Published in final edited form as:

Toxicol Lett. 2019 September 15; 312: 55–64. doi:10.1016/j.toxlet.2019.04.006.

Vasodilatory effect of formaldehyde via the NO/cGMP pathway and the regulation of expression of K_{ATP} , BK_{Ca} and L-type Ca^{2+} channels

Yun Zhao^{a,1}, Jing Ge^{a,1}, Xiaoxiao Li^a, Qing Guo^{a,c}, Yuqing Zhu^d, Jing Song^a, Luoping Zhang^b, Shumao Ding^a, Xu Yang^{a,*}, Rui Li^{a,*}

^aHubei Key Laboratory of Genetic Regulation and Integrative Biology, School of Life Sciences, Central China Normal University, 152 Luoyu Road, Wuhan 430079, PR China

^bDivision of Environmental Health Sciences, School of Public Health, University of California, Berkeley, CA, 94720, USA

^cSchool of Public Health, Huazhong University of Science and Technology, Hangkong Road, Wuhan, 430030, PR China

^dCentre of Stem Cell and Regenerative medicine, School of Medicine, Zhejiang University, 866 Yuhangtang Road, Hangzhou, 310058, PR China

Abstract

Formaldehyde (FA), a well-known toxic gas molecule similar to nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H₂S), is widely produced endogenously via numerous biochemical pathways, and has a number of physiological roles in the biosystem. We attempted to investigate the vasorelaxant effects of FA and their underlying mechanisms. We found that FA induced vasorelaxant effects on rat aortic rings in a concentration-dependent manner. The NO/cyclic guanosine 5' monophosphate (cGMP) pathway was up-regulated when the rat aortas were treated with FA. The expression of large-conductance Ca^{2+} -activated K^+ (BK_{Ca}) channel subunits α and β of the rat aortas was increased by FA. Similarly, the levels of ATP-sensitive K^+ (K_{ATP}) channel subunits $K_{ir6.1}$ and $K_{ir6.2}$ were also up-regulated when the rat aortas were incubated with FA. In contrast, levels of the L-type Ca^{2+} channel (LTCC) subunits, $Ca_v1.2$ and $Ca_v1.3$, decreased dramatically with increasing concentrations of FA. We demonstrated that the regulation of FA on vascular contractility may be via the up-regulation of the NO/cGMP pathway and the modulation of ion channels, including the upregulated expression of the K_{ATP} and BK_{Ca} channels and the inhibited expression of LTCCs. Further study is needed to explore the in-depth mechanisms of FA induced vasorelaxation.

*Corresponding authors. yangxu@mail.ccnu.edu.cn (X. Yang), rui.li@mail.ccnu.edu.cn (R. Li).

Author contributions

R.L., X.Y. and Y.Z. conceived and designed the research. Y.Z., J.G., X.X.L., Q.G. Y.Q.Z. and J.S. performed experiments. Y.Z. and J.G. analyzed data. X.X.L., Q.G., J.G. and Y.Z. interpreted results of experiments and prepared figures. Y.Z. drafted the manuscript. L.P.Z., S.M.D., X.Y. and R.L. edited and revised the manuscript. X.Y. and R.L. approved the final version of the manuscript.

¹These authors contributed equally to this work.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.toxlet.2019.04.006>.

Graphical abstract

Keywords

Formaldehyde; Rat aortas; Vasorelaxation; NO/cGMP pathway; Ion channels

1. Introduction

As the simplest aldehyde, formaldehyde (FA) is widely used in a variety of industries and consumer products, leading to a ubiquitous issue of environmental pollution. In addition to exogenous exposure, FA is also produced endogenously from amine *in vivo* when catalyzed by semicarbazide-sensitive-mineoxidase (SSAO) (Lin et al., 2005; Yu and Deng, 1998). Some other sources of FA generation are DNA methylation and demethylation in the nucleus, and oxidation of exogenous methanol by alcohol dehydrogenase (ADH) or hydrogen peroxide enzyme (catalase) (Pontel et al., 2015).

