or in neurons, for example (Materazzi et al., 2012). As shown previously, deficiency in glutathione-dependent metabolism of acrolein (i.e., GSTP-null mice) increases susceptibility to acrolein-induced endothelial dysfunction *in vivo* and *in vitro* (D. J. Conklin, Haberzettl, et al., 2009a). Whether or not FALDH deficiency or GSH depletion likewise alters vascular sensitivity to FA-induced relaxation (or toxicity) remains to be tested.

In conclusion, we describe a sensitive mechanism of FA-induced vasorelaxation that is sequentially dependent on an endothelium-localized TRPA1 receptor (EDRF/NO) and a VSMC GC/PKG/ K_{IR} pathway in SMA. We hypothesize that this sensitive pathway is only a part of the postprandial hyperemia reflex that increases blood flow to the GI tract to augment digestion and nutrient absorption.

Fig. 15. A cartoon depicting the mechanisms by which formaldehyde (FA) induces vasorelaxation in SMA precontracted by phenylephrine (PE), thromboxane A₂ analog (U46,619) and 60 mM potassium solution (60K). FA appeared to induce a sensitive and sequential mechanism that begins with opening of Transient Receptor Potential Ankyrin 1 (TRPA1) cation channels, Ca²⁺ entry into endothelial cell, eNOS activation, NO formation, GC activation and cGMP formation with Protein Kinase G (PKG) mediated opening of K_{IR} channels leading to VSMC hyperpolarization and relaxation. Several of these steps were significantly impaired by a selective antagonist or treatment, including TRPA1 (A967079), eNOS (LNAME), endothelium (impaired by air perfusion), guanylyl cyclase (GC; ODQ) and K_{IR} channel (BaCl₂). As demonstrated, this sequential pathway was sensitive to exogenous FA (30-150 μM) in the physiological range that was altered at least 2-3x during feeding. Other less sensitive yet equally efficacious pathways of vasorelaxation included VSMC hyperpolarization and a voltage-insensitive mechanism that reversed 60K-induced contraction implicating closure of VSMC Ca²⁺ channels.

CHAPTER IV

ACETALDEHYDE INDUCES AN ENDOTHELIUM-DEPENDENT RELAXATION OF SUPERIOR MESENTERIC ARTERY: POTENTIAL ROLE IN POSTPRANDIAL HYPERMIA

Acetaldehyde (AA) is a small, ubiquitous compound present in foods, beverages, as a gas phase combustion product, and also endogenously-generated from metabolism as from ethanol (EtOH). Acetate is a short chain fatty acid derived from AA oxidation, and acetate levels were significantly higher in urine collected overnight with food provided ad libitum compared with urine collected after 9h fasting. Because feeding increases gastrointestinal blood flow, we explored the direct effects of AA (and acetate) in isolated murine superior mesenteric artery (SMA). Over the concentration range of 1-100 mM, AA strongly and reversibly relaxed agonist-induced contractions of SMA including phenylephrine (PE), thromboxane A₂ analog (U46,619) and high potassium (High K⁺) without toxicity. The sensitivity (EC₅₀) but not the efficacy (>90% relaxation of PE-precontraction) of AA-induced relaxations was dependent on blood vessel (SMA was 3x more sensitive than aorta) and contractile agonist (PE EC₅₀=3.3±0.4 mM; U46,619 EC₅₀ =14.9±1.5 mM; High K⁺ EC₅₀ = 17.7±0.5 mM) yet

independent of circadian cycle and sex. The most sensitive component of the AA-induced relaxation was inhibited significantly by: 1) mechanically-impaired endothelium; 2) nitric oxide synthase (NOS) inhibitor (L-NAME); and, 3) a guanylyl cyclase (GC) inhibitor (ODQ). Both acetate and EtOH stimulated much weaker relaxations in SMA than did AA, yet these relaxations were significantly inhibited by L-NAME as well. Neither EtOH nor acetate relaxed pre-contracted aorta. Although neither cyanamide, a non-specific aldehyde dehydrogenase (ALDH) enzyme inhibitor, nor Alda-1, a specific activator of ALDH2 activity, had any effect on either sensitivity or efficacy of AA-induced relaxation in SMA, cyanamide significantly blocked both EtOH- and acetate-induced relaxations in SMA implicating a role of ALDH activity in vasorelaxation. These data show that AA relaxes SMA via an endothelium- and NO-dependent mechanism supporting the idea that AA may contribute to the physiological reflex of post-prandial hyperemia via vasodilatation of mesenteric vasculature.

1. Introduction

Acetaldehyde (AA) is the second smallest aldehyde, and the concentration of AA in the blood is typically low due to high levels of aldehyde dehydrogenase (ALDH) activity throughout the body. AA is present in food and beverages and is from ethanol (EtOH) metabolism, which can be a major exogenous source of systemic AA and AA may also enter via inhalation of vehicle exhaust-derived aerosols (D. J. Conklin, P. Haberzettl, et al., 2011; Ogunwale et al., 2017). Typically, AA levels range in the blood from 1-100 μ M, and can reach >1 mM with

excessive alcohol ingestion especially in individuals with an aldehyde dehydrogenase 2, ALDH2*2 mutation, e.g., common in SE Asians (Tsukamoto et al., 1991; Vasiliou & Nebert, 2005). Blood levels of AA are constantly changing due to metabolism and exposures (eg., feeding and inhalation) (Hobara et al., 1985) and thus, what physiological role AA plays in vascular control has remained uncertain.

A variety of direct effects of exogenous AA on cardiovascular targets have been described, yet a distinct role of AA in cardiovascular physiology is missing. For example, intra-arterial or intravenous AA reduces heart rate (bradycardia), decreases arterial pressure (hypotension) and causes arrhythmias in animals, but these are considered toxicological effects due to exceedingly high doses administered directly into the blood (Egle, Hudgins, & Lai, 1973; M. A. Green & Egle, 1983). Nonetheless, AA is ubiquitous, and, it is thought that AA generated from EtOH contributes to alcohol-induced cardiomyopathy (Ren & Brown, 2000; Tan et al., 2012). Recently, it was shown that perfusion of a low level of AA (50 μ M) into an isolated rat heart induces a cardioprotective effect against ischemia-reperfusion indicating that endogenous AA levels likely are bioactive (Ueta et al., 2018). It is well-known that AA induces concentration-dependent relaxation in many different isolated blood vessels (Altura & Altura, 1982, 1987), yet the relevance of this relaxation to vascular physiology is unclear. Yet, AA is considered relevant to the well-known phenomenon of EtOH-induced 'flushing' (increased blood flow and redness in facial skin flushing) in people with an

ALDH2*2 mutation that reduces ALDH2 activity, and thus, limits the rate of metabolism of AA to acetate (Scarino, Tardif, & Charbonneau, 2009).

Despite myriad sources and well-known cardiovascular effects of AA, few studies address the physiological role of AA in vascular control. To address this gap in knowledge, we measured urine levels of acetate, the primary metabolite of AA, to assess fluctuations in endogenous AA levels in mice. Urine levels of acetate increased $\approx$ 2-3 times overnight after feeding compared with urine levels during a daytime fast, and thus, it appeared that feeding increased endogenous AA levels (directly or indirectly). The gastrointestinal vasculature, thus, represents a likely target of AA levels after feeding when gut blood flow reflexively increases (Chou & Coatney, 1994; Takagi et al., 1988). To test this idea, isolated superior mesenteric artery (SMA) and aorta were exposed to AA across a physiological to supra-physiological range (1-100 mM). The most sensitive AA-induced relaxation was in the SMA (1-30 mM), and it was dependent on: a functional endothelium, NO formation, and guanylyl cyclase activation. Because EtOH is metabolized to AA by alcohol dehydrogenases, and AA is metabolized by ALDH to acetate, the vascular effects of EtOH and acetate in the absence and presence of an ALDH inhibitor (cyanamide) and ALDH2 activator (Alda1) were also assessed. EtOH and acetate (up to 30 mM) elicited less robust relaxations (less than 50% of AA-induced relaxation), yet these relaxations were NO- and ALDH-dependent in SMA indicating intrinsic overlap with AA's mechanism of action. For many reasons, tobacco and EtOH are often consumed together, so effect of nicotine on AA-induced relaxation also was tested (Abreu-Villaca, Manhaes, Krahe, Filgueiras, &

Ribeiro-Carvalho, 2017). Collectively, these findings support the idea that increased levels of AA due to feeding (and associated drinking of EtOH) likely contribute to reflex-driven increased gastrointestinal blood flow after feeding (aka 'postprandial hyperemia') through a sensitive, robust, reversible, endothelium- and NO-dependent process.

2. Material and Methods

Chemicals and Solutions.

Reagent grade chemicals were purchased from Sigma-Aldrich or other commercial sources as indicated: A967079 (AdooQ); acetaldehyde; acetate; acetylcholine chloride (ACh); Alda1 and cyanamide (gift of Dr. B.G. Hill, Univ. Louisville); 1h-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ); ethanol (100%); N^ω-nitro-L-arginine methyl ester hydrochloride (L-NAME); L-phenylephrine hydrochloride (PE); sodium nitroprusside (SNP); U46,619 (thromboxane A₂ analog); and, 2,3,4,5-pentafluorobenzyle bromide (PFBBBr).

Krebs physiological salt solution (PSS) for SMA was (in mM): NaCl, 119; KCl, 4.7; MgCl₂, 1.2; KH₂PO₄, 1.2; NaHCO₃, 24; glucose, 7.0; pH 7.4. Krebs PSS for aorta was (in mM): NaCl 118, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 12.5, and glucose 5.5; pH 7.4. High K⁺ PSS (High K; 60 mM) was prepared by substituting equimolar K⁺ for Na⁺.

Animals.

Wild type C57BL/6J mice from in house breeding pairs or purchased (The Jackson Laboratory, Bar Harbor, ME) (12-20 weeks old; 25-35g) were used in these studies. Aldose reductase (AR) null mice were from a breeding colony at Univ. of Louisville (Wetzelberger et al., 2010). Mice were treated according to American Physiological Society *Guiding Principles in the Care and Use of Animals*, and all protocols were approved by University of Louisville Institutional Animal Care and Use Committee. Mice were housed under pathogen-free conditions in the Univ. of Louisville vivarium under controlled temperature and 12h light:12h dark cycle. Mice were provided a standard chow diet (Rodent Diet 5010, 4.5% fat by weight, LabDiet; St. Louis, MO).

Urine Collection and Acetate Measurement.

Urine samples were collected, centrifuged (2,500 RPM, 10 min, 4 °C), decanted, and stored at -80 °C until analysis (D. J. Conklin et al., 2017). Each mouse was held (<5 sec) and the mouth was gently touched with the sipper tube of a water bottle filled with D-glucose/saccharin solution (w/v; 3.0%/0.125%; Sigma) immediately prior to a 6h fast. After 6h fast, each mouse was placed in a metabolic cage (Harvard Apparatus) with *ab libitum* access to glucose/saccharin solution in water without access to food for 3h to collect urine (in 4°C water-jacketed organ baths). In total, mice were fasted during daytime for 9-h. After fasted urine collection, mice then were provided food and glucose/saccharin solution *ab libitum* for an overnight urine collection (O/N). For urine samples, volumes were recorded, samples centrifuged to sediment food/feces, decanted, and stored at -80 °C until analysis (D. J. Conklin et al., 2017).

The primary urinary metabolite of AA is acetate, and it was measured using GC-MS as described previously (Kage et al., 2004; Lamarre et al., 2014) with minor modifications (Conklin et al., 2018). Briefly, urine (50 μ L) was mixed with sodium phosphate (20 μ L 0.5M, pH 8.0) containing internal standard (^{13}C -acetate, 2.3 mM). Samples were incubated with PFBBr (130 μ L, 0.1M) for 15 min at 60°C, extracted with hexane (330 μ L), and analyzed by GC-MS in electron ionization (EI) mode. Six-point calibration curve was used to calculate the concentration of acetate. Results were corrected for the natural abundance of ^{13}C isotope. Acetate level was normalized to urinary creatinine (mg) to account for urine dilution (Lorkiewicz et al., 2019).

Isolated Superior Mesenteric Artery (SMA) and Aorta.

Mice were anesthetized with sodium pentobarbital (0.1 ml, 150 mg/kg, i.p.), and SMA and aorta were removed via mid-ventral thoracotomy. Thoracic aorta rings (3-4 mm) were hung on stainless steel hooks in 15-ml water-jacketed organ baths and SMA rings (2 mm) were hung on tungsten wire (75 μ m dia.) in 5-ml heated organ baths (MultiWire Myograph System 620M, DMT, Denmark) in PSS bubbled with 95% O_2 : 5% CO_2 at 37 °C. After 10 min without tension, aorta rings were equilibrated to $\approx$ 1g loading tension over 30 min and SMA rings were equilibrated to $\approx$ 0.25g loading tension over 1h. All rings were stimulated with 60K to test for viability, washed 3 times with PSS over 30 min, and re-equilibrated to appropriate resting tension (Jin, Jagatheesan, et al., 2019).

AA-Induced Relaxation in Isolated Superior Mesenteric Artery (SMA) and Aorta:

Acetaldehyde (1-100 mM) was added to organ baths containing either aorta or SMAs pre-contracted with 1 of 3 contractile agonists: phenylephrine (PE, 10 μ M), U46,619 (0.1 μ M), or High K^+ (60 mM). The efficacy of AA-induced relaxation was calculated as the maximal % reduction in agonist-induced contraction (E_{max}). The sensitivity of agonist-induced relaxation was calculated as the effective concentration producing 50% response (EC_{50}), i.e., cumulative concentration responses were normalized to 100% with interpolation of EC_{50} (D. J. Conklin, Haberzettl, et al., 2009b). To assess whether time of day affected SMA response to AA, mice were euthanized both in day and in night cycle (Jin, Jagatheesan, et al., 2019).

Role of AA Metabolism and Effects of EtOH and Acetate (AA metabolite):

Because EtOH is a known source of AA, we tested whether EtOH alone (up to 1%, equivalent to amount present in 100 mM AA) mimicked AA-induced relaxation. Because AA is oxidized to acetate (primary urinary metabolite), we tested whether acetate (1-100 mM) alone could induce vasorelaxation in PE pre-contracted aorta and SMA. To test the specific role of ALDH to AA-induced vasorelaxation, we used a non-specific ALDH enzyme inhibitor (cyanamide, 1 mM) and an activator of ALDH2 (Alda-1, 25 μ M) (Chen et al., 2008; Srivastava et al., 2001). In addition, to test for role of acetaldehyde reductive metabolism, SMA and aorta were isolated from aldose reductase (AR) null mice.

Role of Endothelium, Nitric Oxide Synthase (NOS), and cGMP:

To evaluate the role of NOS, L-NAME (0.1 mM) was added prior (15 min) to addition of PE (10 μ M), U46,619 (0.1 μ M) or High K⁺ (60 mM). Pre-contracted SMA rings were then relaxed with cumulative concentrations of AA. The role of the endothelium was assessed as vasorelaxation to increasing AA concentrations in aorta and in SMA with intact and injured endothelium. The endothelium was mechanically-injured by air perfusion, and effective impairment was confirmed by near complete abolition (>95%) of ACh-induced dilation of PE pre-contracted blood vessels (Jin, Jagatheesan, et al., 2019). To assess whether cGMP was involved in AA-induced vasorelaxation, isolated blood vessels were pre-incubated with ODQ (3 μ M) to inhibit guanylyl cyclase (GC) and subsequent formation of cGMP (Jiang et al., 2015).

Role of Transient Receptor Potential Ankyrin 1 (TRPA1):

Because formaldehyde induces a sensitive TRPA1-dependent relaxation in isolated SMA, we tested whether the TRPA1 antagonist (A967079, 3 μ M) would alter AA-induced relaxation in PE-precontracted SMA (Jin, Jagatheesan, et al., 2019).

Role of nicotine:

Because most cigarette and e-cigarette contains nicotine, we tested whether nicotine would affect AA-induced relaxation in PE-precontracted SMA.

Statistical Analyses.

Data are expressed as means $\pm$ SE. When comparing two groups, a *t*-test was used (paired or unpaired as appropriate). Multiple groups were compared

using Kruskal-Wallis ANOVA followed by Bonferroni post-hoc tests. Statistical significance was assumed when $P < 0.05$.

3. Results

Acetate: the primary metabolite of acetaldehyde (AA) is increased with feeding.

Acetate is the primary oxidation metabolite of AA, and thus, urinary acetate is a product of endogenous AA metabolism. We measured urinary acetate using GC-MS, and we found that feeding (overnight) significantly increased urinary levels of acetate indicating that a major source of endogenous AA likely is either directly from food or related to feeding, e.g., metabolism of food constituents, intermediary metabolism, etc... In female and male C57BL/6J mice, a daytime fast (9 h) resulted in a basal level of urinary acetate that overnight feeding was increased by 2-3 times (**Table 9**). Thus, we infer that feeding overnight increased AA levels that resulted in increased acetate excretion in urine.

AA-induced relaxation.

Because feeding increases gastrointestinal (GI) blood flow, we investigated whether AA would relax superior mesenteric artery (SMA) – a major conduit for increased GI blood flow with feeding (Chou & Coatney, 1994; Lucchini et al., 1996). In fact, AA (1 to 100 mM) induced a concentration-dependent, robust, repeatable and agonist-dependent relaxation in isolated SMA and aorta of female and male mice (**Fig. 16; Table 10**). Regardless of agonist (PE, U46,619, High K^+), AA relaxed precontraction tension by >90% in SMA (**Fig. 16A, B**) and in aorta (**Table**

10; PE only), yet when added to an uncontracted blood vessel, AA did not affect basal tone (aka myogenic tone; data not shown). Blood vessels sensitivity of male SMA was circadian-independent, and sensitivity of female SMA in daytime was less sensitive to male SMA sensitivity (either day or night cycle) (**Fig. 16 C, D; Table 10**), and thus, subsequent experiments to probe the mechanisms of AA-induced relaxation were conducted using only SMA isolated from male mice in the daytime (lights on). Collectively, AA-induced relaxation was repeatable (even up to 100 mM) without any loss of efficacy or any induced change in endothelium-dependent ACh-induced relaxation indicating little overt vasotoxicity of AA even at supra-physiological levels (data not shown).

Role of endothelium, nitric oxide synthase (NOS), and GC in AA-induced vasorelaxation.

The sensitivity of AA-induced relaxation (but not efficacy) was agonist-dependent (PE<U46,619=High K⁺; **Fig. 16 A, B; Table 10**) and indicated at least 2 distinct mechanisms were operative. We showed the most sensitive component of the AA-induced relaxation was significantly rightward-shifted by mechanical disruption of endothelium in SMA (air perfusion impaired >90% of ACh-induced relaxation) (**Fig. 17 A, B; Tables 11, 12**). This effect was similar to the effect of L-NAME incubation, which further implicated endothelium-derived NO (**Fig. 17 C, D; Tables 11, 12**). Because NO stimulates guanylyl cyclase (GC) to generate cGMP in smooth muscle cells, we used ODQ, an irreversible inhibitor of GC, and it also rightward-shifted the AA-induced relaxation (**Fig. 17 E, F; Tables 11, 12**). Collectively, these data show that the most sensitive component of the AA-induced relaxation was

dependent on a functional endothelium, NOS (likely eNOS) and an NO-mediated activation of GC, but at high concentrations, the relaxation of AA was not affected (**Table 11**). Thus, L-NAME shifted the sensitivity of AA-induced relaxation in SMA pre-contracted with PE (3.3 ± 0.3 vs 6.3 ± 0.6 mM), but did not alter efficacy ($E_{\max}$). Similarly, it neither affected the sensitivity nor efficacy of AA-induced relaxation in U46,619- (17.2 ± 0.3 vs 14.9 ± 1.5 mM) or High K- (18.4 ± 0.4 vs 17.7 ± 0.5 mM) pre-contracted SMA (**Fig. 18A, B; Table 10**). These data support a role of endothelium-derived NO in the most sensitive component of AA-induced relaxation in PE-precontracted SMA.

Ethanol-, acetaldehyde- and acetate-induced relaxations.