Due to its inevitable exposure to public, especially to pathologists, anatomy students and workers involved in the manufacturing of construction materials and other industries with high level of FA exposure, long-term concerns about the harmful effects of FA on human health has been attracting widespread attention (Zhang et al., 2010a, 2010b). Formaldehyde was classified as a Group 1 human carcinogen causing nasopharyngeal cancer and leukemia by the International Agency for Research on Cancer (IARC, 2006, 2012). Meanwhile, a host of studies have reported that formaldehyde induces toxicity not only on the respiratory system, but also on the immune and neural system as well as organs like liver and kidney (Gerin et al., 2016; Katsnelson et al., 2013; Aydin Aydın et al., 2013; Tulpule and Dringen, 2013; Ramos et al., 2017).

A variety of toxic gas molecules generated exogenously and endogenously, such as nitric oxide (NO), carbon dioxide (CO) and hydrogen sulfide (H₂S), are considered to have cardiovascular effects as vasorelaxant substances (Furchgott and Zawadzki, 1980; Furchgott and Jothianandan, 1991; Zhao et al., 2001; Yang et al., 2008; Wang et al., 2015; Mustafa et

al., 2009; Sobrino et al., 2017). Likewise, adverse cardiovascular effects have been related to formaldehyde in many studies. Acute pumping failure caused by FA treatment to hypertrophic and normal hearts probably derives from the impairment of intracellular Ca^{2+} regulation (Takeshita et al., 2009). In addition, FA has been found to significantly inhibit the contraction of smooth muscle mediated by Ca^{2+} , suggesting that the vasorelaxation effect of FA could be a result of the inhibition of the calcium influx (Tani, 1981; Zhang et al., 2010a, 2010b). Zhang et al. revealed that FA at high concentrations ($> 500 \text{ mM}$) could induce vasorelaxation in rats, possibly via the upregulation of iNOS in the aorta and the regulation of the L-type Ca^{2+} channels (Zhang et al., 2018). Unfortunately, due to insufficient research, an explanation of how formaldehyde functions as a vasorelaxant remains elusive.

Based on previous research, we did a preliminary study into the relaxation effect of FA on rat aortas and its possible mechanisms. This study seemed to indicate that the vasorelaxant effect of FA may be related to the NO signaling pathway and the expression of some ion channels, including the BK_{Ca} , K_{ATP} and L-type Ca^{2+} channels. Our results point to the need for further, thorough research. We hope this preliminary research will shine a light on related studies.

2. Materials and methods

2.1. Animals and treatments

Male Wistar rats (weighing 250–300 g) were purchased from the Hubei Research Center of Laboratory Animals (Wuhan, China) and cared for following guidelines set by the Institutional Animal Care and Use Committee of Central China Normal University on January 24, 2016 (Ratification ID: CCNU-IACUC-2016–003) (Supplementary Fig. 8). The rats were raised in our pathogen free laboratory animal room at 24–26 °C with a 12 h light-dark cycle and humidity between 55%–75%. The rats were able to access clean food and water at all times.

2.2. Main reagents, kits and antibodies

Formalin (10%), norepinephrine (NE), acetylcholine (Ach), L-arginine (L-Arg), methylene blue (MB) (0.05%), L-nitro-arginine methylester (L-NAME), tetraethylammonium chloride (TEA), nifedipine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glibenclamide was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rat enzyme-linked immunosorbent assay (ELISA) kits for sGC and cGMP were obtained from Shanghai Blue Gene Biotech Co., Ltd. (Shanghai, China). A modified bicinchoninic acid assay (BCA) protein assay kit was purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China) and the level of NO was measured with a nitric oxide assay kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Mouse monoclonal BK_{Ca} α antibody and rabbit polyclonal BK_{Ca} β antibody were purchased from Abcam (Abcam, Cambridge, MA, UK), while Kir6.1, Kir6.2, $\text{Ca}_v1.2$, $\text{Ca}_v1.3$ antibodies were bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.3. Isolated rat aortic ring assay