Because AA is formed from EtOH by alcohol dehydrogenase (ADH), and AA is metabolized by ALDH to acetate, it is possible that the vascular effects of EtOH or acetate are due to AA. To address this possibility, EtOH and acetate were cumulatively added (up to 100 mM) in PE pre-contracted SMA and aorta. Ethanol is known to induce vasodilation in several blood vessels, yet it is unclear if the effect is due to formation of AA. Ethanol relaxed PE-precontracted SMA weakly ($E_{\max}$ about 40% of PE precontraction) compared with AA (**Fig. 19A, B**). Ethanol did not relax PE pre-contracted aorta even at 100 mM, in fact, it increased tension primarily at 100 mM (**Fig. 20A**). Acetate also induced weak relaxation and a slight contraction at 100 mM in PE-precontracted SMA (**Fig. 19A, B, C**). L-NAME treatment significantly reduced both EtOH- and acetate-induced relaxations of SMA (**Fig. 19D, E; Table 11**). Although both EtOH- and acetate-induced

significantly weaker relaxations in SMA than did AA, the NO dependence of these relaxations was a shared mechanism of action with AA.

Role of AA metabolism: Effects of Cyanamide and Alda1 and AR.

To investigate the possible role of metabolism in AA-induced responses, cyanamide and Alda-1, ALDH inhibitor and ALDH2 activator, respectively, and an aldose reductase (AR) null mouse model were used. Surprisingly, and despite vasoactivity of their own, cyanamide (increased tension, 10-20%) and Alda-1 (decreased tension, 10-20%), neither compound had any significant effect on AA-induced relaxation in PE-precontracted SMA (**Fig. 21A, B; Table 13**). However, the sensitivity of the AA-induced relaxation of SMA was rightward shifted significantly in AR-null mice (EC_{50} , 6.9 ± 1.0 mM, $n=3$) although no effect on maximal relaxation was observed (E_{max} , $78.1 \pm 4.0\%$). Despite lack of a strong effect of ALDH modulators on AA's vasoactivity, cyanamide significantly blocked the relaxation of EtOH, and Alda1 slightly enhanced the EtOH relaxation (**Fig. 21C, D; Table 13**). Similarly, cyanamide significantly blocked the relaxation of acetate, although Alda1 was without effect on the relaxation of acetate (**Fig. 21E, F; Table 13**). These data support a potential regulatory role of ALDH activity in EtOH- and acetate-induced relaxations in SMA (see **Fig. 22**).

Role of Transient Receptor Potential Ankyrin 1 (TRPA1):

Although formaldehyde induces a sensitive TRPA1- and endothelium-dependent relaxation in isolated SMA, AA-induced relaxation in PE-precontracted

SMA was unaffected by addition of the TRPA1 antagonist (A967079, 1 μ M) (**Tables 11, 12**) indicating this pathway is not activated in a similar way by AA.

Nicotine and AA:

In tobacco users, EtOH, and thus, AA, may be present in blood simultaneously with nicotine, so we also tested whether nicotine altered AA-induced relaxation in SMA. Nicotine alone (1 μ M) had no effect in PE-contracted SMA, and also had no effect on subsequent AA-induced vasorelaxation (**Tables 11, 12**).

Table 9. Levels of urinary acetate following a daytime fast and overnight feeding in (A) female and (B) male C57BL/6J mice.

A. Females

	Condition	
Urinary Metabolite	Daytime Fast	Overnight Feeding
Acetate (μg/ml)	2.76±0.27	24.52±4.89*
Creatinine (mg/dl)	3.19±0.41	8.74±1.52
Acetate (μg/mg creatinine)	91.11±6.61	296.82±32.95*

B. Males

	Condition	
Urinary Metabolite	Daytime Fast	Overnight Feeding
Acetate (μg/ml)	4.71±0.66	8.54±2.34*
Creatinine (mg/dl)	6.52±1.16	5.67±1.15
Acetate (μg/mg creatinine)	79.56±7.49	158.57±32.16*

Values = means ± SE. N = 11-12 per group. *, P<0.05 vs fasting.

Table 10. The effective concentration inducing 50% relaxation (EC_{50} ; in mM) of acetaldehyde-induced vasorelaxation in isolated murine superior mesenteric artery (SMA; female and male) and thoracic aorta (male only) with different contractile agonists.

Blood Vessel (sex, time of day)	EC_{50} by precontraction agonist		
	PE	U46,619	High K
SMA (f, day)	6.0±0.6*	--	--
SMA (m, day)	3.3±0.4	14.9±1.5*	17.7±0.5*
SMA (m, day) +LNAME	6.2±0.2*	17.2 ±0.2	18.4 ±0.4
SMA (m, night)	4.3±0.8	--	--
Aorta (m)	15.9±3.0*		

Values = means ± SE. Abbr.: PE, phenylephrine, [10 µM]; U46,619, thromboxane A_2 analog, [0.1 µM]; High K, high potassium solution, [60 mM K^+]; --, did not perform experiment. N=3-10 per group. *, $P<0.05$ vs EC_{50} of "SMA (male, day)" PE agonist (i.e., baseline control).

Fig. 16. Acetaldehyde (AA) induced relaxation in superior mesenteric artery (SMA). **A)** Representative traces of concentration-dependent relaxation induced by cumulative addition of AA (1-100 mM) in isolated SMA pre-contracted by one of 3 agonists: phenylephrine (PE); thromboxane A₂ analog (U46,619); or, high potassium (High K⁺). **B)** Summary data of the efficacy (E_{max} % relaxation) of AA-induced relaxation in isolated PE, U46,619 and High K⁺ pre-contracted SMA. **C)** Summary data of the efficacy (E_{max}) and of the **(D)** sensitivity (EC₅₀) of AA-induced relaxation in isolated PE-precontracted SMA from male mice euthanized either during day or night time. Values are means ± SE of SMA from 3-4 mice.

A.

B.

C.

D.

Fig. 17. Role of the endothelium and nitric oxide (NO) in acetaldehyde-induced (AA) relaxation of SMA. **A, B)** Representative traces and summary data of AA-stimulated relaxation of PE-induced contraction in SMA with intact endothelium or with endothelium impairment due to 15 min of air perfusion. **C, D)** Representative traces and summary data of AA-stimulated relaxation of PE-induced contraction in the absence of and presence of NOS inhibitor, L-NAME. **E, F)** Representative traces and summary data of AA-induced relaxation of PE-induced contraction in the absence of and presence of the guanylyl cyclase antagonist, ODQ (3 μ M), added to bath after PE-induced contraction stabilized and prior to cumulative addition of AA. Values are means $\pm$ SE of SMA from 3-8 mice as indicated. *, $P < 0.05$ vs Control.

A.

B.

C.

D.

E.

F.

Table 11. Efficacy (maximal relaxation, $E_{\max}$) of acetaldehyde- (AA), EtOH- and acetate-induced relaxations of PE-precontracted superior mesenteric artery (SMA) and aorta without and with inhibitor treatments.

Compound	[mM]		Treatment	$E_{\max}$ (% relaxation)	
	SMA	Aorta		SMA	Aorta
AA	1-30	1-100	Control	89.6±1.7	91.6±6.7
AA	1-30	--	ED	83.5±3.4	--
AA	1-30	--	+LNAME	87.1±4.0	--
AA	1-30	--	+ODQ	90.8±1.8	--
AA	1-30	--	+A967079	90.6±4.0	--
EtOH	1-30	1-100	Control	39.4±3.1	0
EtOH	1-30	--	+LNAME	8.1±1.2*	--
Acetate	1-30	1-100	Control	42.9±5.9	0
Acetate	1-30	--	+LNAME	16.0±8.2*	--

Values = means ± SE. Abbr.: PE, phenylephrine, 10 μ M; ED, endothelial dysfunction induced by mechanical air perfusion; LNAME, N^o-nitro-L-arginine methyl ester hydrochloride, 100 μ M; A967079, TRPA1 antagonist, 3 μ M; ODQ, 1h-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. N=3-6 per group. *, P<0.05 vs untreated and matched Control.

Table 12. The effective concentration inducing 50% relaxation (EC₅₀; in mM) of acetaldehyde- (AA), EtOH- or acetate-induced vasorelaxation in isolated murine superior mesenteric artery (SMA; male mice only) pre-contracted with PE in the absence and presence of functional endothelium or selective inhibitors/compounds.

Compound [1-30 mM]	Control EC ₅₀ (vehicle)	Treatment	Treatment EC ₅₀
AA	3.9±0.6	+ED	12.4±2.1*
AA	2.9±0.4	+LNAME	6.2±0.6*
AA	2.1±0.9	+ODQ	7.4±0.9*
AA	2.9±0.6	+A967079	2.8±0.6
EtOH	3.2±1.5	+LNAME	X
acetate	6.9±3.0	+LNAME	X

Values = means ± SE. Abbr.: PE, phenylephrine, 10 µM; ED, endothelium dysfunction induced by mechanical air perfusion; LNAME, N^ω-nitro-L-arginine methyl ester hydrochloride, 100 µM; ODQ, 1h-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; A967079, TRPA1 antagonist, 3 µM; X, could not calculate due to low % relaxation (see **Table 3**). N=3-6 mice used per group. *, P<0.05 vs Control (vehicle).

Fig. 18. Role of the nitric oxide (NO) in acetaldehyde-induced relaxations of SMA. AA (1-100 mM) induced relaxation of isolated SMA pre-contracted by one of 3 agonists: phenylephrine (PE); thromboxane A₂ analog (U46,619); or, high potassium (High K⁺). Summary data of the **(A)** efficacy (% relaxation) and of the **(B)** sensitivity of AA-induced relaxation in isolated PE-precontracted SMA in the absence and presence of L-NAME. Values are means ± SE of SMA from 3-8 mice.

A.

B.

Fig. 19. The vascular effects of ethanol (EtOH), acetaldehyde (AA) and acetate in SMA. Role of the nitric oxide (NO) in ethanol- and acetate-induced relaxations of SMA. **A)** Representative traces of EtOH-, AA- and acetate-induced relaxations in PE pre-contracted SMA. Summary data of EtOH-, AA- and acetate-induced relaxations in PE pre-contracted SMA plotted as measures of efficacy (**B**, % PE contraction) and sensitivity (**C**, converted to 100 % response). Concentration dependent relaxations induced by either (**D**) ethanol or (**E**) acetate of phenylephrine (PE) pre-contracted superior mesenteric artery (SMA) in the absence and presence of L-NAME. Values are means $\pm$ SE of SMA from 3 mice as indicated. *, $P < 0.05$ vs Control.

A.

B.

C.

D.

E.

Fig. 20. The vascular effects of ethanol (EtOH), acetaldehyde (AA) and acetate in aorta. **A)** Representative traces of EtOH-, AA- and acetate-induced responses in PE pre-contracted aorta. Summary data of AA-induced relaxations in PE pre-contracted aorta plotted as measures of efficacy (**B**, % PE contraction) and sensitivity (**C**, converted to 100 % response). Values are means $\pm$ SE of aorta from 5 mice as indicated.

A.

B.

C.

Fig. 21. Role of aldehyde dehydrogenase (ALDH) metabolism in vascular responses to EtOH, AA and acetate. Representative traces and summary data, respectively, of vasorelaxant effects of AA (**A, B**), EtOH (**C, D**) and acetate (**E, F**) in PE pre-contracted SMA of WT mice in the absence and presence of either cyanamide (ALDH enzyme inhibitor, 1 mM) or Alda1 (ALDH2 activator, 25 μ M). The vasorelaxant effects of AA in AR-null mice (**A**). Values are means $\pm$ SE of SMA from 3-8 mice as indicated. *, $P < 0.05$ vs Control.

A.

B.

C.

D.

E.

F.

Fig. 22. Mechanisms of acetaldehyde-induced vasorelaxation and relationship to aldehyde metabolism. **A)** Cartoon depicting the mechanisms by which acetaldehyde (AA) induces vasorelaxation in SMA precontracted with phenylephrine (PE), thromboxane A₂ analog (U46,619) and 60 mM potassium solution (High K⁺). AA induces a sensitive endothelial cell-dependent mechanism that begins with eNOS activation, NO formation, and GC activation leading to VSMC hyperpolarization and vasorelaxation (Pathway designated “1.”). Several steps were confirmed by direct antagonism of NOS (L-NAME), endothelium (impaired by air perfusion) and guanylyl cyclase (GC; ODQ). A less sensitive yet equally efficacious pathway of vasorelaxation included a voltage-insensitive mechanism that similarly reversed U46,619- and High K⁺-induced contraction implicating closure of VSMC Ca²⁺ channels (Pathway designated “2.”). **B)** Cartoon depicting the metabolic pathways of ethanol, acetaldehyde and acetate. Ethanol is oxidized to acetaldehyde by alcohol dehydrogenase (ADH) and/or catalase activity. Acetaldehyde is oxidized to acetate by aldehyde dehydrogenases (ALDH1/ALDH2) or reduced back to ethanol by aldose reductase (AR). Acetate is converted to acetyl coenzyme A (CoA).

Table 13. Role of aldehyde dehydrogenase (ALDH) in AA-induced relaxations of PE-precontracted SMA. The efficacy (**A**; measured as the maximal relaxation, E_{max} ; %) and sensitivity (**B**; measured as the effective concentration inducing 50% relaxation; EC_{50} ; in mM) or the of AA-, EtOH-, or acetate-induced vasorelaxation in isolated murine superior mesenteric artery (SMA; male) pre-contracted with PE in the presence of cyanamide or Alda1 (ALDH2 activator).

A. Efficacy

SMA	E_{max} (%Relaxation of PE-precontraction)		
Compound (1-30 mM)	Control (vehicle)	+Cyanamide	+Alda1
Acetaldehyde	89.5±4.8	85.9±5.9	93.4±3.5
Ethanol	40.6±3.1	13.6±6.8*	52.2±4.4
Acetate	38.1±5.1	8.2±9.1*	43.1±8.0

B. Sensitivity

SMA	EC_{50}, [in mM]		
Compound (1-30 mM)	Control (vehicle)	+Cyanamide	+Alda1
Acetaldehyde	2.5±0.4	3.7±0.7	2.0±0.3
Ethanol	1.5±0.2	X	1.2±0.4
Acetate	2.4±0.8	X	3.6±0.9

Values = means ± SE. Abbr.: PE, phenylephrine, 10 µM; X, could not calculate due to low % relaxation. Cyanamide, 1 mM; Alda1, 25 µM. N=3-6 mice per group. *, $P < 0.05$ vs Vehicle Control.

4. Discussion

The physiological vascular action of AA is unclear but our study provides evidence for a potential contribution of AA in postprandial hyperemia in SMA via an EDRF-mediated dilatory pathway. In support, we show that SMA is significantly more sensitive than the aorta to AA, and importantly, the SMA is a known major arterial blood supply to the GI tract that dilates postprandially in response of a variety of stimuli e.g., nutrients, vago-vagal reflex, NO, substance P, etc... (Chou & Coatney, 1994; Lucchini et al., 1996). Moreover, because rodents feed at night and sleep (fast) during the day, we observed a circadian difference in urinary acetate levels indicating endogenous AA level likely increases with feeding (see **Table 9**). Although, AA is oft considered an inhaled toxicant, its natural abundance in food, water and beverages (Aldehydes, 1981; D. J. Conklin, P. Haberzettl, et al., 2011) and as an abundant metabolic product, e.g., EtOH, make it a plausible stimulus for increasing GI blood flow. As this is the first suggestion of this specific physiological role that we know of, more research will be needed to establish AA as a *bona fide* stimulus of postprandial hyperemia *in vivo*.

We found there are two distinct pathways of AA-induced vasorelaxation in SMA (**Fig. 22A**), and, we focused on the mechanism of the most sensitive pathway as it is likely a physiological pathway. The sensitive AA-induced vasorelaxation requires a functional endothelium, NOS (presumably eNOS) and guanylyl cyclase (GC/cGMP). The endothelial cells likely contribute to the most sensitive components, whereas the latter component is likely present in vascular smooth muscle cells (VSMC). These components are also activated by formaldehyde in

rat aorta and murine SMA (Jin, Jagatheesan, et al., 2019; Q. Zhang et al., 2018), yet the TRPA1 channel is not stimulated by AA indicating a different 'activating step' in the endothelium for AA. Functionally, L-NAME, the eNOS inhibitor, significantly blocks the most sensitive component of the AA-induced relaxation and shifts the EC₅₀ to the right. But this shift is less strong than that of endothelium disruption and ODQ (a GC inhibitor) suggesting a second endothelium-dependent component likely is operative. We also cannot rule out the possibility that additional components in the vascular wall (perivascular nerves, SubP, CGRP, etc...) may contribute to the AA-induced relaxation (Nilius et al., 2012).

AA induced a much stronger relaxation in SMA (and aorta) than either an upstream precursor, EtOH, or its downstream metabolite, acetate – both of which stimulate vasorelaxation in SMA but not in aorta indicating important similarities and differences with AA. EtOH-induced vasorelaxations have been well-documented for over 4 decades, yet the complete mechanism of this phenomenon is only now becoming clearer. EtOH induces vascular relaxation by acting on both endothelial cells and VSMCs to dilate mesenteric and cutaneous blood vessels; yet EtOH also contracts cerebral, coronary, pulmonary and renal vascular beds and skeletal muscle arterioles (Altura & Altura, 1982, 1987; Altura, Altura, & Carella, 1983; Greenberg et al., 1993; Rocha et al., 2012; Ru et al., 2008; Toda, Konishi, Miyazaki, & Komura, 1983). Our data show that EtOH (and acetate) relax SMA at concentrations <100 mM after which there is an onset of contraction. In contrast, EtOH and acetate only increase tension in precontracted aorta. Nonetheless, L-NAME significantly blocks the most sensitive component of both EtOH- and

acetate-induced relaxation in SMA reflecting NO-dependence (endothelium) -- as observed for AA (see shared Pathway 1. in **Fig. 22A**). Because the relaxations induced by EtOH and acetate in SMA are qualitatively similar to AA, this may reflect a role of AA in both EtOH- and acetate-induced relaxations. As AA induces relaxation in both SMA and aorta this likely reflects a shared yet less sensitive pathway (#2.; see **Fig. 22A**) that extracellular AA but not EtOH or acetate.

The concentration of AA in human blood is kept very low due to fast acting aldehyde dehydrogenase 2 (ALDH2) enzyme. The capacity of the liver to eliminate AA formed from EtOH is so efficient that measurable levels of AA are typically undetected in peripheral blood (Lindros, Stowell, Pikkarainen, & Salaspuro, 1980) but AA is detected in saliva. In an alcohol drinking model, salivary AA concentration was approximately 150 μ M (maximum 260 μ M) over the first 20 to 40 min followed by a rapid decrease to 20 to 30 μ M for the remainder of the follow-up period (350 min)(Salaspuro, 2017). Moreover, ALDH2*2 enzyme deficiency is present in 35-45% of Asian people (8% of world's population), and results from *ALDH2**2 gene mutation that decreases ALDH activity by 20% (heterozygous mutation) and 50% (homozygous), and thus, people with *ALDH2**2 gene mutation are susceptible to facial flushing and nausea after alcohol consumption presumably due to high AA levels (Chen, Ferreira, Gross, & Mochly-Rosen, 2014; Gross et al., 2015). Consistent with epidemiological findings, alcohol drinking in ALDH2-deficient subjects markedly elevates AA concentrations in saliva (Vakevainen, Tillonen, Agarwal, Srivastava, & Salaspuro, 2000; Yokoyama et al., 2008) and in blood of EtOH- or AA-injected ALDH2-deficient mice (Jamal et al., 2016). Interestingly, high

levels of AA are associated with increased risk of GI tract cancers (Salaspuro, 2017) indicating that AA is likely elevated in the GI tract as well.

Although concentrations of salivary and blood AA and those used in our study are difficult to compare, theoretically, consuming as little as one glass of wine (5 oz or 150 ml) containing 15-20 g of EtOH (11-13% ABV) would increase blood EtOH concentration up to 65 mM (assuming 5 l blood volume, 70 kg adult). As EtOH is rapidly metabolized to AA by ADH in liver and in blood vessels, it is plausible that AA levels increase quite quickly in endothelial cells and VSMC with corresponding intracellular/membrane levels being likely in the bioactive range described in our study (1-30 mM). Despite this theoretical calculation, there is little evidence for circulating AA levels in the active dilation range presumably because of rapid intracellular oxidative metabolism of AA by ALDHs to acetate (see **Fig. 22B**). To test the role of SMA-dependent ALDH metabolism on AA's bioactivity, we use an ALDH inhibitor, cyanamide, and an ALDH2 activator, alda-1, to decrease and increase ALDH activity, respectively, and in turn, potentially augment and diminish AA's action. Interestingly, alda-1 (25 μ M) and cyanamide (1 mM) are vasoactive in SMA with opposing effects: alda-1 relaxed; cyanamide contracted (see **Fig. 21E**). Because of these opposing effects, we infer that the pharmacological target – (ALDH2 enzyme activity) may be an active regulator of SMA vascular tone. Perhaps more surprising, neither compound significantly alters AA-induced relaxation, yet cyanamide effectively blocks both EtOH- and acetate-induced relaxations in SMA. In contrast to our hypothesis, wherein we expect cyanamide (or AR-null) to enhance EtOH-induced relaxation by increasing AA

level, we observe inhibition of EtOH-induced relaxation. Notably, this result is not without precedent. For example, EtOH combined with cyanamide inhibits rather than enhances EtOH-induced increases in blood flow (Carmichael et al., 1987). Interestingly, AA alone did not increase portal blood flow either. Equally provocative is our finding that alda-1 increases the relaxation of EtOH but does not alter relaxation of either AA or acetate. Collectively, these data are both compelling (*ALDH activity likely is involved in vascular effects of EtOH and acetate.*) and vexing (*Why are vascular effects of AA unaltered by modulators of ALDH activity?*). The latter may be a function of addition of extracellular addition of AA whereas both EtOH and acetate need to be intracellular in order to be metabolized to bioactive AA. The cyanamide-mediated inhibition of EtOH- and acetate-induced relaxations further indicates dependence on ALDH activity for initiation of relaxation. There is, of course, precedent for such a role of ALDH2 activity in nitroglycerin-induced vasorelaxation (Beretta et al., 2012; Hink et al., 2007), however, others show that cyanamide alone induces relaxation in isolated and precontracted rabbit aorta (Fukuto, Gulati, & Nagasawa, 1994). Moreover, as nicotine did not alter AA-induced SMA relaxation, we infer nicotine does not inhibit vascular ALDH activity. To dissect out the complex biochemical interplay between AA vascular action and ALDH activity will require further studies.

In conclusion, we describe a sensitive component of an AA-induced vasorelaxation in SMA that is sequentially dependent on an EDRF/NO and a GC/cGMP pathway. We suggest this relaxation is a likely contributor to a broader, multifaceted postprandial hyperemic reflex that increases blood flow to the GI tract

to augment digestion and nutrient absorption. Moreover, EtOH and/or acetate (a short chain fatty acid metabolite of gut microbiota) may contribute to local vascular AA levels (via ADH and ALDH activities) to further augment the reflex increase in gut blood flow related to feeding (and drinking).

CHAPTER V

CROTONALDEHYDE-INDUCED VASCULAR RELAXATION AND TOXICITY:

ROLE OF ENDOTHELIUM AND TRANSIENT RECEPTOR POTENTIAL

ANKYRIN-1 (TRPA1)

Introduction: Crotonaldehyde (CR) is an electrophilic α,β -unsaturated aldehyde present in foods and beverages, and as a minor metabolite of 1,3-butadiene. CR is a product of incomplete combustion, and thus, it is measured at relatively high levels (ppm) in cigarette smoke and structural fires. Exposure to CR has been linked to cardiopulmonary toxicity and cardiovascular disease including atherosclerosis. **Objective:** The purpose of this study was to examine the direct effects of CR in isolated murine blood vessels (aorta and superior mesenteric artery, SMA) in vitro, and dissect the underlying mechanisms. **Method and Results:** CR induced concentration-dependent (1-300 μ M) and equally robust relaxations (75-80%) in phenylephrine (PE) precontracted aorta and SMA, yet the SMA was 20x more sensitive than aorta to CR (SMA EC₅₀ 3.8 $\pm$ 0.5 μ M; aorta EC₅₀ 76.0 $\pm$ 2.0 μ M; $p < 0.05$). To probe the mechanisms of CR-induced relaxation and toxicity, we used the sensitive SMA. The CR-induced relaxation was inhibited in

the presence of: 1) mechanically-impaired endothelium; 2) N ω -Nitro-L-arginine methyl ester hydrochloride (L-NAME); 3) guanylyl cyclase (GC) inhibitor (ODQ); 4) transient receptor potential ankyrin-1 (TRPA1) antagonist (A967079); and, 5) non-vasoactive level of nicotine (1 μ M). Similarly, a known TRPA1 agonist, allyl isothiocyanate (AITC; mustard oil) also stimulated SMA relaxation dependent on: endothelium, TRPA1, NO, and GC. Moreover, TRPA1 was co-localized with isolectin by positive immunofluorescent staining of the SMA endothelium. Thus, CR (1-30 μ M) induces relaxation in SMA via an endothelium-, NO-, GC-, and TRPA1-dependent pathway. Additionally, CR was toxic at high concentrations (100-300 μ M) where it stimulated tension oscillations and significantly depressed subsequent PE-induced contractions after washout. CR-induced vasotoxicity was significantly enhanced in SMA with impaired endothelial function. **Conclusions:** The sensitive relaxation of CR depends on a functional endothelium and TRPA1 whereas vasotoxicity was enhanced under conditions of endothelium dysfunction. CR is both vasoactive and vasotoxic along a concentration continuum with elevated CR likely encountered in smoke exposures.

1. Introduction

Crotonaldehyde (CR) is a highly reactive α,β -unsaturated aldehyde that is environmentally ubiquitous, e.g., found in foods such as fish, meat, fruits and vegetables and beverages such as wine and whiskey (Eder, Schuler, & Budiawan, 1999; IARC., 1995). Sources of CR exposure are mainstream and side-stream tobacco smoke (IARC., 1995). CR is also formed endogenously during lipid

peroxidation (Chung, Chen, & Nath, 1996; Chung et al., 1999; Duescher & Elfarra, 1993; Filser et al., 2001; Kawaguchi-Niida et al., 2006; Nair, Bartsch, & Nair, 2007; Voulgaridou, Anestopoulos, Franco, Panayiotidis, & Pappa, 2011) and in the metabolism of 1,3-butadiene (Duescher & Elfarra, 1993; Filser et al., 2001) and N-nitrosopyrrolidine (Hecht, Upadhyaya, & Wang, 1999; M. Y. Wang, Chung, & Hecht, 1988). CR can also be formed from acetaldehyde by aldol condensation and dehydration (Tuma, Hoffman, & Sorrell, 1991). CR is an important industrial chemical used in the synthesis of tocopherols, sorbitol, and 3-methylbutanol, but it is also a contaminant and a byproduct of various chemical processes (IARC., 1995).

CR can rapidly penetrate cell membranes and induce adverse biological effects, such as inflammation in alveolar macrophages of smokers (Facchinetti et al., 2007; Yang et al., 2013a; Yang et al., 2013b); oxidative stress and apoptosis in epithelial cells (Liu, Yang, Pan, Zhu, & Xie, 2010a, 2010b); and toxicity to respiratory tract tissues (Reddy et al., 2002). Glutathione S-transferase P (GSTP) accelerates metabolism of short chain unsaturated aldehydes such as acrolein (3-C) and CR (4-C) via conjugation with glutathione (GSH) (Berhane, Widersten, Engstrom, Kozarich, & Mannervik, 1994; D. J. Conklin, Haberzettl, et al., 2015a). The major metabolite of CR and a urinary biomarker of CR exposure is 3-hydroxy-1-methylpropyl mercapturic acid (HPMMA) (Gray & Barnsley, 1971). Urinary levels of HPMMA are significantly increased after cigarette smoking and are significantly higher (3x) in U.S. cigarette smokers than in non-smokers (Jain, 2015; Lorkiewicz et al., 2019). Moreover, levels of both HPMMA and 3HPMA (major metabolite of

acrolein) are similar in smokers yet as well as in non-smokers indicating similar levels of these two aldehydes are found in the environment, derived from endogenous metabolic processes and in cigarette smoke (Jain, 2015).

CR is chemically similar to acrolein, and thus, the lung toxicity of cigarette smoke has been attributed in part to both acrolein and CR. It has been listed as one of nine constituents recommended for disclosure and monitoring by the World Health Organization (WHO) study group on tobacco product regulation (Haussmann, 2012). Moreover, because the primary acrolein metabolite, 3HPMA, is associated with cardiovascular and pulmonary disease risk (DeJarnett et al., 2014; Srivastava et al., 2011; Zhang et al., 2018), it is expected that CR also contributes to disease risk. Similarly, occupational exposure to 1,3-butadiene, a precursor of CR, is associated with cardiovascular disease, and in an animal model, exposure to 1,3-butadiene increases arteriosclerotic plaque development in cockerels (Penn & Snyder, 1996b). Nonetheless, little research has been dedicated to assess the direct vascular actions of CR.

Because CR and acrolein are known to activate the transient receptor potential ankyrin 1 (TRPA1) channel and because TRPA1 is present in the vasculature, we explored the TRPA1-dependence of CR vasoreactivity in isolated blood vessels. TRPA1 is widely distributed in the nervous and cardiovascular systems especially sensory neurons (unmyelinated C-fibers) (Anand et al., 2008; Nagata, Duggan, Kumar, & Garcia-Anoveros, 2005), endothelial cells (Earley et al., 2009a), and heart and cardiomyocytes (Andrei, Sinharoy, Bratz, & Damron, 2016; D. J. Conklin, Y. Guo, et al., 2019). Activation of TRPA1 by a variety of

agonists including acrolein, CR and allyl isothiocyanate (AITC, mustard oil) leads to pain (Trevisan et al., 2016), neurogenic tissue inflammation (Schwartz et al., 2011), pulmonary edema and airway hyper-reactivity (Andre et al., 2008; D. J. Conklin, Haberzettl, Jagatheesan, Kong, & Hoyle, 2016), vasodilation (Jin, Jagatheesan, et al., 2019) and hypotension (Earley, 2012; Pozsgai et al., 2010). Thus, TRPA1 could be important in the vasoactive effects of CR.

Consistent with our hypothesis, our research showed that CR induced a robust, concentration-dependent relaxation that in superior mesenteric artery (SMA) was dependent on: 1) a functional endothelium; 2) NO formation; 3) guanylyl cyclase (GC); and, 4) TRPA1 activation. High concentrations of CR, however, led to tension oscillations and irreversible suppression of subsequent contractility reflecting acute vasotoxicity. These data indicate that CR is vasoactive with TRPA1 at low concentrations and vasotoxic at high concentrations.

2. Material and Methods

Chemicals and Solutions.

Reagent grade chemicals were purchased from Sigma-Aldrich or other commercial sources as indicated: A967079 (AdooQ); acetylcholine chloride (ACh); crotonaldehyde (CR); allyl isothiocyanate (AITC); 1h-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ); N^ω-nitro-L-arginine methyl ester hydrochloride (L-NAME); L-phenylephrine hydrochloride (PE); sodium nitroprusside (SNP).

Animals.

Male or female wild type C57BL/6J (WT), TRPA1-null and GSTP-null mice (12-20 weeks old; 25-35g) were purchased (The Jackson Laboratory, Bar Harbor, ME) or used from in house breeding (D. J Conklin et al., 2017; D. J. Conklin, Haberzettl, et al., 2009b). Mice were treated according to American Physiological Society *Guiding Principles in the Care and Use of Animals*, and all protocols were approved by University of Louisville Institutional Animal Care and Use Committee. Mice were housed under pathogen-free conditions in the University of Louisville vivarium under controlled temperature and 12h light:12h dark cycle. Mice were provided water and a standard chow diet *ad libitum* (Rodent Diet 5010, 4.5% fat by weight, LabDiet; St. Louis, MO).

Urine Collection and CR Metabolite Analysis

Urine Collection. To measure total urine HPMMA, mice were exposed to HEPA-filtered air or mainstream cigarette smoke (MCS; KY Reference 3R4F cigarettes: 50% of smoke of 12 cigarettes over 6h) and then placed singly per metabolic cage with glucose/saccharin solution (w/v; 3.0%/0.125%) to collect urine (without food) up to 3h post-exposure followed by urine collection overnight (O/N; with food). Urine samples were centrifuged in cold immediately after collection (600×g, 5 min; to pellet any feces and food particles), decanted, and stored at -80 °C (D. J. Conklin et al., 2018).

CR Metabolite Analysis. The major urine metabolite of CR, 3-hydroxy-1-methylpropylmercapturic acid (HPMMA), was quantified by ultrafast performance liquid chromatography-mass spectrometry (UPLC-MS) as described (D. J. Conklin et al., 2018). Briefly, urine (25 µl) was mixed with 15 mM ammonium acetate (975

μl) containing HPMMA-d3 standard (667 ng) and filtered through a 0.2 mm PTFE membrane. Samples (2 μl) were applied on UPLC and the eluate was analyzed for the MRM traces by MS. To account for urinary dilution, all values for urinary metabolites were normalized to urinary creatinine (mg/dl) (D. J. Conklin et al., 2018).

CR-Induced Vasoreactivity in Isolated Aorta and Superior Mesenteric Artery (SMA)

Isolation and Organ Bath Conditions. After removal and cleaning of the aorta and SMA, thoracic aorta rings (3-4 mm) were hung on stainless steel hooks in organ baths and SMA rings (2 mm) were hung on tungsten wire in 5-ml heated organ baths (MultiWire Myograph System 620M, DMT, Denmark) in PSS bubbled with 95% O₂:5% CO₂ at 37 °C. After 10 min without tension, aorta and SMA rings were equilibrated to respective loading tension over 30 min (aorta, 1g; SMA, 0.25g). All rings were stimulated with High K⁺ (60 mM) to test for viability, washed 3 times with PSS over 30 min, and re-equilibrated to appropriate resting tension (Jin, Jagatheesan, et al., 2019). Krebs physiological salt solution (PSS) for SMA was (in mM): NaCl, 119; KCl, 4.7; CaCl₂, 2; MgCl₂, 1.2; KH₂PO₄, 1.2; NaHCO₃, 24; glucose, 7; pH 7.4. Krebs PSS for aorta was (in mM): NaCl, 119; KCl, 4.7; CaCl₂, 1.2; KH₂PO₄, 1.2; MgSO₄, 1.2; NaHCO₃, 25; glucose, 5.5; pH 7.4. High K⁺ PSS (High K⁺; 60 mM) substituted equimolar K⁺ for Na⁺ in SMA PSS.

Crotonaldehyde (CR) Experimental Series:

CR-Induced Relaxation. Crotonaldehyde (1, 3, 10, 30, 100, and 300 μM) was added to organ bath containing uncontracted and pre-contracted (PE, 10 μM; PE₁) aorta and SMA. Following exposure to the highest concentration of CR, organ

baths were washed (3x) with fresh PSS over 30 min, and then vessels were precontracted again with PE (PE₂) and relaxed sequentially with ACh (10 μ M) and then SNP (100 μ M) to test for endothelium-dependent and -independent relaxations, respectively. The efficacy of CR-induced relaxation was calculated as the % reduction in agonist-induced contraction. The sensitivity of CR-induced relaxation was the effective concentration producing 50% response (EC₅₀), i.e., cumulative concentration responses normalized to 100% with interpolation of EC₅₀ (Jin, Jagatheesan, et al., 2019). EC₅₀ values were calculated only if total relaxation % exceeded 50% of PE₁. Contractile toxicity was quantified as the PE₂/PE₁ tension ratio (as %) by dividing PE-induced tension post-CR-exposure (PE₂) by PE-induced tension pre-CR (i.e., PE₁) multiplied by 100. Tension oscillations were quantified by their frequency (oscillations/min) and their amplitude (mN) as averaged after high CR concentration exposure (300 μ M) (D. J. Conklin, Boyce, Trent, & Boor, 2001a).

Role of the Endothelium, Nitric Oxide Synthase (NOS), and cGMP. The role of the endothelium in CR-induced vasorelaxation was studied in SMA with intact and injured endothelium. The endothelium was mechanically injured by air perfusion and damage confirmed by near complete abolition (>95%) of ACh-induced dilation of PE pre-contracted SMA (Jin, Jagatheesan, et al., 2019). To evaluate the role of NOS, L-NAME (100 μ M) was added after addition of PE (10 μ M; 15 min), and, pre-contracted SMA were then relaxed with cumulative concentrations of CR (1, 3, 10, 30, 100 and 300 μ M). To assess whether cGMP was involved in CR-induced

vasorelaxation, PE-precontracted SMA were exposed to ODQ (3 μ M) to inhibit guanylyl cyclase (GC) and subsequent formation of cGMP (Jiang et al., 2015).

Role of TRPA1. The role of the TRPA1 channel was assessed by incubation of SMA with TRPA1 antagonist (A967079, 1 μ M) followed by cumulative addition of CR (1-300 μ M). Similarly, effects of CR in SMA of TRPA1-null mice were measured without and with the TRPA1 antagonist (A967079, 1 μ M) present. Likewise, effects of a known TRPA1 agonist, allyl isothiocyanate (AITC, 1-300 μ M), were measured in PE-precontracted SMA.

Role of GSTP on CR Relaxation. Because CR is primarily metabolized by glutathione S-transferase P (Berhane et al., 1994), we assessed whether GSTP deficiency could enhance effects of CR in isolated blood vessels of GSTP-null mice. Blood vessels were PE-precontracted and exposed to increasing concentrations of CR (1-300 μ M) as described above.

Role of Nicotine. Because exposure to CR via tobacco smoke also will necessarily contain nicotine, we tested whether a super-pharmacological level of nicotine (1 μ M) would affect CR-induced relaxation in PE-precontracted SMA.

Histology and TRPA1 Immunofluorescence. Thin sections (4 μ m) of formalin-fixed, paraffin-embedded SMA, aorta and dorsal root ganglia (DRG; positive control) of WT mice were stained with H&E or TRPA1 antibody (1:200; Alomone Labs, Israel; Cat. #: ACC-037). To label endothelial cells, blood vessel sections were co-stained with isolectin GS-IB4 Alexa Fluor 594 conjugate (1:200 dilution; Invitrogen; Cat. #: I21413). Images of H&E-stained sections were made using a digital Spot camera

mounted on an Olympus microscope. Immunofluorescence microscopy was performed with rabbit polyclonal antibody against TRPA1 without or with a blocking peptide of the TRPA1 antibody and Alexa Fluor 647 goat anti-rabbit secondary antibody (1:400 dilution; Invitrogen; Cat. #: 21244). Slides were covered with DAPI containing Slow Fade® Gold anti-fade reagent (Invitrogen; Cat. #: S36938), and fluorescence was visualized on a Nikon eclipse Ti fluorescence Microscope using NIS-Elements (Nikon; Japan) at 200X. A DAPI filter was used for nuclear staining and a Cy5 Red filter for TRPA1 staining (D. J. Conklin, Y. Guo, et al., 2019; Jin, Jagatheesan, et al., 2019).

Statistical Analyses. Data are expressed as means $\pm$ SE. When comparing two groups, a *t*-test was used (paired or unpaired as appropriate). Multiple group testing was done with One-Way ANOVA with Bonferroni post hoc. If either equal variance or normality test failed, One-Way ANOVA on Ranks (Kruskal-Wallis) and Dunn's post hoc was used (SigmaPlot, ver. 12). Statistical significance was assumed where $P < 0.05$.

3. Results

Urinary CR Metabolite, HPMMA, is Elevated in Mice Exposed to Mainstream Cigarette Smoke (MCS).

The total urinary HPMMA (μg) excreted from C57BL/6 (WT) mice exposed to KY Reference cigarette MCS (3R4F) was increased 2.5x over that in urine of air-exposed mice (**Fig. 23A**). As expected, urinary nicotine level also was

increased significantly after MCS exposure (**Fig. 23A**). Baseline excretion of HPMMA (about 1.8 $\mu\text{g}/16\text{h}$) is equivalent to 0.025 μmol CR (MW = 70.07), and as blood volume of a 25g mouse is about 2 ml, this amount of CR approximates to 12.5 μM in blood. Moreover, as the HPMMA level was increased 2.5x after MCS exposure, a potential blood level of CR >30 μM was estimated. So, we tested for direct vascular effects of CR from 1-300 μM to cover a broad systemic concentration range.

Concentration-Dependent Effects.