In general, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (approximately 30 mg/kg). After removing the thoracic aortas, the adherent connective tissues and fat were dissected and the aortas were cut into several rings (2–3 mm). The above steps were very carefully carried out to avoid damage to the endothelial cells. All rings were bathed in tubes in Krebs solution (120.6 mM NaCl, 5.9 mM KCl, 1.2 mM NaH_2PO_4 , 1.2 mM MgCl_2 , 15.4 mM NaHCO_3 , 2.5 CaCl_2 , 11.5 mM glucose, pH = 7.4) at 37 °C, while 95% O_2 and 5% CO_2 were bubbled through, and the tension, which was recorded with a MedLab Biological Signal Collection System (Medeas Science and Technology, Nanjing, China), was about 1.5 g at optimum resting conditions. A 1-hour-equilibration was administered to the aortic rings during which time the buffer was replaced with fresh Krebs solution every 15 min. The aorta rings were treated with 60 mM KCl before experiments were carried out, to assess their viability. 10^{-6} M acetylcholine was used to examine the integrity and viability of the vascular endothelium. Saline and formalin with a range of final concentrations (0.04 mM, 0.08 mM, 0.16 mM, 0.32 mM, 0.64 mM, 1.28 mM, 2.56 mM) were added to the buffer when the rings reached their maximum constriction due to the administration of NE. To investigate the relative signaling pathways involved in the vasorelaxation effects of FA, several chemicals that block the NO signaling pathway and some ion channels, such as L-NAME (100 μM), MB (10 μM), TEA (10 mM), glibenclamide (10 μM), nifedipine (1 μM), were incubated for about 10 min before contraction induced by NE.

2.4. Rat aortic slice assay

Rat isolated aortic slices were prepared as described (Schachter, 2007; Li et al., 2010). Briefly, the entire thoraco-abdominal aorta (7–8 cm) was removed and immediately transferred to a sterile, ice-cold phosphate-buffered saline solution (PBS, pH = 7.5). The vessel was cut into segments (1 mm) longitudinally and horizontally after dissecting the adherent and adventitial tissues. The operations need to be very carefully performed so as not to damage the endothelial cells. The slices were transferred to 24-well plates and suspended in Krebs-Ringer bicarbonate (KRB) incubation solution (pH = 7.2, 1 mL) containing 0.05 mM L-Arg. 95% O_2 - 5% CO_2 was bubbled through the tissue suspension at 37 °C with 60 beats/minute-shaking. Saline or formalin were added to the wells to make final concentrations of 0, 0.64, 1.28, 2.56 mM. The tissues were incubated for 2 h and then harvested from the media and rapidly weighed. Using a glass homogenizer, all tissues were homogenized on ice with 10 mL/g PBS to make a 10% tissue homogenate. This was used for the measurement of NO production and sGC and cGMP levels.

2.5. Determination of NO, sGC, cGMP and protein

The manufacturer's instructions for the ELISA and other relevant kits were strictly followed to determine the levels of NO production, sGC, cGMP and protein in tissues.

2.6. Histopathological examination

The isolated aortic rings were prepared followed the procedures described above. The rings were suspending in KRB incubation solution (1 mL) after being transferred to two 6-well

plates. 95% O₂ – 5% CO₂ was bubbled through the tissue suspensions at 37 °C with 60 beats/minute-shaking. Formalin was added to the wells to reach final concentrations of 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56 mM and the wells were then incubated for 2 h. As for the KRB incubation solution containing 0.05 mM L-arg, the final concentrations of formaldehyde in the wells were 0.64, 1.28, 2.56 Mm. Saline was used as the control. After incubation, the rings were collected and fixed in 4% formalin for hematoxylin and eosin (H&E) staining analysis.

2.7. Immunohistochemical assay

The thoracic aorta tissue sections were incubated with 0.3% H₂O₂ to quench endogenous peroxides. The sections were boiled in sodium citrate (0.01 M, pH 6.0) for antigen retrieval. After 10-minute-permeation with 0.2% Triton X-100, they were blocked for 30 min with 5% bovine serum albumin (BSA) in PBS at room temperature. The following diluted primary antibodies: mouse anti-BK_{Ca} α (1:200, Abcam, Cambridge, MA, UK), rabbit anti-BK_{Ca} β (1:1000, Abcam, Cambridge, MA, UK), rabbit anti-Kir6.1 (1:200, Santa Cruz, CA, USA), mouse anti-Kir6.2 (1:50, Santa Cruz, CA, USA), rabbit anti-Ca_v1.2 (1:100, Santa Cruz, CA, USA), goat anti-Ca_v1.3 (1:50, Santa Cruz, CA, USA), rabbit anti-endothelial nitric oxide synthase (eNOS) (1:500, Wuhan GoodBio Technology CO., Ltd. Wuhan, China), were used to incubate sections overnight at 4 °C. The slides were then incubated at 37 °C with biotinylated immunoglobulins and avidin-biotin peroxidase complex after washing with PBS, while H₂O₂ (3%) and diaminobenzidine tetrahydro chloride (DAB, 5 mg/10 mL) (Sigma-Aldrich, St. Louis, MO, USA) were used as chromogens to visualize reaction products. The immunostaining results were examined under a DM 4000B Microscope (Leica), and the average optical intensities of the relevant proteins were determined using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