CR (1-300 μM) produced robust (around 75-80%) concentration-dependent relaxations in precontracted aorta and SMA (**Fig. 23B-C**), but relaxations did not reach 100% in SMA due to a consistent reversal of relaxation and induction of tension oscillations (“spasms”) at 300 μM (**Fig. 23B**). CR induced equally robust vasorelaxation in both isolated aorta and SMA, but the SMA was significantly more sensitive (20x) than aorta to CR (SMA EC_{50} 3.8 ± 0.5 μM ; aorta EC_{50} 76.0 ± 2.0 μM ; $p < 0.05$) (**Fig. 23D**; **Tables 14-15**). Tension oscillation frequencies were low (<2/min) and of modest amplitude (<2.5 mN) (**Fig. 23B**; **Table 16**). Endothelial impairment abolished CR induction of oscillations, and L-NAME treatment decreased oscillation frequency by 35% and amplitude by 60% although neither reduction was statistically significant (**Table 16**).

CR (1-300 μM) added to uncontracted SMA had no effect on basal tension (myogenic tone; **data not shown**) nor on PE_2 -generated tension ($84.0 \pm 12.8\%$ of PE_1 ; $n=3$) nor on ACh-induced relaxation ($-91.1 \pm 1.3\%$; $n=3$) in the continued

presence of CR. However, after 3 bath exchanges over 30 min, CR-treated SMA were largely refractory to PE₃-induced tension by $74.4 \pm 15.0\%$ of PE₁ (n=3; P=0.0666); yet, retained functional ACh-induced relaxation ($-84.6 \pm 2.4\%$; n=3).

Mechanisms of CR-Induced Vasorelaxation.

The most sensitive component of CR-induced vasorelaxation (1-30 μ M) was significantly inhibited in PE-pre-contracted SMA by a mechanically disrupted endothelium (>90% loss of ACh-induced relaxation) (**Fig. 24A-B; Tables 14-15**). Similarly, CR-induced vasorelaxation in pre-contracted SMA was equally inhibited in the presence of L-NAME, and the EC₅₀ shifted rightward (less sensitive) (**Fig. 24C-D; Tables 14-15**). This L-NAME effect indicated a role of NO (likely eNOS) as critical in the most sensitive part of the CR-induced relaxation in PE-precontracted SMA. Similarly, ODC treatment, an irreversible guanylyl cyclase (GC) inhibitor, significantly blocked only the most sensitive part of the CR-induced relaxation (**Fig. 24E-F; Tables 14-15**). Collectively, these data show that the sensitive portion of the CR-induced relaxation was dependent on the endothelium, NOS (likely eNOS) and NO-mediated activation of GC (and formation of cGMP in VSMC). The female SMA was marginally less sensitive to CR than male SMA (**Table 15**).

Role of TRPA1 channel in the CR-induced relaxation.

Because CR is a known agonist of TRPA1, we hypothesized that the TRPA1 channel contributes to endothelium-dependent CR-induced vasorelaxation in SMA. In support, the TRPA1 antagonist, A967079 (1 μ M), significantly inhibited

the most sensitive part ($<10\ \mu\text{M}$) of the CR-induced relaxation in PE-contracted SMA (**Fig. 25A-B; Tables 14-15**). We also tested CR-induced relaxation with or without A967079 ($1\ \mu\text{M}$) in PE-contracted SMA of male TRPA1-null mice (**Fig. 25C-D; Tables 14-15**). In contrast, presence of A967079 appeared to enhance slightly the relaxation of CR in TRPA1-null SMA (**Fig. 25D**). Because CR is a substrate of GSTP, we tested for a metabolic role of GSTP in SMA treated with CR. There was no difference in the CR-induced relaxation in PE-contracted SMA of GSTP-null mice compared with WT SMA (**Fig. 25E-F; Tables 14-15**), and there was no difference in the CR-induced relaxation with or without ezatiostat which was a GSTP inhibitor (**Fig. 25G-H; Tables 14-15**). Because AITC is a known TRPA1 agonist, it, unsurprisingly, induced a concentration- and TRPA1-dependent relaxation in SMA (**Fig. 26A**). This relaxation, as with CR, was inhibited by the TRPA1 antagonist (A976709), and the AITC-induced relaxation was similarly shifted rightward in SMA of TRPA1-null mice (**Fig. 26B**), supporting the idea that TRPA1 mediates this sensitive vasorelaxation (see EC_{50}s in **Table 15**).

In immunofluorescent-stained cross-sections of mouse SMA, TRPA1 (green; **Fig. 26D**) was co-localized with isolectin (red) in the endothelium (see yellow in **Fig. 26E**). Modest yet specific TRPA1 staining also was present in the media:adventitia border of SMA (green; **Fig. 26D**). Although aorta (**Fig. 26F-H**) had positive TRPA1 staining localized in the media (green; **Fig. 26G**), there was no staining observed in the endothelium. Positive TRPA1 staining was confirmed by use of a TRPA1 blocking peptide in aorta (**Fig. 26H**) and in dorsal root ganglion

(DRG; **Fig. 27**), which also served as a positive control for TRPA1 (D. J. Conklin, Y. Guo, et al., 2019).

Vascular smooth muscle toxicity of CR.

The PE₂/PE₁ tension (%) was significantly decreased after CR exposure (CR PE₂/PE₁: 27.0±2.2%) compared with the untreated control group (PE₂/PE₁: 133.6±12.3%) (**Fig. 28A, Table 17**). The PE₂/PE₁ tension (%) was further decreased in endothelial dysfunction (ED) and ODQ treatment groups compared with the CR only exposed group (**Fig. 28A**) but there were no differences with any other treatment group. Although CR treatment alone depressed PE₂ contraction, it had no effect on subsequent ACh-induced relaxation (**Fig. 28B, Table 16**). In fact, the only 2 groups with impaired ACh-induced relaxation following CR exposure were by design: ED and L-NAME treatments (**Fig. 28B, Table 17**) suggesting that CR did not induce acute endothelial cell injury despite causing VSMC dysfunction.

Nicotine and CR.

In users of combustible tobacco, CR and nicotine exposure occur simultaneously; therefore, we tested whether nicotine altered CR-induced relaxation in SMA. Nicotine (>10 µM) induced robust relaxation in SMA (**Fig. 29A**). Nicotine alone (1 µM) had no effect on tension in PE-contracted SMA, yet decreased slightly and significantly efficacy of subsequent CR-induced vasorelaxation (**Table 14; Fig. 29B**).

Fig. 23. Crotonaldehyde-induced (CR) relaxation in aorta and superior mesenteric artery (SMA). **A)** Cumulative quantification of mass spectrometry analyses of primary crotonaldehyde (CR) metabolite (3-hydroxy-1-methylpropylmercapturic acid; HPMMA) and nicotine in urine of mainstream cigarette smoke-exposed (MCS; 12 cigarettes/6 h) male mice 16h post-exposure. (Graph derived from data in Conklin et al., 2018). **B)** Representative trace of CR-stimulated (1-300 μ M) relaxation and tension oscillations of phenylephrine (PE)-contracted SMA, and post-CR exposure vasoreactivity. **C)** Representative trace of CR-stimulated (1-300 μ M) relaxation of phenylephrine (PE)-contracted aorta, and post-CR exposure toxicity (no contraction to PE). **D)** Summary sensitivity data of CR-induced relaxation in PE pre-contracted SMA and aorta. Values = means $\pm$ SE of 3-4 preparations. *, $P < 0.05$ vs aorta.

A.

B.

C.

D.

Table 14. Efficacy (maximal relaxation, $E_{\max}$) of crotonaldehyde-induced relaxations of PE-precontracted superior mesenteric artery (SMA; male and female) without and with inhibitor treatments.

SMA (+treatment, N)	$E_{\max}$ (Control, PE+vehicle)	$E_{\max}$ (ED or PE+inhibitor)
WT+ED	-74.3±6.6	-63.6±7.1
WT+L-NAME	-62.7±5.3	-66.8±2.4
WT+ODQ	-73.4±6.9	-54.7±15.2
WT+A967079	-57.2±3.5	-61.6±6.8
TRPA1-null+A967079	-70.5±6.4	-79.0±4.2
WT+Ezatiostat	-56.5±7.2	-61.5±2.5
GSTP-null	-52.6±2.9	--
WT+Nicotine	-67.0±4.2	-55.0±6.5 [#]
WT (f)	-59.8±3.9	--

Values = means ± SE. Abbr.: PE, phenylephrine, 10 μ M; ED, endothelial dysfunction induced by mechanical air perfusion; L-NAME, N^o-nitro-L-arginine methyl ester hydrochloride, 100 μ M; A967079, TRPA1 antagonist, 1 μ M; ODQ, 1h-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, 3 μ M; Ezatiostat 1 μ M; Nicotine 1 μ M; --, experimental group not performed; (f), female. All SMA were from male mice except where indicated female, (f). N=3-6 per group. *, P<0.05 vs matched Control (PE+vehicle); #, 0.05<P<0.10 vs matched Control (PE+vehicle).

Table 15. The sensitivity (effective concentration inducing 50% relaxation; EC₅₀; in μ M) of crotonaldehyde (CR)-induced vasorelaxation in PE pre-contracted murine superior mesenteric artery (SMA) in the absence and presence of functional endothelium or selective inhibitors.

SMA (+treatment, N)	EC₅₀ (Control, PE+vehicle)	EC₅₀ (ED or PE+inhibitor)
WT+ED	5.7 $\pm$ 1.0	68.2 $\pm$ 13.9*
WT+L-NAME	6.0 $\pm$ 2.0	43.6 $\pm$ 17.9*
WT+ODQ	3.9 $\pm$ 1.6	35.3 $\pm$ 1.6*
WT+A967079	7.2 $\pm$ 2.3	12.1 $\pm$ 2.1*
TRPA1-null	17.9 $\pm$ 7.4	14.1 $\pm$ 2.7
WT+Ezatiostat	4.3 $\pm$ 0.7	4.2 $\pm$ 0.6
GSTP-null	5.9 $\pm$ 0.8	--
WT+Nicotine	3.3 $\pm$ 0.8	5.7 $\pm$ 1.2
WT (f)	6.6 $\pm$ 0.3*	--

Values = means $\pm$ SE. Abbr.: PE, phenylephrine, 10 μ M; ED, endothelial dysfunction induced by mechanical air perfusion; L-NAME, N^o-nitro-L-arginine methyl ester hydrochloride, 100 μ M; A967079, TRPA1 antagonist, 1 μ M; ODQ, 1h-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, 3 μ M; Ezatiostat 1 μ M; Nicotine, 1 μ M; --, experimental group not performed; (f), female. All SMA were from male mice except where indicated (f). N=3-6 per group. *, P<0.05 vs matched Control (PE+vehicle).

Table 16. Characteristics of tension oscillations induced by a high concentration of crotonaldehyde (CR; 300 μ M) in PE-precontracted superior mesenteric artery (SMA).

SMA (+treatment)	Frequency (oscillations/min)	Amplitude (mN)
WT+CR	2.0 $\pm$ 0.4	2.0 $\pm$ 0.3
WT+CR+ED	0*	--
WT+CR+L-NAME	1.3 $\pm$ 0.2	0.9 $\pm$ 0.1
WT+CR+ODQ	2.1 $\pm$ 0.6	1.2 $\pm$ 0.5
WT+CR+A967079	1.7 $\pm$ 0.3	1.9 $\pm$ 0.5
TRPA1-null+CR	1.7 $\pm$ 0.3	2.0 $\pm$ 0.2
WT+CR+Ezatiostat	1.9 $\pm$ 0.1	2.1 $\pm$ 0.2
GSTP-null+CR	1.9 $\pm$ 0.6	3.4 $\pm$ 0.5
WT+Nicotine	1.8 $\pm$ 0.2	1.6 $\pm$ 0.3
WT (f) +CR	1.4 $\pm$ 0.6	2.9 $\pm$ 0.6

Values = means $\pm$ SE. Abbr.: PE, phenylephrine, 10 μ M; WT, wild type; ED, endothelial dysfunction; L-NAME, N^o-nitro-L-arginine methyl ester hydrochloride, 100 μ M; ODQ, 1h-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, 3 μ M; A967079, TRPA1 antagonist, 1 μ M; Ezatiostat 1 μ M; Nicotine, 1 μ M; (f), female. All SMA were from male mice except where indicated (f). N=3-10 per group. *, P<0.05 vs WT+CR.

Fig. 24. Mechanisms of crotonaldehyde-induced (CR) relaxation in superior mesenteric artery (SMA). **A)** Representative traces of CR-stimulated relaxation of PE-induced contraction without and with endothelium impairment due to 5 min of air perfusion. **B)** Summary data of CR-induced relaxation in isolated SMA pre-contracted with PE in the presence and absence of functional endothelium. **C)** Representative traces of CR-stimulated relaxation of PE-induced contraction in the absence and presence of L-NAME (100 μ M). **D)** Summary data of CR-induced relaxation in isolated SMA pre-contracted with PE in the absence and presence of L-NAME. **E)** Representative traces of CR-stimulated relaxation of PE-induced contraction in the absence and presence of the guanylyl cyclase (GC) antagonist, ODQ (3 μ M), added to bath after PE-induced contraction plateaued and prior to cumulative addition of CR. **F)** Summary data of CR-induced relaxation in isolated SMA pre-contracted with PE in the absence and presence of ODQ. Values = means $\pm$ SE of 3-4 preparations. *, $P < 0.05$ vs control.

Fig. 25. Role of the TRPA1 channel in crotonaldehyde (CR)-induced relaxation in superior mesenteric artery (SMA). **A)** Representative traces of CR-stimulated relaxation of phenylephrine (PE) pre-contracted SMA in the absence (Control) and presence of the TRPA1 antagonist, A967079 (1 μ M) added after PE-induced contraction plateaued and prior to cumulative addition of CR in wild type (WT) SMA. **B)** Summary data of CR-induced relaxation in isolated SMA pre-contracted with PE in the absence and presence of TRPA1 antagonist. **C)** Representative traces of CR-stimulated relaxation of PE pre-contracted SMA in the absence (Control) and presence of the TRPA1 antagonist, A967079 (1 μ M) added after PE-induced contraction plateaued and prior to cumulative addition of CR in TRPA1-null SMA. **D)** Summary data of CR-induced relaxation in PE pre-contracted TRPA1-null SMA in the absence and presence of TRPA1 antagonist. **E)** Representative traces of CR-stimulated relaxation of PE pre-contracted SMA of WT and GSTP-null mice. **F)** Summary data of CR-induced relaxation in PE pre-contracted WT and GSTP-null SMA. **G)** Representative traces of CR-stimulated relaxation of phenylephrine (PE) pre-contracted SMA in the absence (Control) and presence of the GSTP antagonist, Ezatiostat (1 μ M) added after PE-induced contraction plateaued and prior to cumulative addition of CR in wild type (WT) SMA. **H)** Summary data of CR-induced relaxation in isolated SMA pre-contracted with PE in the absence and presence of GSTP antagonist. Values = means $\pm$ SE of 3-4 preparations. *, $P < 0.05$ vs Control.

Fig. 26. Role of TRPA1 in AITC-induced relaxation of superior mesenteric artery (SMA), and localization in SMA and aorta. A) Representative traces of AITC-stimulated, concentration-dependent relaxation of PE-contracted SMA. **B)** Summary data of AITC-induced relaxation in PE-contracted SMA of WT and TRPA1-null mice in the absence (DMSO) and presence of TRPA1 antagonist, A967079 (1 μ M). Formalin-fixed, paraffin-embedded, cross-sections of murine SMA (**C-E**) and aorta (**F-H**) stained with H&E (**C, F**). Immunofluorescent localization of TRPA1 alone in SMA (green, **D**) or with isolectin (red, endothelium) and DAPI (**E**). Immunofluorescent localization of TRPA1 alone in aorta (green, **G**) or with isolectin (red) and a TRPA1 blocking peptide and DAPI (**H**). All images were taken at 200x magnification (scale bar = 100 μ m). Values = means $\pm$ SE of 3-4 preparations. *, $P < 0.05$ vs Control. Abbr.: L, lumen.

Fig. 27. Immunofluorescent localization of TRPA1 in dorsal root ganglion (DRG). Formalin-fixed, paraffin-embedded sections of murine DRG were stained with H&E (**A**); DAPI only (blue) (**B**); TRPA1 antibody (red) and DAPI (**C**); or, TRPA1 antibody, TRPA1 blocking peptide, and DAPI (**D**). All images were imaged at 200x magnification (scale bar = 100 μ m).

Fig. 28. Toxicity of crotonaldehyde (CR) exposure in superior mesenteric artery (SMA). A-B) Ratio of PE₂ (PE-induced tension post-CR [300 µM] exposure) to PE₁ (PE-induced tension pre-CR) (as %) across treatment groups with untreated control group for comparison. **B)** Effects of CR and treatments on ACh-induced relaxation. Values = means ± SE of 3-6 preparations. *, *P*<0.05 vs untreated Control or vs CR control (B only). Abbr.: ED, endothelial dysfunction.

Table 17. Vasotoxicity of crotonaldehyde (CR) in superior mesenteric artery (SMA).

SMA (+treatment)	PE₂/PE₁ (%)	ACh (%)
WT (control)	133.6±12.3	-95.4±1.6
WT+CR	27.0±2.2*	-92.6±2.5
WT+CR+ED	10.0±2.5*#	-16.7±3.3*
WT+CR+L-NAME	21.0±1.7*	-64.2±4.8*
WT+CR+ODQ	9.5±3.2*#	-83.9±2.4
WT+CR+A967079	34.0±2.4*	-89.9±2.3
TRPA1-null+CR	34.0±4.7*	-97.1±1.8
WT+CR+Ezatiostat	43.5±4.5*#	-91.9±3.4
GSTP-null+CR	37.5±2.5*	-95.1±1.3
WT+CR+Nicotine	26.6±4.5*	-87.3±2.9
WT (f) +CR	26.1±6.2*	-93.9±3.3

Values = means ± SE. Abbr.: PE, phenylephrine, 10 µM; ACh, acetylcholine chloride, WT, wild type; ED, endothelial dysfunction; L-NAME, N^ω-nitro-L-arginine methyl ester hydrochloride, 100 µM; ODQ, 1h-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, 3 µM; A967079, TRPA1 antagonist, 1 µM; Ezatiostat 1 µM; Nicotine, 1 µM, (f), female. The PE₂/PE₁ tension (%) was calculated by dividing PE-induced tension post-CR-exposure (PE₂) by PE-induced tension pre-CR (i.e., PE₁) and multiplied by 100. ACh-induced relaxation was calculated as the % reduction in PE₂-induced tension. All SMA were from male mice except where indicated (f). N=3-10 per group. *, P<0.05 vs WT (male) (control). #, P<0.05 vs WT+CR.

Fig. 29. Effects of nicotine on CR-induced relaxation in SMA. A) Representative trace of concentration dependent (0.1 μ M – 1 mM) nicotine-induced relaxation of PE pre-contracted SMA. **B)** Nicotine at 1 μ M had no effect on tension of PE-precontracted SMA yet significantly decreased efficacy (10, 30 and 100 μ M) of subsequent CR-induced vasorelaxation (**Table 1**). Values = means $\pm$ SE of 6 preparations. *, $P < 0.05$ vs matched Control; #, $0.05 < P < 0.10$ vs matched Control.

4. Discussion

To our knowledge, this is the first study to show the vasoreactivity of CR and its mechanisms of action that include TRPA1. The mechanisms of CR-induced vasorelaxation are similar to formaldehyde, including dependence on TRPA1, endothelium, NO formation and guanylyl cyclase activation (Jin, Jagatheesan, et al., 2019). The robust and sensitive relaxation is reminiscent of actions described for acrolein at low concentrations in perfused rat mesenteric bed (Awe et al., 2006), isolated rat coronary artery (D. J. Conklin et al., 2001a), and isolated mouse aorta (D. J. Conklin, Haberzettl, et al., 2009a). Moreover, we show that, also like acrolein (D. J. Conklin et al., 2001a), CR at higher concentrations induces tension oscillations (“spasms”) and suppresses contractility (toxicity) in SMA. This continuum from potent relaxation to vascular toxicant belies the importance of studying these responses as CR is abundant in foods, beverages, occupational settings, and especially in combustion-derived smoke, i.e., tobacco smoke, wildfires, etc... Thus, appreciating the avidity of unsaturated aldehydes for altering vascular wall function is a first step toward understanding how exposure to CR may increase cardiovascular disease risk.

It is unsurprising that vascular TRPA1 is involved in CR-induced vasorelaxation. Activation of TRPA1 can lead to release of vasoactive peptides, substance P (SubP) and CGRP (Trevisan et al., 2016) that can induce vasodilation and inflammatory responses (Achanta, Chintagari, Brackmann, Balakrishna, & Jordt, 2018; D. J. Conklin, 2016). The mechanism of CR-induced TRPA1-mediated relaxations in SMA, however, appears to be dependent largely on endothelium-

localized TRPA1 as we show that endothelial impairment either by air perfusion or L-NAME shifts the sensitivity of the CR response rightward to a degree similar to that of either TRPA1 antagonist treatment or by use of TRPA1-null SMA. This is mirrored by relaxation of known TRPA1 agonist AITC that is equally shifted by both A967079 and TRPA1 deletion. Nevertheless, we cannot fully rule out that other cells in the isolated SMA, e.g., perivascular nerves, contribute to CR's mechanism of relaxation. Moreover, there are at least 2 mechanisms mediating the full relaxation of CR (see **Fig. 30**): 1) endothelium; and, 2) time-dependent, irreversible change in VSMC contractile machinery, e.g., closure of VSMC calcium channels. Although the latter conclusion has limited experimental support, we infer it because PE contraction is significantly impaired both pre- and post-CR exposure and washouts; endothelium is intact because ACh-induced relaxation is unaffected post-CR exposure (no endothelium dysfunction); and, previous experience with similar vascular toxicity of acrolein led to findings consistent with a role of external calcium (D. J. Conklin et al., 2006).

Cigarette smoke is a major source of exogenous CR (Goniewicz et al., 2017; Lorkiewicz et al., 2019). We show that exposure of mice to MCS increases excretion of the CR metabolite, HPMMA, by 2.5x, indicating there is a fair amount of CR produced endogenously in mice. Our estimation of circulating blood levels of CR puts it in the low end of the vasoactive range - a novel consideration. Given that acrolein and CR are highly reactive unsaturated aldehydes, it is hard to fathom these molecules floating as free aldehydes in the blood, and we do not think that. However, the metabolites of CR and acrolein certainly are carried in the blood, and

we hypothesize that these metabolites may be vasoactive, and this possibility should be investigated further. Nonetheless, many unsaturated aldehydes are noted potent vasodilators including acrolein (Awe et al., 2006), 4-hydroxynonenal (4HNE) (Martinez et al., 1994; Romero et al., 1997) and cinnamaldehyde (Pozsgai et al., 2010; Yanaga et al., 2006), and now CR -- all these are known ligands of TRPA1. These potent dilatory effects may contribute to a variety of physiological processes such as postprandial hyperemia or flow-mediated dilation (FMD) or response to injury/infection, but too much of any of these aldehydes may lead to pathological changes including vasospasm, atherosclerosis and vascular toxicity (Boor & Conklin, 2008; D. J. Conklin et al., 2006; D. J. Conklin et al., 2001a). Although these parent aldehydes can covalently modify intracellular amino-terminus free cysteines to activate TRPA1 (Macpherson et al., 2007), it is unclear if their metabolites can do this as well or if there are other mechanisms of activating TRPA1 channels.

Nicotine is also an important component of tobacco smoke, and nicotine activates the sympathetic nervous system that can increase cardiovascular disease risk factors such as heart rate and blood pressure and decrease heart rate variability (HRV). These changes, in turn, affect myocardial remodeling, impair lipid metabolism, and stimulate inflammation (Benowitz & Burbank, 2016; Dutta et al., 2012). The plateau concentration of nicotine in the blood of smokers throughout the day averages 40 µg/L (0.25 µM) (Moyer et al., 2002), and thus, we tested whether effects of super-pharmacological level of nicotine (1 µM) could alter CR effects in PE pre-contracted SMA. Although nicotine induces relaxation at high

concentrations (≥ 10 μM), it is inactive at 1 μM (see **Fig. 29A**); yet, it modestly suppresses CR-induced relaxation. The mechanism of this effect is unclear, but inhaled nicotine at a high concentration (1 mM) is thought to activate sensory TRPA1 channel and stimulate airway constriction reflex (Talavera et al., 2009). Our previous studies show that nicotine (1 μM) has no effect on formaldehyde- or acetaldehyde-induced relaxations in PE-contracted SMA (Jin, Jagatheesan, et al., 2019; Jin, Lorkiewicz, et al., 2019), indicating this effect appears specific for CR. Inhibition of CR vasorelaxation may promote the sympathomimetic effects of nicotine alone, e.g., increased blood pressure, perhaps counteracting vasodepressor effects of CR.

In conclusion, this study describes a sensitive mechanism of CR-induced vasorelaxation that is sequentially dependent on endothelium-localized TRPA1 channel, NO, and a VSMC GC pathway in SMA (but not in aorta), which may play an important role in regulating blood flow/blood pressure at low endogenous levels of CR. Smooth muscle impairment at high concentrations (300 μM) of CR evokes tension oscillations reminiscent of “spasms” observed with acrolein *in vitro* – another vasotoxic and TRPA1 agonist compound. Future studies of the *in vivo* contribution of CR and TRPA1 to cardiovascular physiology and pathophysiology are required to better understand how exposure to these reactive aldehydes confer increased cardiovascular disease risk.

Fig. 30. Cartoon depicting the mechanisms of crotonaldehyde (CR) induced vasorelaxation in SMA. CR induces a sensitive and sequential mechanism dependent on activation of Transient Receptor Potential Ankyrin 1 (TRPA1) cation channels, endothelial cell-based eNOS, NO-mediated GC activation leading to VSMC hyperpolarization and relaxation. These steps were significantly impaired by a selective antagonist or treatment, including endothelium impairment (by air perfusion), eNOS inhibition (L-NAME), guanylyl cyclase inhibitor (GC; ODQ), and TRPA1 inhibitor (A967079). An additional, but less sensitive pathway of CR-mediated relaxation likely involves closure of VSMC calcium channels. Potential role of VSMC-localized TRPA1 channel activation (dashed line) in CR-induced relaxation, tension oscillations or vascular toxicity is uncertain. Glutathione S-transferase P (GSTP) deletion had no effect on CR actions in SMA.

CHAPTER VI

VASCULAR EFFECTS OF INHALED ALDEHYDES IN VIVO:

ROLE OF ENDOTHELIUM- AND TRANSIENT RECEPTOR POTENTIAL ANKYRINE-1 (TRPA1) - DEPENDENT MECHANISM

Introduction Formaldehyde (FA) and acetaldehyde (AA) are small, saturated aldehydes, and crotonaldehyde (CR) is a highly electrophilic α , β -unsaturated aldehyde, they are all abundant in the environment and in the aerosols of tobacco as well as in many foods and beverages. Because of high-level exposures in users of tobacco products and are well-known associated with the development of cardiovascular disease (CVD), including atherosclerosis. We explored the effects of FA, AA and CR exposures in isolated murine blood vessels. **Objective** The purpose of this study was to explore the acute and chronic cardiovascular toxicity of inhaled aldehydes in mice in vivo; and explore the possible mechanisms. **Method and Results** C57BL/6J (wild type, WT) mice were chronically exposed to CR or filtered room air for 12 weeks (1 ppm, 5 d/week). Blood pressure was significantly lower in the CR group than in the air control group, while heart rate was unchanged. Hemodynamic changes

were accompanied by changes in aortic vasoreactivity ex vivo. Following 12-wk CR chronic exposure, there was no change in aortic response to phenylephrine (PE), however, the sensitivity of acetylcholine- (ACh) and sodium nitroprusside-induced relaxation were significantly altered in the CR-exposed mice. To dissect the mechanism of CR-induced vascular changes, we performed acute 4-day CR exposures (1 and 3 ppm). Acute CR exposures also altered aortic responses to ACh and SNP. Based on these findings, we tested for the role of the transient receptor potential ankyrin 1 (TRPA1) channel. In CR-exposed TRPA1-null mice (4-days, 1ppm), there were no changes in aortic responses to any agonist (PE, ACh, and SNP) compared with air group, indicating a distinct role of TRPA1. Interestingly, there also were no differences in aortic responses in acutely-exposed female C57BL/6J mice (1 ppm, 6h/day, 4days), indicating sex-dependence. WT mice were exposed to FA or AA gas (5ppm, 6h/day) for 4days, FA from 30-1,500 Mm or AA from 1-100 mM strongly and reversibly inhibited agonist-induced contractions. There was no difference in the sensitivity and relaxation of acetylcholine (ACh) and sodium nitroprusside (SNP). So, short-term inhalation exposure to FA or AA at levels present in tobacco product aerosols did not alter the direct effects of these aldehydes. **Conclusions** These consistent vascular effects indicate that CR is vasoactive at low concentrations and chronic exposure to CR may lead to compensatory changes in the vasculature to offset excessive smooth muscle relaxation and hypotension, which may lead to vascular disease. Future studies are required to better understand the mechanisms by CR exposure modifies cardiovascular pathophysiology, and the

specific role of TRPA1 and sex in CVD such as atherosclerosis. Those two saturated aldehydes, FA and AA, have limited direct toxicity in large arteries – prior inhalation exposure indicating these agents may not be involved in tobacco-related endothelium dysfunction.

1. Introduction

Smoking is the most significant preventable factor in the development of cardiovascular disease (CVD) (Association., 2014; Bhatnagar, 2006), has been shown to increase the risk of myocardial infarction, coronary artery disease, atherosclerosis and stroke (Ambrose & Barua, 2004). Tobacco use is responsible for an estimated 7 million global deaths each year (Organization., 2017), most deaths due to CVD (Hausmann, 2012). Extensive evidence demonstrates that exposure to both mainstream cigarette smoke (MCS) (Administration., 2012; U.S. Department of Health and Human Services, 2014) or second-hand tobacco smoke (Prevention., 2017; U.S. Department of Health and Human Services, 2014) releases high concentration of aldehydes and increases the risk for CVD.

More than 90% of the non-cancer health risk associated with smoking (i.e., cardiovascular and pulmonary disease risk) is attributable to aldehydes (Hausmann, 2012). The unsaturated aldehydes (Administration., 2012; Smith & Fischer, 2001; van Andel I, 2017), including acrolein and crotonaldehyde (CR), some studies showed that MCS contains acrolein (50-70 ppm) and

crotonaldehyde (CR) (1-53 µg/cigarette), these units are very different (Alwis, deCastro, Morrow, & Blount, 2015; Pazo et al., 2016), though they are classified as the most significant toxins in tobacco smoke (Hausmann, 2012; Stabbert et al., 2017). Our previous study showed that e-cigarette contains very high concentrations of FA and AA (Ogunwale et al., 2017). Aldehyde also generated by burning coal and gasoline, FA and AA approximately 71.4% of the gasoline exhaust from cars (Committee on, 1981). As we know, it's very difficult to measure aldehyde directly since it's highly reactive. So, even if it is formed in relatively small amounts, are likely to be of greater toxicological because of their high toxicity.

Because CR is physically and chemically similar to acrolein, we expected that the cardiovascular toxicity of CR in vivo may be similar to acrolein exposure, but surprisingly there are limited data on CR toxicity in vivo. CR has been shown to induce inflammation, oxidative stress and apoptosis in epithelial cells (Liu et al., 2010a, 2010b) and to cause cardiomyocyte dysfunction (Pei et al., 2014), but CR inhalation studies have not been performed. Both acute and chronic inhalation exposure to acrolein has been shown and induce endothelium dysfunction in mice (D. J. Conklin, Haberzettl, Lesgards, et al., 2009). Acrolein ingestion promotes pro-atherosclerotic changes and atherosclerosis (D. J. Conklin et al., 2010; Srivastava et al., 2011). Similarly, inhalation exposure to 1,3-butadiene, an upstream precursor of CR, accelerates arteriosclerotic plaque development in cockerels (Penn & Snyder, 1996b). Another epidemiological study expressed nearly 50% increase in the standardized mortality ratio (SMR)

for atherosclerotic heart disease that was associated with environmental butadiene exposure (Matanoski et al., 1990). However, whether CR could promote the atherosclerotic effects of 1,3-butadiene exposure has not been studied.

The direct vascular toxicity of CR inhalation remains unexplored, not to mention about the mechanisms by which inhaled CR may induce cardiovascular effects. From our previous studies, we show that the transient receptor potential ankyrin 1 (TRPA1) receptor is a pulmonary irritant receptor, mediate responses to inhaled compounds such as tobacco smoke and acrolein and blocked the relaxation of FA in vitro (D. J. Conklin, 2016; D. J Conklin et al., 2017; Jin, Jagatheesan, et al., 2019). Furthermore, the negative cardiovascular effects of inhaled acrolein (at levels in cigarette smoke) are TRPA1-dependent (Kurhanewicz, Ledbetter, Farraj, & Hazari, 2018; Kurhanewicz et al., 2017). Yet the role of TRPA1 in potential cardiovascular toxicity of inhaled CR is unknown. To address these gaps in knowledge regarding potential cardiovascular toxicity (and its mechanisms) of CR, healthy adult male and female C57BL/6 mice were exposed acutely or chronically (12 weeks; 1 ppm) by inhalation to CR, and biomarkers of cardiovascular system harm were measured. To probe the role of TRPA1, we performed select CR exposures in TRPA1-null mice (D. J. Conklin et al., 2017).

2. Material and Methods

Chemicals and Solutions.

Reagent grade chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or other commercial sources as indicated: A967079 (AdooQ); acetylcholine chloride (ACh); 4-aminopyridine (4-AP); crotonaldehyde (CR); allyl isothiocyanate (AITC); 1h-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ); N^o-nitro-L-arginine methyl ester hydrochloride (L-NAME); L-phenylephrine hydrochloride (PE); sodium nitroprusside (SNP).

Krebs physiological salt solution (PSS) for aorta was (in mM): NaCl, 119; KCl, 4.7; CaCl₂, 1.6; MgSO₄•7H₂O, 1.2; KH₂PO₄, 1.2; NaHCO₃, 25; glucose, 5.5; pH 7.4. High K⁺ PSS (60 mM) substituted equimolar K⁺ for Na⁺.

Mice and Crotonaldehyde Exposure

Mice.

Male and female C57BL/6 mice were obtained from The Jackson Laboratories (Bar Harbor, ME). Male TRPA1-null mice (on a C57BL/6 background) were from a breeding colony at the Univ. Louisville (D. J Conklin et al., 2017). All mice were treated according to the *Guiding Principles for the Care and Use of Animals in Research and Teaching* as adopted by the American Physiological Society, and all protocols were approved by the Univ. Louisville Institutional Animal Care and Use Committee. Before and during exposures, mice were housed under pathogen-free conditions, controlled temperatures, and a 12h:12h light:dark cycle.

Mice were maintained on a standard chow diet (Rodent Diet 5010, 4.5% fat by weight, LabDiet; St. Louis, MO).

Aldehydes exposures.

To parallel our previous study of chronic acrolein toxicity in mice at 1 ppm exposure in mice (Daniel J. Conklin et al., 2017), we used 1 ppm CR. Mice were exposed to either HEPA- and charcoal-filtered air or CR (1 or 3 ppm, 6h/day) for 4 days or 12 weeks (1 ppm; 6h/day, 5 days/week) using a custom exposure system and certified permeation tubes (Kin-Tek; LaMarque, TX) as previously described (Daniel J. Conklin et al., 2017). Immediately following the final exposure, mice were anesthetized with sodium pentobarbital (≈ 150 mg/kg, i.p.) followed by ventral thoracotomy and exsanguination via cardiac puncture for blood collection in EDTA-coated syringes. Organs were removed, weighed, and snap frozen in N₂ and stored at -80°C until further analysis. Mice were exposed to either HEPA- and charcoal-filtered air, FA or AA (1 or 5 ppm, 6h/day) for 4 days using the same exposure system and methods.

Non-invasive blood pressure.

Blood pressure was measured in male mice using the CODA tail-cuff blood pressure system (Kent Scientific; Torrington, CT) as described (Daniel J. Conklin et al., 2019). Mice were acclimated for 5 consecutive days to the CODA system prior to collecting data. During the chronic exposure study, blood pressure measurements were made immediately following an exposure period once per week beginning in the second week of exposure and continuing for the remainder

of the study. Briefly, mice (n=15 in both control and exposure groups) were put in a holding chamber on a warming platform, and 25 measurement cycles were performed on each mouse with a five second interval between each cycle (approx. 30 min).

Hemodynamics by telemetry.

To measure blood pressure and heart rate real-time in CR exposure, mice were implanted with pressure cannula in aortic arch for radiotelemetry (PA-C10; DSI, St. Paul, MN). Naïve mouse was given 1-week post-surgery to recover before being placed in an exposure chamber for 15 min of baseline recording *prior to onset of CR exposure (9 min; 1 ppm; 2x over 1h)*, and *systolic blood pressure (mmHg), diastolic blood pressure (mmHg), mean blood pressure (mmHg), pulse pressure (mmHg), and heart rate (bpm) were continuously recorded (1 kHz).*

Echocardiography.

Echocardiography was performed in chronically-exposed male C57BL/6 mice using a Vevo-770 echocardiography system (FUJIFILM Visual Sonics; Toronto, Canada). Body temperature was maintained (36.5-37.5°C) using a rectal thermometer interfaced with a servo-controlled heat lamp. Mice were anesthetized with 2% isoflurane and maintained under anesthesia with 1.5% isoflurane during the procedure. The 707-B (30MHz) scan head was used to obtain 2D images (100 fps) of the parasternal long axis. M-modes were taken from the same anatomical position. An apical four-chamber view of the heart was obtained, and pulse wave Doppler readings were taken of the mitral valve. Pulse wave Doppler readings

were also taken in the ascending aorta, the innominate artery, and the left common carotid artery and in the descending aorta distal the left subclavian artery. Additional pulse wave Doppler readings were taken at the branches of the descending aorta and both the right and left renal arteries. Pulse wave velocity was calculated by dividing the distance (mm) between the left subclavian artery and the right and left renal arteries by the pulse wave travel time (ms).

Vascular Reactivity

Aorta isolation and preparation. Exposed mice were anesthetized (sodium pentobarbital, ≈ 150 mg/kg, i.p.) and the aorta was removed via mid-ventral thoracotomy. Aortas were cleaned of perivascular adipose tissue and cut into rings. Thoracic aorta rings (3-4 mm) were hung on stainless steel hooks in 15-ml water-jacketed organ baths or in 5-ml heated organ baths of a MultiWire Myograph System 620M (DMT; Hinnerup, Denmark). The organ baths contained PSS bubbled with 95% O₂: 5% CO₂ at 37°C. After 10 min without tension, aorta rings were equilibrated to ≈ 1 g loading tension over 1h. All rings were stimulated with High K⁺ to test for viability, washed three times with PSS over 30 min, re-equilibrated to 1g (aorta) of resting tension. The rings were then stimulated again with High K⁺ followed by three bath changes and re-equilibration to resting tension. Transducer signals were recorded using LabChart software.

Post-aldehydes exposure evaluation. To test whether CR exposure altered vascular reactivity, the following responses were measured: 1) contractions induced by High K⁺; 2) concentration-dependent contractions of phenylephrine

(PE); 3) concentration-dependent relaxations of acetylcholine (ACh) in PE-precontracted rings; and, 4) concentration-dependent relaxations of sodium nitroprusside (SNP; nitric oxide donor) in PE-precontracted rings. Measures of efficacy (E_{max} : contractions normalized to aortic length; percentage relaxation of PE contraction)(Jin, Lipinski, & Conklin, 2018) and sensitivity (EC_{50} , effective concentration producing 50% response, i.e., cumulative concentration responses normalized to 100% with interpolation of EC_{50}) were calculated (Conklin et al., 2009).

Histology and TRPA1 Immunofluorescence.

Thin sections (4 μ m) of formalin-fixed, paraffin-embedded SMA, aorta and dorsal root ganglia (DRG; positive control) of WT mice were stained with H&E or TRPA1 antibody (1:200; Alomone Labs, Israel; Cat. #: ACC-037). To label endothelial cells, blood vessel sections were co-stained with isolectin GS-IB4 Alexa Fluor 594 conjugate (1:200 dilution; Invitrogen; Cat. #: I21413). Images of H&E-stained sections were made using a digital Spot camera mounted on an Olympus microscope. Immunofluorescence microscopy was performed with rabbit polyclonal antibody against TRPA1 without or with a blocking peptide of the TRPA1 antibody and Alexa Fluor 647 goat anti-rabbit secondary antibody (1:400 dilution; Invitrogen; Cat. #: 21244). Slides were covered with DAPI containing Slow Fade® Gold anti-fade reagent (Invitrogen; Cat. #: S36938), and fluorescence was visualized on a Nikon eclipse Ti fluorescence Microscope using NIS-Elements (Nikon; Japan) at 200X. A DAPI filter was used for nuclear staining and a Cy5 Red

filter for TRPA1 staining (D. J. Conklin, Y. Guo, et al., 2019; Jin, Jagatheesan, et al., 2019).