2.8. Immunofluorescent assay

For immunofluorescent staining of the aortic tissue sections, we used sodium citrate (0.01 M, pH 6.0) for antigen retrieval. Sections were then blocked for 30 min with 5% BSA in PBS at room temperature. The following diluted primary antibodies: mouse anti-BK_{Ca} α (1:200, Abcam, Cambridge, MA, UK), rabbit anti-BK_{Ca} β (1:1000, Abcam, Cambridge, MA, UK), rabbit anti-Kir6.1 (1:200, Santa Cruz, CA, USA), mouse anti-Kir6.2 (1:50, Santa Cruz, CA, USA), rabbit anti-Ca_v1.2 (1:100, Santa Cruz, CA, USA), goat anti-Ca_v1.3 (1:200, Santa Cruz, CA, USA), were used to incubate sections overnight at 4 °C. Slides were then incubated at 37 °C with secondary antibodies Alexa Fluor 488 (donkey anti-goat, 1:400; Life Technologies), Alexa Fluor 488 (donkey anti-rabbit, 1:400; Life Technologies), Alexa Fluor 488 (donkey anti-mouse, 1:400; Life Technologies), Alexa Fluor 594 (donkey anti-rabbit, 1:400; Life Technologies), Alexa Fluor 594 (donkey anti-mouse, 1:400; Life Technologies) and Alexa Fluor 594 (donkey anti-goat, 1:400; Life Technologies) for 1 h. 4',6-diamidino-2-phenylindole (DAPI) (blue) was used to counterstain all sections. The slides were then mounted and viewed under a fluorescent microscope (Olympus BX53).

2.9. Statistical analysis

Data are presented as mean $\pm$ SEM. One-way analysis of variance (ANOVA) followed by Student's two-tailed *t*-test was used to test the significance of differences between groups. *p*

< 0.05 was considered to be statistically significant. GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA) was used for the statistical analysis, while Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA) was used for the quantification of all protein expression in the immunohistochemical assays.

3. Results

3.1. Vasorelaxant effect induced by FA on rat aortic rings

To test if FA played a role in regulating vascular tone, the aortic rings were pre-contracted using 10^{-6} M NE and then incubated with saline or FA (0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56 mM), and the vascular tension recorded at different times. Compared with the control group, no vasodilatory effect was observed in the aortic rings exposed to FA (0.04 mM to 0.64 mM), while such responses were increased when exposed to higher levels of FA (1.28 mM and 2.56 mM) for 2 min. (Fig. 1a). Exposure to FA (0.32 mM–2.56 mM) for 6–10 min. induced significant vasodilation in the aortic rings (Fig. 1a) ($p < 0.001$). The relaxation induced by FA (0.64 mM–2.56 mM) was more than 90%, and almost stable after 10 min (Fig. 1a). All these responses were dose-dependent. The vasorelaxation levels induced by FA (0.04 mM and 0.16 mM) were lower than those for H_2S at the same concentrations, while the vasodilatory effect of 0.64 mM FA was significantly stronger than that of 0.64 mM H_2S (Fig. 1) ($p < 0.001$). The results of H&E staining revealed that the aortas incubated with saline and FA (0.04 mM to 0.16 mM) had normal histological features (Fig. 1b1–b4), while aortas incubated with FA (0.32 mM–2.56 mM) group (Fig. 1b4–b8) appeared to be abnormal compared to the control. These abnormalities mainly manifested in the tunica medias of the aortas incubated with FA (0.32 mM–2.56 mM). The middle elastic plates were loose, broken, or had disappeared, whereas they were arranged concentrically when treated with low concentrations of FA (0.04 mM to 0.16 mM) (Fig. 1b2–b4).