Statistics

Data are presented as mean $\pm$ standard error of mean (SEM). Mann-Whitney U Rank sum tests were used for the data comparison between two groups, and two-way Repeated Measures ANOVA with Bonferroni's post-test was used when comparing more than two groups with repeated measures (SigmaPlot, ver. 12.5; Systat Software, Inc., San Jose, CA). Statistical significance was set at $p < 0.05$.

3. Results

Crotonaldehyde and Hemodynamics

Non-invasive blood pressure and heart rate were measured in both air- and CR-exposed male mice once a week during the chronic study (**Figs. 31A, B**). Compared with the air-exposed control group, CR-exposed mice had significantly lower systolic, mean, and diastolic blood pressures at the 7th, 8th, and 10th weeks of exposure. Diastolic and mean blood pressures also were decreased significantly in the CR-exposed mice in the fifth week compared with the baseline measurements made during the second week of exposure (**Fig. 31A**). Heart rate was significantly higher in CR- than in air-exposed mice by echocardiography (under isoflurane anesthesia; **Table 18**). In addition to HR change, isovolumic relaxation time (IVRT) was significantly decreased in CR-exposed mice compared

with air controls (**Table 18**). There was no effect, however, of chronic CR exposure on aortic stiffness as indexed by pulse wave velocity (PWV) measured by ultrasound (**Table 18**).

Chronic Exposure to Crotonaldehyde and Vascular Reactivity

Because chronic exposure to CR lowered blood pressure but not pulse wave velocity, we investigated potential acute CR-induced blood pressure changes. For this a naïve mouse with an indwelling blood pressure cannula in the aortic arch and acutely exposed to CR (1 ppm) for a 9-min session. Onset of CR exposure was followed by a delayed yet robust decreases in diastolic blood pressure (15 mm Hg) and heart rate (100 bpm) – both effects returned to baseline within 10 min after CR exposure ended and filtered air returned (**Figs. 32 A, B**). These data provide plausibility that even an acute and brief CR exposure at 1 ppm can rapidly and reversibly depress systemic arterial blood pressure.

Although the aorta does not directly regulate blood pressure, the aorta is an important marker of systemic vascular dysfunction especially of endothelial dysfunction and atherosclerosis risk. CR had no effect on the efficacy of PE, ACh or SNP action in the aorta of chronically-exposed male mice (**Table 19**) and there also were no differences in aortic sensitivity or contractility to PE between exposure groups, however the ACh-induced, endothelium-dependent relaxations were significantly stronger (% relaxation; **Fig. 32A**) and more potent (i.e., enhanced sensitivity; EC50 shifted to right; **Fig. 32B**) in the CR group vs air group (**Table 20**). By comparison, the endothelium-independent relaxations of SNP were not different between groups (**Fig. 32C, D; Table 20**) indicating a preferential CR

effect on the endothelium. Interestingly, this effect may have contributed to the lower overall arterial blood pressure measured in the CR-exposed group compared with air group.

Acute Exposure to Crotonaldehyde and Vascular Reactivity

Because of the endothelium-dependent changes seen in the aortas of chronic CR-exposed WT male mice, vascular function was also measured post-exposure in isolated aorta of WT male mice after 4 days of CR exposure (1 and 3 ppm). In the aorta of CR-exposed mice after both 1 and 3 ppm, there was a significant increase in relaxation and a slight increase in sensitivity of aorta in response to ACh (**Fig. 33A, B; Fig. 34A, B**). Similarly but, modestly there was also an increased relaxation (**Fig. 33C; Fig. 34C**) and sensitivity (**Fig. 33D; Fig. 34D**) in response to SNP that resulted in a significantly decreased EC_{50} (at 3 ppm only; **Table 20**).

Acute Exposure to Crotonaldehyde and Role of TRPA1

Because of the known relationship between the TRPA1 channel and neurogenic inflammation induced by MCS or acrolein, we performed acute exposures to CR (4 days; 1 ppm) in TRPA1-null male mice. In contrast to the WT mice, measures of vascular reactivity in TRPA1-null mice showed no significant changes in relaxation or sensitivity in response to ACh or SNP (**Fig. 35**), nor was there was a significant change in any EC_{50} (**Table 20**). These data indicate that the vascular responses observed in male WT mice in response to CR were likely mediated by TRPA1.

Acute Exposure to Crotonaldehyde and sex dependence

Because acrolein-induced toxicity showed protective in female mice in our previous study. We performed acute exposures to CR (4 days; 1 ppm) in female mice. In contrast to the WT mice, measures of vascular reactivity in female mice showed no significant changes in relaxation or sensitivity in response to ACh or SNP (**Fig. 36**), nor was there was a significant change in any EC50 (**Table 20**). These data indicate that the vascular effects of CR at 1 ppm were sex-dependent.

Acute Exposure to Formaldehyde or Acetaldehyde and Vascular Reactivity

We also measured vascular function in isolated aorta of WT male mice after 4 days of FA (5 ppm), there was no difference in the relaxation and the sensitivity in response to ACh or SNP (**Fig. 37**). Mice were exposed to 2 weeks of AA (5 ppm), there was no difference in the relaxation and the sensitivity in response to ACh or SNP (**Fig. 38**). These results indicated that there was limited vascular dysfunction after acute FA or sub-acute AA exposure.

Compare the vascular toxicity of aldehydes

The PE2/PE1 tension (%) which was calculated by dividing PE-induced tension post-CR-exposure (PE2) by PE-induced tension pre-CR (i.e., PE1) and multiplied by 100. The PE2/PE1 tension (%) was significantly inhibited after CR exposure in vitro compared to FA or AA exposure (**Fig. 39**). The effective concentration inducing 50% relaxation (EC50) of CR-induced vasorelaxation was significantly lower than FA and AA in PE precontracted superior mesenteric artery (SMA) (**Table 21**). The unsaturated aldehyde (i.e., CR) caused more toxic to vascular

smooth muscle than saturated aldehydes (i.e., FA and AA). However, both FA (0~300 μ M), AA (0~30 mM) and CR (0~300 μ M) showed limited endothelial dysfunction after exposure in vitro.

Fig.31. Hemodynamic effects of inhaled crotonaldehyde (CR) in mice. Blood pressure was measured non-invasively by tail cuff method in C57BL/6J male mice weekly (within 1h after exposure) during the chronic air and CR exposures (1 ppm; 6h/d, 5 d/week, 12 weeks) (**A**). Blood pressure (mm Hg) was significantly lower in CR-exposed mice compared with air-exposed control mice over several weeks of the latter half of the exposure (**A**), but there was no consistent change in heart rate (HR, bpm) throughout (**B**). Aortic arch blood pressure was measured via invasive radiotelemetry in a male C57BL/6J mouse during an acute CR exposure (9 min, 1 ppm). Diastolic blood pressure (DBP; **C**) and HR (**D**) fell rapidly upon onset of CR exposure, and both parameters recovered quickly to baseline levels within 10-12 min after ceasing CR exposure. Aortic arch blood pressure was measured via invasive radiotelemetry in male C57BL/6J mice during an acute CR exposure (3h, 1 ppm; 3h, 3 ppm) to simulate our acute and chronic exposure conditions. Although only modest drops in DBP (**E**) and HR (**F**) were observed upon beginning 1 ppm CR exposure, there were much more noticeable drops in DBP (**E**) and HR (**F**) when the CR level was increased to 3 ppm. As with brief CR exposure (9 min, 1 ppm), HR recovered quickly to baseline levels within 5 min of ceasing the CR exposure, however, DBP was slow to recover and did not reach baseline levels after 15 min. Summary data of two mice exposed to CR (1 and 3 ppm, 3h each) showed that all blood pressures (systolic, mean, diastolic; **G**) were suppressed equally by CR exposure whereas mean HR was not (**H**). Values = mean $\pm$ SE (n=9-10 mice per group, **A, B**; n=2, **G, H**); a, $p < 0.05$ vs air control; b, $p < 0.05$ vs baseline.

Table 18. Echocardiography measures in male mice chronically exposed to air or crotonaldehyde.

Measurement	Exposure	
	Air Control (5)	1 ppm CR (5)
Body Weight (g)	29.1 ± 0.5	28.6 ± 0.5
HR (bpm)	476 ± 20	536 ± 13*
EDV (μL)	42 ± 7	41 ± 2
ESV (μL)	12 ± 2	11 ± 1
SV (μL)	30 ± 4	30 ± 2
EF (%)	72 ± 1	74 ± 2
CO (mL/min)	13.9 ± 1.4	16.2 ± 0.7
LVIDd (mm)	3.7 ± 0.1	3.4 ± 0.1
LVIDs (mm)	2.1 ± 0.1	1.9 ± 0.2
LVPWd (mm)	0.8 ± 0	1.0 ± 0.1
LVPWs (mm)	1.3 ± 0.1	1.4 ± 0.1
LVAWd (mm)	1.1 ± 0.1	1.1 ± 0
LVAWs (mm)	1.6 ± 0.1	1.6 ± 0
LVM (mg)	105.4 ± 6.9	103.6 ± 6.8
IVRT (ms)	13.3 ± 0.9	9.6 ± 1.2*
Aortic AVG	2587.0 ± 698.8	1806.8 ± 71.0
Innominate AVG	1209.8 ± 78.6	1216.0 ± 67.1
LCC_AVG	744.6 ± 69.8	770.8 ± 95.4
PWV (Right & Left; mm/s)	2.8 ± 0.1	2.9 ± 0.1
RWT (mm)	0.46 ± 0.03	0.56 ± 0.05

Values = mean±SE. (n), number of mice; *, p<0.05 compared to air control; #, 0.05≤p≤0.10 compared to air control. Abbr.: CO, cardiac output; CR, crotonaldehyde; EDV, end-diastolic volume; EF, ejection fraction; ESV, end-systolic volume; HR, heart rate; IVRT, isovolumic relaxation time; LCC, left coronary cusp; LVAWd, left ventricular anterior wall thickness at end-diastole; LVAWs, left ventricular anterior wall thickness at end-systole; LVIDd, left ventricular internal dimension at end-diastole; LVIDs, left ventricular internal dimension at end-systole; LVM, left ventricular mass; PWV, pulse wave velocity; RWT, relative wall thickness; SV, stroke volume.

Fig. 32. Vascular toxicity of chronic crotonaldehyde (CR) inhalation exposure in mice. Aortic function was measured *ex vivo* in male C57BL/6J mice following 12 weeks of exposure to CR (1 ppm, 6h/d, 5 d/week, 12 weeks). Acetylcholine (ACh) was used to assess aortic endothelium-dependent relaxation efficacy (% relaxation; **A**) and sensitivity (EC_{50} ; **B**) in phenylephrine (PE)-precontracted aortic rings by isometric myography. CR exposure significantly enhanced ACh sensitivity (**B**). There were, however, no changes in aortic efficacy (% relaxation; **C**) and sensitivity (EC_{50} ; **D**) to the endothelium-independent vasorelaxant sodium nitroprusside (SNP). Values = mean $\pm$ SE (n=10 mice per group); *, $p < 0.05$ vs air control.

Fig. 33. Vascular toxicity of acute crotonaldehyde (CR) inhalation exposure in C57BL/6 mice. Aortic function was measured ex vivo following 4 days of exposure of male C57BL/6J mice to either air or CR (1 ppm, 6h/d, 4 days). Acetylcholine (ACh) was used to assess aortic endothelium-dependent relaxation efficacy (% relaxation; **A**) and sensitivity (EC_{50} ; **B**) in phenylephrine (PE)-precontracted aortic rings by isometric myography. CR exposure significantly enhanced ACh efficacy (**A**) and slightly shifted sensitivity (**B**). There were, however, no changes in aortic efficacy (% relaxation; **C**) and sensitivity (EC_{50} ; **D**) to the endothelium-independent vasorelaxant sodium nitroprusside (SNP). Values = mean $\pm$ SE (n=8-10 mice per group); *, $p<0.05$ vs air control. #, $0.05\leq p\leq 0.10$ vs air control.

Fig. 34. Vascular toxicity of acute crotonaldehyde (CR) inhalation exposure in C57BL/6 mice. Aortic function was measured ex vivo following 4 days of exposure of male C57BL/6J mice to CR (3 ppm, 6h/d, 4 days). Acetylcholine (ACh) was used to assess aortic endothelium-dependent relaxation efficacy (% relaxation; **A**) and sensitivity (EC_{50} ; **B**) in phenylephrine (PE)-precontracted aortic rings by isometric myography. CR exposure significantly enhanced ACh efficacy (**A**) and slightly shifted sensitivity (**B**). There was no change in aortic efficacy (% relaxation; **C**) but there was an enhanced sensitivity (EC_{50} ; **D**) in response to the endothelium-independent vasorelaxant sodium nitroprusside (SNP). Values = mean $\pm$ SE (n=10 mice per group); *, $p < 0.05$ vs air control. #, $0.05 \leq p \leq 0.10$ vs air control.

Table 19. Effects of either air or crotonaldehyde (CR; 1 or 3 ppm) exposure on efficacy responses of isolated thoracic aorta of C57BL/6J (wild type, WT) and TRPA1-null mice to pharmacological agents in the absence and presence of nitric oxide synthase inhibitor (L-NAME).

Exposure Conditions			PE, Emax [mN/mm]		ACh, Emax [%]			
Strain (sex)	Level (ppm)	Duration	Air	CR	Air	CR		
WT	1	4 days	4.1 ± 0.7	3.9 ± 0.6	-53.8 ± 4.4	-65.2 ± 5.2*		
TRPA1-null	1	4 days	2.4 ± 0.5	2.1 ± 0.3	-64.6 ± 3.4	-59.8 ± 4.0		
WT	3	4 days	4.0 ± 0.7	3.7 ± 0.7	-49.3 ± 4.6	-57.8 ± 4.4		
WT	1	12 weeks	3.2 ± 0.6	3.6 ± 0.7	-51.2 ± 5.6	-62.6 ± 3.8		
WT (f)	1	4 days	3.5 ± 0.6	4.0 ± 0.6	-76.4 ± 3.8	-77.4 ± 4.6		
Exposure Conditions			PE Ratio, +L-NAME		ACh, [%], +L-NAME		SNP, [%], +L-NAME	
Strain (sex)	Level (ppm)	Duration	Air	CR	Air	CR	Air	CR
WT	1	4 days	1.3 ± 0.03	1.5 ± 0.05*	-1.4 ± 0.5	-0.9 ± 0.5	-96.9 ± 0.7	-98.4 ± 0.6
TRPA1-null	1	4 days	1.8 ± 0.1	2.0 ± 0.2	-1.7 ± 1.1	0 ± 0	-99.7 ± 0.2	-99.6 ± 0.2
WT	3	4 days	1.4 ± 0.04	1.5 ± 0.04 [#]	0 ± 0	0 ± 0	-98.6 ± 1.3	-99.1 ± 0.7
WT	1	12 weeks	1.5 ± 0.1	1.5 ± 0.1	-0.3 ± 0.2	-0.8 ± 0.5	-97.3 ± 1.3	-98.3 ± 0.6
WT (f)	1	4 days	1.5 ± 0.1	1.5 ± 0.05	-0.5 ± 0.4	-1.9 ± 1.5	-99.4 ± 0.5	99.3 ± 0.3

Values = mean ± SE (n=10-11 mice per group); Abbr.: ACh, acetylcholine; CR, crotonaldehyde; EC50, half maximal effective concentration; PE, phenylephrine; SNP, sodium nitroprusside; TRPA1, transient receptor potential ankyrin 1; *, p<0.05 compared with air control based on Bonferroni' s or Dunn' s post-test; #, 0.05≤p≤0.10 compared with air control based on Bonferroni' s or Dunn' s post-test; †, p<0.05 for WT vs TRPA1-null based on Dunn' s post-test; ¶, 0.05<p<0.10 for WT vs TRPA1-null based on Dunn' s post-test.

Table 20. Effects of either air or crotonaldehyde (CR; 1 or 3 ppm) exposure on sensitivity responses of isolated thoracic aorta of C57BL/6J (wild type, WT) and TRPA1-null mice to pharmacological agents.

Exposure Conditions			PE, EC ₅₀ [nM]		ACh, EC ₅₀ [nM]		SNP, EC ₅₀ [nM] ¹	
Strain (sex)	Level (ppm)	Duration	Air	CR	Air	CR	Air	CR
WT	1	4 days	128 ± 30	201 ± 55	285 ± 79	135 ± 31 [#]	11.5 ± 0.7	9.2 ± 0.9 [#]
TRPA1-null	1	4 days	198 ± 34	252 ± 61	156 ± 30	296 ± 124	9.9 ± 0.4	9.9 ± 0.5
WT	3	4 days	139 ± 31	167 ± 26	236 ± 100	102 ± 9	18.8 ± 2.3	12.7 ± 2.8*
WT	1	12 weeks	108 ± 26	231 ± 109	238 ± 53	78 ± 8*	12.0 ± 2.0	8.6 ± 0.6
WT (f)	1	4 days	157 ± 45	234 ± 70	100 ± 15	68 ± 13	8.5 ± 1.9	7.5 ± 1.0

Values = mean ± SE (n=9-11 mice per group); all values are of male mice except where (f) = female mice. ¹, SNP EC₅₀ measured in presence of L-NAME, 100 µM. **Abbr.:** ACh, acetylcholine; CR, crotonaldehyde; EC₅₀, half maximal effective concentration; PE, phenylephrine; SNP, sodium nitroprusside; TRPA1, transient receptor potential ankyrin 1; *, p<0.05 compared with air control based on Bonferroni's or Dunn's post-test; #, 0.05≤p≤0.10 compared with air control based on Bonferroni's or Dunn's post-test.

Fig. 35. Vascular toxicity of acute crotonaldehyde (CR) inhalation exposure in TRPA1-null mice. Aortic function was measured ex vivo following 4 days of exposure of male TRPA1-null mice to CR (1 ppm, 6h/d, 4 days). Acetylcholine (ACh) was used to assess aortic endothelium-dependent relaxation efficacy (% relaxation; **A**) and sensitivity (EC_{50} ; **B**) in phenylephrine (PE)-precontracted aortic rings by isometric myography. CR exposure had no effect on efficacy (**A**) or sensitivity (**B**) of ACh-induced relaxations. Similarly, there were no CR-induced changes in aortic efficacy (% relaxation; **C**) or sensitivity (EC_{50} ; **D**) in response to the endothelium-independent vasorelaxant sodium nitroprusside (SNP). Values = mean $\pm$ SE (n=10-11 mice per group).

Fig. 36. Vascular toxicity of acute crotonaldehyde (CR) inhalation exposure female mice. Aortic function was measured ex vivo following 4 days of exposure of female mice to CR (1 ppm, 6h/d, 4 days). Acetylcholine (ACh) was used to assess aortic endothelium-dependent relaxation efficacy (% relaxation; **A**) and sensitivity (EC_{50} ; **B**) in phenylephrine (PE)-precontracted aortic rings by isometric myography. CR exposure had no effect on efficacy (**A**) or sensitivity (**B**) of ACh-induced relaxations. Similarly, there were no CR-induced changes in aortic efficacy (% relaxation; **C**) or sensitivity (EC_{50} ; **D**) in response to the endothelium-independent vasorelaxant sodium nitroprusside (SNP). Values = mean $\pm$ SE (n=9-11 mice per group).

A.

B.

C.

D.

Fig. 37. The changes of aorta function after 4 days of exposure of FA 5ppm. **A)** The relaxation of aorta to ACh. **B)** The relaxation of aorta to SNP. **C)** The sensitivity of aorta to ACh. **D)** The sensitivity of aorta to SNP. *, $P < 0.05$ vs Control.

A.

B.

C.

D.