3.2. FA up-regulates the NO/cGMP pathway in the rat aorta

L-NAME and MB, blockers of eNOS and sGC, respectively, were used to explore the effects of FA on the aortic NO/cGMP pathway. The results showed that both could dramatically reduce the relaxation of aortic rings induced by FA (0.64 mM to 2.56 mM) (Fig. 2a, b). H&E staining was used on the aortic rings incubated with FA in the presence of L-arg, a type of NO donor. Similarly, the middle elastic plates in the tunica media were normal in the control and L-arg groups (Supplementary Fig. 2a, b), whereas they become loose, broken, or even disappeared in the L-arg + FA (0.64 mM to 2.56 mM) groups (Supplementary Fig. 3c–e). Further studies were conducted to test the downstream pathway by immunohistochemical assay and to determine the production of NO, sGC and cGMP. Positive expressions of eNOS, resulting from the combination of a specific antibody with eNOS, were observed to mainly distribute in the endothelial cells as shown by the immunohistochemical assay (Fig. 2c). According to the analysis, eNOS expression in the L-arg + 2.56 F A group was markedly increased in comparison to both the control and L-arg groups (Fig. 2d) ($p < 0.01$; $p < 0.001$). Similar results were obtained from the measurement of NO production (Fig. 1e). In addition, the expression levels of sGC in the L-arg + FA (0.64 mM to 2.56 mM) groups were higher than those in the control and L-arg groups (Fig. 2f), and both cGMP and PKG

levels showed an upward trend with increasing doses of FA (Fig. 2g, h). Protein levels in the aortic hemi-segments didn't change significantly among the groups (Supplementary Fig. 3).

3.3. High concentrations of FA promote the expression of K_{ATP} and BK_{Ca} channel subunits in rat aortas

To determine which ion channel on the cytomembrane of vascular smooth muscle cells might be involved in the vasodilation induced by FA, an in-depth study was conducted to investigate if there was any relationship between the vasorelaxant effect of FA and the BK_{Ca} and K_{ATP} channels by using TEA and glibenclamide. Our results indicated that the vasodilatory effects of FA (0.64 mM–2.56 mM) on rat aortic rings were markedly inhibited when these blockers were present (Figs. 3a and 4a). The expression of BK_{Ca} α and BK_{Ca} β , two main subunits of the BK_{Ca} channels, was further evaluated by immunohistochemical assay. We observed that expression of these two subunits in the vascular smooth muscle cells was up-regulated by FA (Fig. 3b, c, e, f), with 0.64 mM FA inducing the strongest up-regulation in comparison to 1.28 mM and 2.56 mM FA (Fig. 3c, f). Immunofluorescence techniques were used to compare any alterations to BK_{Ca} α and BK_{Ca} β in the blood vessels of animals treated with saline and 0.64 mM FA, respectively. These investigations demonstrated that the expression of both BK_{Ca} α and BK_{Ca} β was considerably enhanced in the vascular smooth muscle, which is consistent with the immunohistochemical staining result (Fig. 3d, g). In addition, we investigated the effects of FA (0.64 mM–2.56 mM) on Kir6.1 and Kir6.2, two major subunits of the K_{ATP} channels, using the same methods. In general, the results resembled those for the BK_{Ca} channels, the stimulus of FA at high concentrations (0.64 mM–2.56 mM) dramatically promoted the expression of Kir6.1 and Kir6.2 subunits (Fig. 4b, c, e, f). Interestingly, the effect of 0.64 mM FA on the expression of K_{ATP} channels was noteworthy in that it resulted in the highest increase in the expression of the two subunits (Fig. 4c, d, f, g).