Fig. 38. The changes of aorta function after 2 weeks of exposure of AA 5ppm. **A)** The relaxation of aorta to ACH. **B)** The relaxation of aorta to SNP. **C)** The sensitivity of aorta to ACH. **D)** The sensitivity of aorta to SNP. *, $P < 0.05$ vs Control.

4. Discussion

Aldehydes are ubiquitous pollutants and toxic. Extensive epidemiological and experimental data show that aldehyde affect cardiovascular system. However, the processes by which aldehydes impair cardiovascular function and accelerate the cardiovascular disease are not known. This study especially show the vascular toxicity of CR *in vivo* and the possible mechanisms that would help us to study the toxicity of tobacco smoking and environmental pollution, and could also help government regulate the policies.

Crotonaldehyde is a hazardous air pollutant of significant human concern, especially as a component of cigarette smoke (IARC., 1995), which could induce apoptosis and necrosis in epithelial cells (Liu et al., 2010a, 2010b), oxidative stress, inflammation in alveolar macrophages (Facchinetti et al., 2007). In spite of these well-recognized toxicity of crotonaldehyde, the mechanisms underlying adverse effects of CR inhalation are not well understood. To our knowledge, this is the first study to evaluate the cardiovascular effects of CR exposure *in vivo* and propose that CR may contribute to vasodilation via a TRPA1- and endothelium-mediated pathway.

Isovolumic relaxation time (IVRT) is an interval in the cardiac cycle, from the aortic component of the second heart sound, that is, closure of the aortic valve, to onset of filling by opening of the mitral valve. The IVRT likely decreased with CR exposure because of the increased of heart rate or the decreased of the afterload as a result of low blood pressure (see **Table. 18**). We measure the blood pressure of the chronically-exposed mice immediately following an exposure

period once a week for the duration of the study. The blood pressure significantly decreased in CR group compared with air group, although there was no difference in heart rate between these two groups (see **Fig. 32**); this might be the reason of decreased IVRT. Many studies have showed that shear stress is critically important in regulating the vascular physiology and pathobiology of the vessel wall by modulating endothelial cell (EC) function (C. Cheng et al., 2004; Malek, Alper, & Izumo, 1999). In particular, low and oscillatory shear stress, but not increased shear stress, contributes to the development of atherosclerotic lesions (C. Cheng et al., 2006). Shear stress and stretch could also modulate EC functions by activating mechano-sensors and signaling pathways. Low shear stress in vitro can downregulate endothelial nitric oxide synthase (eNOS) and upregulate endothelin-1 [Ziegler et al., 1998] and adhesion factors (Chappell, Varner, Nerem, Medford, & Alexander, 1998), which results in an increase in the adhesion of monocytes. Of course, strong irritation in the airways can cause airway hyperresponsiveness and respiratory depression, and which are associated with systemic hypotension, though hypotension may also be caused by systemic cardiovascular depression due to parasympathetic vagal reflexes (B. P. Lee, Morton, & Lee, 1992; Polverino et al., 2012).

Regardless of the mechanism, coronary vasospasm or peripheral vasodilation can cause ischemia and heart attack or hypotension and syncope, and/or even death and thus could be responsible for the drop in blood pressure after CR exposure. Furthermore, in isolated aorta of 4-day 3 ppm exposed mice, the sensitivity and relaxation of aorta to SNP increased significantly in CR group,

and there was a trend that the sensitivity and relaxation of ACh also was increased in CR group (see **Table 19; Fig. 34**). We thus decreased the dose of CR to 1ppm to test for the effects at a lower level of exposure. In isolated aortas of 4-day and 12-week 1 ppm exposed mice, the sensitivity and relaxation of aorta to ACh significantly increased in CR group. What's more, there was a trend that the sensitivity and relaxation of SNP increased in the exposed CR group after 4 days of exposure (see **Table 19 and Fig. 32**). These data show that the vasculature becomes more sensitive to both direct additions and chronic exposures to CR, with the resulting effects being augmented smooth muscle relaxation. These consistent vascular effects indicate that chronic exposure to CR may lead to compensatory changes in the vasculature to offset excessive smooth muscle relaxation and hypotension.

Endothelium is important regulation of vascular tone through the production of a variety of mediators such as nitric oxide, reactive oxygen species, prostanoids, and endothelin. It not only acts as a barrier but also acts as an active signal transducer (Vita & Keaney, 2002). Alteration in endothelial function precedes the development of morphological atherosclerotic changes and also contributes to the initiation and evolution of prothrombotic, proinflammatory and proliferative states. Endothelial dysfunction refers to impairment of endothelium-dependent vasodilatation and implies widespread abnormalities in endothelial integrity and homeostasis; this dysfunction might occur at an early stage of hypertension (Deanfield et al., 2007). Evaluation of endothelial function has been critical to our understanding of the significance of endothelium to cardiovascular system.

Exposure to FA (5 ppm), AA (5 ppm) and CR 1 ppm showed limited endothelial effect in our study, but, CR exposure showed significant difference between WT mice and TRPA1-null mice.

TRPA1 is a cationic (calcium) channel and a promiscuous receptor concentrated in sensory fibers (unmyelinated C-fibers) that mediates pain reception/transmission (nociceptors). The distribution of TRPA1 receptors has expanded to non-neuronal system, such as heart, endothelium, urothelium and in the vasculature (Bautista et al., 2006; Bessac et al., 2008; Earley et al., 2009a; Sinharoy et al., 2017). Activation of peripheral sensory fiber TRPA1 leads to pain signaling and release of vasoactive peptides, substance P (SubP) and cGRP (Russell, King, Smillie, Kodji, & Brain, 2014; Trevisan et al., 2016), which may contribute to increased vasodilation and inflammatory response such as increased blood flow, increased vascular permeability (edema), increased white blood cell binding, and extravasation and pain (D. J. Conklin, 2016). TRPA1 is in the pulmonary airways and it plays a role in the protective physiological maneuver called “respiratory braking” elicited by inhaled toxins. Our previous study showed that TRPA1-null mice are more sensitive than WT mice to acrolein exposure and that TRPA1 antagonist injection post-acrolein exposure reduces acrolein-induced mortality (D. J Conklin et al., 2017; D. J. Conklin et al., 2017). This is consistent with the results that combined with TRPA1 antagonists (HC-030031) and TRPV1 antagonists (AMG 9810) can prevent pain and inflammation in pancreatic cancer mice (Schwartz et al., 2011). Interestingly, compared to the augmentation vasodilation in WT mice after CR exposure, there was no difference both in the

sensitivity and relaxation of ACh and SNP in CR group and air group in TRPA1-null mice after CR exposure (see **Table 19** and **Fig. 36**). This disappearance of augmented smooth muscle relaxation in the vasculature indicate that TRPA1 plays an specific role in the toxicity of CR. Future study still needs to make clear whether TRPA1 plays an protective function or induces impairment to the blood vessels.

The study of sex difference is very important in the exposure-related cardiovascular effects. Our previous study showed that female mice exposed to a high level of acrolein showed both pulmonary and systemic protection compared to the male mice, demonstrating reduced morbidity and a significantly lower sensitivity to acrolein-induced mortality (D. J Conklin et al., 2017). Although both acute and chronic exposure to CR induced vascular changes in male mice, females showed no changes in aortic sensitivity or reactivity in response to CR exposure in vivo (see **Fig. 36**). These differences between males and females post-CR exposure seem to support our previous findings that females are somewhat protected against α,β -unsaturated aldehyde-induced toxicity and injury.

An aldehyde is a compound containing a functional group with the structure $-\text{CHO}$, which is a carbon double-bonded to oxygen. Numerous studies show that unsaturated aldehydes are more toxic than saturated aldehydes in Pharmacodynamics. Our study also show that the concentrations of aldehydes cause relaxation of SMA is much higher in saturated aldehydes than in unsaturated aldehydes in vitro (see Chapter III, IV, V). The PE2/PE1 tension (%) was significantly inhibited after CR exposure compared to FA or AA exposure in vitro

(see **Fig. 39**). The effective concentration inducing 50% relaxation (EC₅₀) of CR-induced vasorelaxation was significantly lower than FA and AA in PE precontracted superior mesenteric artery (SMA) (see **Table 22**). CR induce more toxic to vascular smooth muscle than FA or AA. However, both FA (0~300 μ M), AA (0~30 mM) and CR (0~300 μ M) didn't cause endothelial dysfunction after exposure in vitro. Future studies are needed to show the mechanism and pathology of aldehydes-induced endothelial dysfunction in vasculature.

In conclusion, this study indicates that chronic exposure to CR may lead to compensatory changes in the vasculature to offset excessive smooth muscle relaxation and hypotension that may lead to vascular disease. Because the vascular changes were not seen in TRPA1-null or in female WT mice, we conclude that these CR-induced changes within the vasculature are TRPA1- and sex-dependent. However, additional studies are needed to further clarify these findings as well as to provide a better mechanistic understanding of CR-induced cardiovascular pathophysiology. As exposure to CR through both ambient and personal means continues to be a potential health threat to the global population, our current results provide novel insights into the cardiovascular effects of CR and implicate chronic low levels of exposures as a potential inducer of CVD. Future studies are required to test the mechanisms and the toxicology of aldehydes.

Table 21. The effective concentration inducing 50% relaxation (EC50) of aldehyde-induced vasorelaxation in isolated murine (male) superior mesenteric artery (SMA) with different contractile agonists in the absence and presence of the nitric oxide synthase (NOS) inhibitor, LNAME.

EC50 (SMA)	PE (10 ⁻⁵ M)		U46619(10 ⁻⁷ M)		High K ⁺ (60mM)	
		+L-NAME		+L-NAME		+L-NAME
Formaldehyde (μ M)	52.4 $\pm$ 13.9	206.9 $\pm$ 10.4	514.5 $\pm$ 74.4	568.9 $\pm$ 56.6	1093.2 $\pm$ 50.4	984.1 $\pm$ 52.8
Acetaldehyde (mM)	3.3 $\pm$ 0.3	6.2 $\pm$ 0.6	14.9 $\pm$ 1.5	17.2 $\pm$ 0.2	17.5 $\pm$ 0.5	18.4 $\pm$ 0.4
Crotonaldehyde (μ M)	3.8 $\pm$ 0.5	43.9 $\pm$ 17.7				
Cinnamaldehyde (μ M)	11.9 $\pm$ 3.1	327.6 $\pm$ 96.4				
AITC (μ M)	3.9 $\pm$ 1.0					

Fig.39. Vascular smooth muscle toxicity of aldehydes (**A**) and endothelium function change after aldehydes exposure (**B**). PE, phenylephrine; the PE2/PE1 tension (%) was calculated by dividing PE-induced tension post-CR-exposure (PE2) by PE-induced tension pre-CR (i.e., PE1) and multiplied by 100.

A.

B.

CHAPTER VII

SUMMARY AND CONCLUSIONS

A. Major findings in this dissertation

1. Aldehydes exposure and vascular response *in vivo* and *in vitro*

Aldehydes could be delivered either via inhalation, oral gavage, or intravenous injections. All these pathways would increase the concentration of aldehydes in the blood, which cause vasorelaxation of the blood vessels, including the blood vessels in the gastrointestinal (GI) tract (D. J. Conklin et al., 2004; Jin, Jagatheesan, et al., 2019). Although the effects of aldehydes have been studied for several decades, the vascular effects of aldehydes are still unclear. This study mainly focuses on the vascular toxicity of inhaling aldehydes (FA, AA, and CR) and the underlying mechanisms.

Mice were exposed to CR (1 ppm) for 12 weeks, in the isolated aortas of exposed mice, the efficacy and sensitivity of ACh significantly increased in PE-precontracted aortic rings by isometric myography (see **Chapter VI, Fig. 32; Table 19**). In isolated aortas of 4-days CR 1 ppm exposed mice, CR exposure significantly enhanced Ach efficacy and slightly shifted sensitivity. However, no

changes in aortic efficacy and sensitivity to SNP (see **Chapter VI, Fig. 33; Table 19**). These data show that the vasculature becomes more sensitive to both direct additions and chronic exposures to CR, with the resulting effect being augmented smooth muscle relaxation. Acute exposure to CR 1 ppm significantly decreased the blood pressure during CR exposure by telemetry, did not recover shortly after CR exposure stop; these results were very robust and consistent (see **Chapter VI, Fig. 31**). Our study showed that inhalation of low concentrations of CR might affect the function of smooth muscles and induce hypotension. Many studies showed that hypotension could affect the quality of life and also induce cardiovascular diseases, such as myocardial infarction, syncope, heart failure, etc. (Duschek, Hoffmann, & Reyes Del Paso, 2017; Owens & O'Brien, 1999; Shibasaki, Ogawa, Yamada, Ouchi, & Akishita, 2018; Tsujimoto & Kajio, 2018), mice might also be hurt by hypotension. Chronic exposure to CR may lead to compensatory changes in the vasculature to offset excessive smooth muscle relaxation and hypotension, which might promote the release of neuronal hormones to increase blood pressure that would cause disease. We could not rule out that inhalation of CR might induce cardiovascular disease after long exposure.

FA and AA are contained in cigarette smoke. Our previous study showed that the levels of aldehydes were high in cigarettes (Lorkiewicz et al., 2019; Schick et al., 2017). In our study, after acute exposure to FA or subacute exposure to AA, there were no differences in the sensitivity or relaxation of ACh and SNP in response to isolated aortas between the control and exposed groups

(see **Chapter VI, Fig. 38**). The effect of FA or AA exposure on endothelium function is very limited at this exposure level. Many studies showed that FA is a carcinogen and an irritant, and we could not exclude the possibility that cancer would present an earlier risk than cardiovascular impairment (Nielsen, Larsen, & Wolkoff, 2017; Tyihak, Bocsi, Timar, Racz, & Szende, 2001). However, the risks of chronic inhalation of FA or AA at the levels found in tobacco or e-cigarette aerosols are still unknown.

An aldehyde is a compound containing a functional group with the structure -CHO , which is a carbon double-bonded to oxygen, and unsaturated aldehydes are more active than saturated aldehydes. Our study shows that the concentrations of aldehydes required to cause relaxation of SMA is much higher in saturated aldehydes than in unsaturated aldehydes *in vitro* (see **Chapter III, IV, V**); FA and AA relaxed agonist-induced tension by >90% in SMA and aorta. The PE2/PE1 tension (%) was significantly inhibited after CR exposure compared to FA or AA exposure *in vitro* (see **Chapter VI, Fig. 39A**). The effective concentration inducing 50% relaxation (EC_{50}) of CR-induced vasorelaxation was significantly lower than FA and AA in PE-precontracted SMA (see **Chapter VI, Table 22**). Future studies are needed to show the mechanism and pathology of aldehyde-induced toxicity in the vasculature. CR stimulated isometric tension oscillations at high concentrations ($\geq 300 \mu\text{M}$) (see **Chapter V, Fig.23B**), though FA and AA did not, the mechanism of the oscillations is thus

unclear. It might be dependent on the toxicity since it only showed in unsaturated aldehydes.

In conclusion, this study indicates that CR is more toxic to vasculature *in vitro* than FA and AA. Chronic exposure to CR may lead to compensatory changes in the vasculature to offset excessive smooth muscle relaxation and hypotension that may lead to vascular disease. However, additional studies are needed to further clarify these findings as well as to provide a better mechanistic understanding of CR-induced cardiovascular pathophysiology. As exposure to CR through both ambient and personal means continues to be a potential health threat to the global population, our current results provide novel insights into the cardiovascular effects of CR and implicate chronic low levels of exposures as a potential inducer of CVD.

2. Aldehydes and endothelium function

It is well known that the endothelium is very important in the regulation of vascular tone in the blood vessels (Furchgott & Zawadzki, 1980; Palmer, Ferrige, & Moncada, 1987). Endothelial dysfunction refers to impairment of endothelium-dependent vasodilatation, which implies widespread abnormalities in endothelial integrity and homeostasis and might occur at an early stage of hypertension (Deanfield et al., 2007), and it is the precursor of atherosclerosis and inflammation (Vanhoutte, Shimokawa, Feletou, & Tang, 2017; Versari, Daghini, Viridis, Ghiadoni, & Taddei, 2009). Our previous study showed that acrolein

feeding increased atherosclerotic lesion formation in the aorta (Srivastava et al., 2011), and 5ppm of acrolein derived from tobacco smoke induced endothelium dysfunction in GSTP-null mice (D. J. Conklin, Haberzettl, et al., 2009b).

In contrast to the idea that FA induces oxidative stress in endothelial cells (Yu, 1998), our data indicate that in our endothelial dysfunction model *in vitro*, endothelial dysfunction blocks only the physiological (sensitive) component of FA-induced vasorelaxation. We did not observe FA-, AA-, or CR-induced endothelial dysfunction even after exposing SMA to 1,000 μ M FA, 300 mM AA or 300 μ M CR. The relaxation of ACh was not different from vehicle group after CR exposure (see **Chapter VI, Fig. 39B**). This is consistent with our previous study: in isolated human coronary artery bypass graft (CABG) blood vessels with endothelial dysfunction, FA still induces relaxation, but in a far less sensitive manner (EC_{50} , 315 ± 0 mM) (D. J. Conklin et al., 2004) than that present in healthy mice as seen in our study.

Acute exposure to FA or CR, subacute exposure to AA, and chronic exposure to CR showed limited endothelial dysfunction in the isolated aortas. This could be because the length and dose of exposure were not enough to see an effect or because arteries other than the aorta are more sensitive to aldehydes exposure *in vivo* study. It is well known that the effect of aldehydes is time- and dose-dependent (D. J. Conklin et al., 2004). The velocity of blood flow in the aorta is so fast that the amount of time the aldehydes affect endothelial cells in aorta is limited, or the little damaged endothelial cells might be repaired very fast.

3. Aldehydes and postprandial hyperemia

Our study showed that urinary levels of the metabolites of FA and AA increase with feeding such that FA and AA likely contribute to the physiological reflex of post-prandial hyperemia via SMA vasodilatation. The SMA is a major arterial blood supply to the GI tract, and it dilates postprandially in response to many stimuli e.g., nutrients, vago-vagal reflex, NO, and substance P (Chou & Coatney, 1994; Lucchini et al., 1996). These aldehydes are often considered to be toxicants, but they are naturally abundant in food, water, and other beverages (Aldehydes, 1981; D. J. Conklin, P. Haberzettl, et al., 2011) and their abundant metabolic products make them plausible stimuli for increasing GI blood flow. This is the first suggestion of these physiological roles; more research will be needed to establish these aldehydes as bona fide contributors to postprandial hyperemia *in vivo*.

4. The mechanisms of vasodilation of aldehydes

L-NAME, an NO inhibitor, totally blocked the relaxation of the aorta (about 90%), but the relaxation of the SMA is much less (approximately 15%) (data not shown). We conclude that endothelium-derived hyperpolarizing factor (EDHF) is the dominant mechanism in the relaxation of the SMA, which is consistent with a previous study showing that EDHF is much more important in the relaxation of smaller blood vessels than in large conduit vessels (Tomioka et al., 1999).

Testing the function changes in SMA is therefore a good way to study the smaller blood vessels as well as resistance arteries. Although the aorta does not directly regulate blood pressure, the aorta is an important marker of systemic vascular dysfunction, especially of endothelial dysfunction and atherosclerosis risk. We thus test the changes of aorta function in our *in vivo* studies and explore the mechanisms of vasodilation in SMA in our *in vitro* studies. The mechanisms of the vascular effects of aldehydes have been studied for several decades, but the complex mechanisms of this phenomenon are only now becoming clear.

We show that there are 3 distinct pathways of FA- and CR-induced vasorelaxation in SMA and 2 distinct pathways of AA-induced vasorelaxation in SMA (see **Chapter III, Fig. 15; Chapter IV, Fig. 22; Chapter V, Fig. 29**) and while this is fascinating in its own right, we focused on the mechanism of the most sensitive, and thus, most likely physiological pathway. The sensitive FA-, AA- and CR-induced vasorelaxations require a functional endothelium, NOS (presumably eNOS), and guanylyl cyclase (GC/cGMP). The endothelial cells likely contribute to the most sensitive components, whereas the latter component is likely present in vascular smooth muscle cells (VSMC). We propose a model for each of these three aldehydes featuring a stepwise sequence of events that is quite typical of agonists that elicit EDRF/NO. However, we also cannot rule out the possibility that additional components in the vascular wall (perivascular nerves, SubP, CGRP, etc...) may contribute to the AA-induced relaxation (Nilius et al., 2012).

The EC₅₀ of FA, AA and CR are 52.4 ± 13.9 μM, 3.3 ± 0.3 mM and 3.8 ± 0.5 μM, respectively, which show that CR is much more toxic than FA and AA in the vasculature. As we know, it is very difficult to test the actual concentrations of aldehydes in the blood and in the cells since the aldehydes are so active and metabolism of aldehydes occurs quickly. One study showed the concentration of FA was in the range of 1.27 ×10⁻² to 2.28 μg/mL in the blood (Y. S. Wang et al., 2011), which looked much lower than the EC₅₀ of relaxation in our study. The sensitivity of FA- and AA-induced relaxation (but not efficacy) was agonist-dependent (PE<U46,619=High K⁺) (see **Chapter VI, Table 22**) and indicated K⁺ channel plays an important role in the relaxation of FA and AA. What's more, we show that Kir channel regulate the relaxation of FA.

5. Aldehydes and TRPA1 Receptor

Our study show several distinct pathways of FA-, AA-, and CR-induced vasorelaxation in SMA (see figures in **Chapter III, IV, V**). We focus on the mechanism of the most sensitive, and thus, most likely, physiological pathway. FA-, AA-, and CR-induced vasorelaxation requires a functional endothelium, NOS (presumably eNOS), and guanylyl cyclase (GC/cGMP), and what's more, FA- and CR- induced vasorelaxation in a similar pathway via the TRPA1 channel. It is likely that the TRPA1 and NOS components are in endothelial cells, whereas the latter is also present in vascular smooth muscle cells (VSMC).

In addition, several unsaturated aldehydes including acrolein, cinnamaldehyde, and 4HNE induce sensitive and robust vasodilation in mesenteric arteries (Awe et al., 2006; Romero et al., 1997) and other blood vessels (Yanaga et al., 2006) as well as tracheal smooth muscle (Cheah et al., 2014), perhaps indicating a shared (universal) aldehyde-TRPA1 pathway. TRPA1 is a cation (calcium) channel and a promiscuous receptor concentrated in sensory fibers (unmyelinated C-fibers) that mediate pain reception/transmission (nociceptors), and it is also present in the vasculature (Earley et al., 2009a; Sinharoy et al., 2017). It is a well-known target of unsaturated aldehydes, such as acrolein and crotonaldehyde (Andre et al., 2008; Bessac et al., 2008; D. J. Conklin, 2016) and cinnamaldehyde (Pozsgai et al., 2010), but its activation by FA is somewhat surprising given the proposed mechanism of unsaturated aldehyde-mediated activation via conjugation with TRPA1 N-terminal free cysteines (Macpherson et al., 2007). Activation of peripheral sensory fiber TRPA1 leads to pain signaling and release of vasoactive peptides, substance P (SubP), and cGRP (Trevisan et al., 2016), which may contribute to increased vasodilation. We confirmed localization of TRPA1 in mouse dorsal root ganglion (DRG) and showed Immunofluorescent staining in the endothelium of murine SMA but not in aorta.

In our *in vivo* study, compared to the augmentation vasodilation in WT mice after CR exposure, there was no difference in the sensitivity or relaxation of ACh and SNP in CR group and air group in TRPA1-null mice after CR exposure; the compensatory changes in the vasculature were missing in TRPA1-null mice

(see **Chapter VI, Table 19,20 and Fig. 35**). This disappearance of augmented smooth muscle relaxation in the vasculature indicates that TRPA1 plays a specific role in the toxicity of CR.

TRPA1 is found in the pulmonary airways, and it plays a role in the protective physiological maneuver called “respiratory braking” elicited by inhaled toxins. Our previous study showed that TRPA1-null mice are more sensitive than WT mice to acrolein exposure because of its “respiratory braking”, and that TRPA1 antagonist injection post-acrolein exposure reduces acrolein-induced mortality (D. J Conklin et al., 2017; D. J. Conklin et al., 2017). Our results show that TRPA1 receptor plays a much more important role in the vascular toxicity of the unsaturated aldehydes than saturated aldehydes. Future studies are needed to make clear whether TRPA1 plays a protective function or induces impairment to the blood vessels.

In our *in vitro* study, SMA is the main artery in the GI system, and food is a main source of endogenous aldehydes. Cho et al. showed that TRPA1 is present in sensory nerve endings and in some gastrointestinal enteroendocrine cells in the mouse gastrointestinal tract (Cho, Callaghan, Bron, Bravo, & Furness, 2014). Our study also showed the immunofluorescence staining of TRPA1 present in the endothelium of the blood vessels in SMA. The specific TRPA1 antagonist, A967079 ($IC_{50} = 289$ nM), significantly blocked the most sensitive component of the FA- and CR-induced relaxation and shifted the EC_{50} to the right quantitatively like that of endothelium disruption but less so than L-NAME treatment (see

Chapter III, Fig. 11; Chapter V, Fig. 25). At high concentrations of FA and CR—likely at unphysiological levels of FA and CR—the TRPA1 antagonist did not affect relaxation, reflecting a non-TRPA1 pathway(s), perhaps via TRPV1 or TRPV4 channels (Ma et al., 2008). We cannot rule out the possibility that additional components in the vascular wall (perivascular nerves, SubP, CGRP, etc.) also may contribute to overall FA- and CR-induced relaxation (Nilius et al., 2012).

6. Aldehydes and Nicotine

Nicotine is an important component of tobacco smoke, and extensive research has shown nicotine is a main reason for increased cardiovascular events via activation of the sympathetic nervous system, increased myocardial remodeling, impaired lipid metabolism, and induction of inflammation (Benowitz & Burbank, 2016; Dutta et al., 2012). However, some papers show that nicotine is a double-edged sword which promotes angiogenesis and arteriogenesis on the contrary (Heeschen, Weis, & Cooke, 2003; J. Lee & Cooke, 2012).

Few studies show the interaction between nicotine and aldehydes *in vivo*, especially the effect of nicotine on the cardiovascular effects of aldehydes. Nicotine could metabolize to methylamine and then metabolize to FA by semicarbazide-sensitive amine oxidase (SSAO) (D. J. Conklin et al., 2004; Yu, 1998). Previous studies have shown a dose-related interaction between nicotine

and AA in conditioned taste-aversion (CTA) paradigm (Kunin, Latendresse, Gaskin, Smith, & Amit, 2000).

One purpose in our study is to investigate the effect of nicotine on the function of aldehydes. The plateau concentration of nicotine in the blood averaging 40 µg/L throughout the day in smokers (Moyer et al., 2002). In this study, we aim to mimic the vascular effect of nicotine and aldehydes when smoking or smoking as well as drinking. Our study showed that low concentrations of nicotine as well as 1 µM does not affect the relaxation of FA, AA, or CR in SMA (see **Chapter III, Chapter IV, Table 11,12; Chapter V, Fig. 29**). Nicotine does not affect the vascular function of aldehydes *in vitro*.

Nicotine mainly binds and activates nicotine acetylcholine receptor (nAChR), but it is also a sensory agonist that activates TRPA1 and is responsible for the airway constriction reflex (Talavera et al., 2009). TRPA1 is a well-known target of acrolein, cinnamaldehyde, FA, and other aldehydes (Andre et al., 2008; Bessac et al., 2008; D. J. Conklin, 2016; Pozsgai et al., 2010). However, the function of TRPA1 in nicotine exposure and the effect of nicotine on aldehydes *in vivo* are still unclear.

7. Aldehydes and sex difference

This data in our study was consistent with our previous study, in which female mice exposed to a high level (210-250 ppm) of acrolein showed both

pulmonary and systemic protection compared to their male counterparts, demonstrating reduced morbidity and a significantly lower sensitivity to acrolein-induced mortality female mice showed a protective effect under acrolein exposure (D. J Conklin et al., 2017). Although both acute and chronic exposure to CR induced vascular changes in male mice, females showed no changes in aortic sensitivity or reactivity in response to CR exposure in vivo (see **Chapter VI, Fig. 36**). These differences between males and females post-CR exposure seem to support our previous findings that females are somewhat protected against α,β -unsaturated aldehyde-induced toxicity and injury. This is consistent with the human studies, rodent studies have also found that females are less sensitive than males in tobacco-induced atherosclerotic damage in part because of protective effects of female hormones (Bolego, Poli, & Paoletti, 2002). Females had greater pain in some inflammatory conditions than males, which is under the control of prolactin (Patil, Green, Henry, & Akopian, 2013). Prolactin receptor regulates transient receptor potential (TRP) channels in neurons including TRPA1, TRPV1, and TRPM8 and has a sex difference (Patil, Ruparel, Henry, & Akopian, 2013). The mechanism of female-dependent protection in mice is not clear. Future studies are needed to explore the sex-dependent differences in respiratory, cardiovascular, and systemic toxicity in electronic cigarette aerosols or aldehyde exposures.

8. Normalization of aorta contractility in testing vascular function

Vascular function measurements are widely used in the study of pathogenesis of cardiovascular, cerebrovascular, pulmonary vascular, and kidney diseases (D. J. Conklin, 2016; D. J. Conklin et al., 2004; Earley, Gonzales, & Crnich, 2009b; Jin, Jagatheesan, et al., 2019; Jin et al., 2018). In our *in vitro* studies, isolated vascular rings were hung on the organ bath systems and were in the Krebs-Henseleit equilibrium solution bubbling with a mixture of gases (95% O₂ + 5% CO₂) to mimic physiological conditions. The blood vessels were given a certain preload by pulling by two wires to mimic a physiological or pathological condition *in vivo*. We then studied the contractility and relaxation of the blood vessels. Our method proved to be a reliable method for the comparison of contractility outcomes from different studies. We developed a simple 2-step aorta normalization method: (1) measure blood vessel segment length (mm), area (mm²) and calculate volume (mm³); then, (2) normalize isometric contraction (mN) by segment length and volume. We recommend that aortic contractions be normalized to segment length and/or volume to reduce variability, enhance efficiency, and to foster universal comparisons across isometric myograph platforms, laboratories, and experimental settings.

B. Strength

First, we show that the vascular toxicity of aldehydes *in vivo* exposure, which are absolutely associated with cigarette smoking. Second, this is the first study to show the vascular effects of CR exposure *in vivo* exposure, although the effect of endothelium dysfunction is limited. We also test the vasodilation of CR *in vitro* and showed the possible mechanism. Third, FA and AA are all very

sensitive to the vasodilation of aorta and SMA. However, FA and AA at our exposure levels showed limited toxicity to blood vessels *in vivo* exposure or the direct effect to the blood vessels *in vitro* even at very high concentrations. Fourth, we tested the direct vascular effects of three aldehydes from low concentrations to high concentrations *in vitro*, which help us study the real biological changes to aldehydes exposure in life. Fifth, we show a good pharmacological model of endothelial dysfunction which is caused by air perfusion. Sixth, since the 85% vasorelaxation of SMA is EDHF dependent, it is a good choice to select SMA as a representative to study the function of small arteries, and it is the most important artery to control the blood flow to GI system. Seventh, we show the function of TRPA1 receptor in the mediation of cardiovascular toxicity of CR. And, we show TRPA1 staining in the endothelium. TRPA1 might be the target for medical treatment after aldehydes exposure.

C. Limitations

There are important caveats to consider in this study. First, mice are obligate nose breathers and are “involuntary smokers,” meaning that they decrease breathing rate and depth to reduce the inhalation of toxins such as acrolein (D. J Conklin et al., 2017). This reflex is known as the “respiratory braking response” (Alarie, 1973). The nose is very sensitive to aldehydes exposure, it would be swelling because of edema, and the mice even die of hypoxia because nose congestion. Second, low levels of FA, AA, and CR did not induce endothelial dysfunction, even in the chronic exposure of CR. This could

be because the level of exposure was too low and/or because and the length of exposure was not sufficient to see significant changes. It is difficult to decide the exposure time for these inhalation experiment as several factors can complicate our desired outcomes. As we know, aldehydes are carcinogenic as well as irritants, and it is possible that cancer could develop before the development of negative cardiovascular effects during exposure. Third, we only tested the vascular function changes in aorta and SMA; we have not tested functional changes in other arteries such as the pulmonary artery and the third or fourth segments of mesenteric artery that might more sensitive to inhalation exposure. Fourth, we did not test whether TRPA1 antagonist had protective yet nonspecific effects, especially in the chronic exposure of CR. Testing the function of TRPA1 antagonist is needed in order to further explore the function of TRPA1 receptor. Fifth, perivascular adipose tissue (PVAT) is very important to the function of blood vessels, and there is a lot of TRPA1 receptor staining in the PVAT. We have not yet tested the effect of TRPA1 on the PVAT on the relaxation of blood vessels. Sixth, in vivo exposure study, the concentrations of aldehydes are continuous though the real smoking is intermittent since it's hard to prudence intermittent pattern during aldehyde exposure in our exposure system. Seventh, aldehydes are very active and the metabolisms of aldehydes are very fast. It's difficult to measure the concentration of aldehyde in the blood.

D. Future direction

Additional studies are still needed to further explore the toxicity of e-cigarettes and cigarettes. We still have not identified the actual constituent that is responsible for the toxicity of smoking or how to cure the toxicity of smoking. We could test other possible mechanisms that mediate cardiovascular toxicity of aldehydes. In the future, we could increase the dose of aldehydes or the length of exposure to test whether it induces endothelial dysfunction. We could also test the vascular function changes of other arteries such as the pulmonary artery and smaller arteries such as the third or fourth segments of mesenteric artery. Additional studies could also be performed to determine the effect of TRPA1 antagonists during aldehydes exposure in mice as well as the effect of PVAT on the relaxation of blood vessels, including testing the function of TRPA1 receptor in PVAT. We could test CR exposure in atherosclerosis models to test whether TRPA1 could block atherosclerosis. We could test CR exposure in TRPA1-null mice. Finally, more studies are still needed to examine the effect of e-cigarette flavors on cardiovascular function such as testing blood pressure, heart rate, and body temperature changes during flavor exposures in mice.

E. Conclusion

These studies indicate that short-term exposures of mice to FA or AA have limited direct toxicity in aorta. However, CR exposures (acute and chronic) lead to altered vascular function that may lead to cardiovascular disease. Moreover, we describe direct effects of FA, AA, and CR that are dependent on an EDRF/NO and VSMC GC/PKG pathway in SMA, with FA- and CR-induced

relaxations also dependent on an endothelium-localized TRPA1 receptor. From our *in vitro* study, we conclude that aldehydes play a key role in the regulation of blood flow in the gastrointestinal (GI) tract to augment digestion and nutrient absorption and induce postprandial hyperemia, what's more, the TRPA1 receptor plays an important role in the aldehyde-induced vasodilation. Future studies are required to better understand how CR exposure modifies cardiovascular pathophysiology and the specific roles that TRPA1 and sex play in contributing to the burden of tobacco-related CVD including pathophysiological processes of atherosclerosis.

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ABBREVIATIONS

AA	Acetaldehyde
ACh	Acetylcholine chloride
ADH	Alcohol dehydrogenase
AITC	Allyl isothiocyanate
ALDH2	Acetaldehyde dehydrogenase 2
ALT	Alanine transaminase
ANOVA	Analysis variation
AR	Aldose reductase
AST	Aspartate transaminase
BaCl ₂	Barium chloride
CACs	Circulating angiogenic cells
CK	creatinine kinase
CR	Crotonaldehyde
CVD	Cardiovascular disease
CO	Cardiac output
EC	Endothelial cell
EC ₅₀	Half maximal effective concentration

EDHF	Endothelium-Derived Hyperpolarizing Factor
EDRF	Endothelium-Derived Relaxing factor
EPC	Endothelial progenitor cell
FA	Formaldehyde
FALDH2	Formaldehyde dehydrogenase
GC	Guanylyl cyclase
GI	Gastrointestinal
GSH	Glutathione
GSTP	Glutathione S-transferase P
GTT	Glucose tolerance test
HbA1c	Whole blood hemoglobin A1c
HPMMA	3-hydroxy-1-methylpropylmercapturic acid
3-HPMA	3-hydroxypropyl mercapturic acid
4HNE	and 4-hydroxynonenal
HR	Heart rate
IL-6	Interleukin 6
IVRT	Isovolumic relaxation time
LDH	Lactate dehydrogenase
LDLR-KO	Low-density lipoprotein receptor deficient

L-NAME	N&-Nitro-Larginine methyl ester hydrochloride
MCS	Mainstream cigarette smoke
NO	Nitric oxide
NOS	Nitric oxide synthase
ODQ	1 h-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
PE	L-phenylephrine hydrochloride
PKG	Protein Kinase G
PMA _s	Platelet-monocyte aggregates
PSS	Phosphate-buffered solution
PWV	Pulse wave velocity
SMA	Superior mesenteric artery
SNP	Sodium nitroprusside
TEA	Tetraethylammonium chloride
TNF α	tumor necrosis factor alpha
TRPA1	Ttransient receptor potential ankyrin 1
VSMC	Vascular smooth muscle cells
WT	Wild type

CURRICULUM VITAE

Lexiao Jin

505 South Hancock Street, Room 552G

Louisville, KY 40202

Phone: 502-936-2316

Email: l0jin003@louisville.edu

Present appointment:

2016.6-current PhD student in Pharmacology and Toxicology

University of Louisville, Louisville, KY

2009-current Attending doctor at the Department of Anesthesiology

The Second Affiliated Hospital & Yuying Children's Hospital of

Wenzhou Medical College, Zhejiang Province.

P.R.China. 325027

Education

06/2003 M.D. in Clinical Medicine

Wenzhou Medical University, China

12/2008 M.S. in Anesthesiology

Wenzhou Medical University, China

Publication

1. **Jin L**, Jagatheesan G, Guo L, Nystoriak M, Malovichko M, Lorkiewicz P, Bhatnagar A, Srivastava S, Conklin DJ. Formaldehyde Induces Mesenteric Artery Relaxation via a Sensitive Transient Receptor Potential Ankyrin-1 (TRPA1) and Endothelium-Dependent Mechanism: Potential Role in Postprandial Hyperemia. *Front Physiol.* 2019 Mar 28;10:277. doi: 10.3389/fphys.2019.00277. eCollection 2019.
2. **Jin L**, Lipinski A, Conklin DJ. A Simple Method for Normalization of Aortic Contractility. *J Vasc Res.* 2018;55(3):177-186. doi: 10.1159/000490245. Epub 2018 Jul 5.
3. **Jin L**, P. Lorkiewicz, M.V. Malovichko, A. Bhatnagar, S. Srivastava, D.J. Conklin*. Acetaldehyde Induces an Endothelium-Dependent Relaxation of Superior Mesenteric Artery: Potential Role in Postprandial Hyperemia. (accepted).
4. **L. Jin**, G. Jagatheesan, L. Guo, and D.J. Conklin*. Crotonaldehyde-induced Vascular Relaxation: Role of Endothelium and Transient Receptor Potential Ankyrin-1 (TRPA1). (be submitted).
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Poster and Platform

1. Poster, 9/2016. "A Simple Method for Normalization of Aorta Contractility." Research! Louisville, Louisville, KY.
2. Poster, 09/2017. Mechanism of Formalin-Induced Vasorelaxation. Research! Louisville, Louisville, KY.
3. Poster, 3/2018. Formaldehyde-induced TRPA1- and EDRF-Dependent Contractile Inhibition in Murine Blood Vessels. SOT annual meeting, San Antonio, Texas.
4. Poster, 09/2018. Diverse Aldehydes Stimulate a Common Endothelium-, Transient Receptor Potential Ankyrin1-, and Nitric Oxide-dependent Pathway in Isolated Murine Blood Vessels. Research! Louisville, Louisville, KY.
5. Presentation, 11/2018. The Cardiovascular Effects of Crotonaldehyde in Vivo and in Vitro. OVSOT student meeting, Louisville, KY.
6. Poster, 3/2019. The Cardiovascular Effects of Crotonaldehyde in Vivo and in Vitro. SOT annual meeting, Baltimore, Maryland.
7. Poster, 09/2019. Vascular Toxicity of Mainstream Cigarette Smoke in Mice: Role of crotonaldehyde and TRPA1. Research! Louisville, Louisville, KY.
8. Poster, 09/2019. Vascular Toxicity of Mainstream Cigarette Smoke in Mice: Role of crotonaldehyde and TRPA1. OVSOT meeting, Cincinnati, OH.