3.4. High concentrations of FA down-regulate the expression of the relevant subunits of L-type Ca^{2+} channels in rat aortas

We also investigated the involvement of the L-type Ca^{2+} channel in the process of FA-induced vasorelaxation. This channel is one of the main ion channels in the membrane of the vascular smooth muscle cells (VSMCs) that regulates vascular contractility. We examined the effect of nifedipine, an L-type Ca^{2+} channel blocker, to determine whether the vasodilatory effect induced by high concentrations of FA is related to the L-type Ca^{2+} channel. The results from the rat aortic ring assay suggested that the vasodilatory effects of FA (0.64 mM to 2.56 mM) on aortic rings could be inhibited by nifedipine (Fig. 5a). Thereafter, the immunohistochemistry assay and immunofluorescence assay were used to detect expression of $Ca_v1.2$ and $Ca_v1.3$, two prime subunits of L-type Ca^{2+} channels, in the vascular smooth muscle. Increasing FA doses led to an obvious decline in the expression levels of both subunits (Fig. 5b, c, e, f) in a dose-dependent manner. Accordingly, 2.56 mM FA (the highest concentration) reduced the expression of $Ca_v1.2$ (Fig. 5b, c) to the greatest extent, hence, we examined how much 2.56 mM FA affected $Ca_v1.2$ and $Ca_v1.3$ expression using immunofluorescence techniques. The results showed a similar decrease in expression to a large extent compared with the control group, when the aortic rings were relaxed by 2.56 mM FA (Fig. 5d, g).

3.5. Low concentrations of FA enhanced the expression of subunits of BK_{Ca} channels and Kir6.2 for K_{ATP} channels in rat aortas

Although results from the first round of immunochemical staining and analysis showed a marked improvement on the expression of the BK_{Ca} and K_{ATP} channels and a decrease of L-type Ca²⁺ channel expression. 0.64 mM FA, instead of 2.56 mM FA, was shown to induce the biggest increase in BK_{Ca} and K_{ATP} channel expression. We therefore conducted a second round of tests trying to understand the effects of FA at low concentrations (0.08 mM to 0.32 mM). The relevant blockers used in the first round were used in this second round, and the results obtained suggest that TEA could completely inhibit the vasorelaxation induced by FA (0.16 mM and 0.32 mM) (Supplementary Fig. 4a). Glibenclamide and nifedipine blocked the vasodilation caused by 0.32 mM FA to a large extent (Supplementary Fig. 4b, c). With increasing FA doses, expression of the BK_{Ca} α and BK_{Ca} β subunits increased. In addition, only FA at 0.32 mM could lead to a marked up-regulation (Fig. 6a–d), which is consistent with the regulatory effect of FA on Kir6.2 (Fig. 6g, h). As for Kir6.1, no significant change was found after exposure to FA (0.08 mM to 0.32 mM) (Fig. 6e, f). Treatment with low concentrations of FA (0.08 mM to 0.32 mM) had no apparent influence on the expression of Ca_v1.2 and Ca_v1.3 in the vascular smooth muscle (Fig. 6i–l).

4. Discussion

In this study we investigated the vasorelaxant effects of FA *in vitro*. Since H₂S is known to be a gaseous vasorelaxant (Yang et al., 2008), we used H₂S as a positive control for our study on FA. The findings suggested that the vasodilation induced by both H₂S and FA, increased as the respective concentrations rose, so we inferred that FA, at least to some extent, might play a role in the regulation of the vascular tone in a similar manner to H₂S (Supplementary Fig. 1). From the data, we found that the reaction-response curve had shifted to the left, which means that the vasorelaxation induced by FA is concentration-dependent, and FA functions as an endogenously vasoactive gaseous factor within a physiological concentration range.

To elucidate the mechanisms behind the FA-triggered vasorelaxation, we explored some of the classical signaling pathways and ion channels that are known to mediate the vasorelaxant effects. The resultant aortic ring assays showed that: (i) Inhibition by L-NAME and MB, blockers of eNOS and sGC respectively, significantly attenuated the FA-induced vasodilation. Various physiological responses involving relaxation of blood vessels, could be triggered by NO when sGC is activated to produce the second messenger c-GMP (Bucci et al., 2012). The findings above suggest that FA might, in part, relax vascular tissue via the NO/cGMP signaling pathway. (ii) The modulation by FA on the K_{ATP} channels, BK_{Ca} channels in particular and the L-type Ca²⁺ channels in VSMCs were proved by the pre-treatment of rat aortic rings with glibenclamide, TEA and nifedipine, respectively. (iii) FA-triggered vasorelaxation is not mediated by cyclic adenosine monophosphate (cAMP) or prostaglandin, since the pre-incubation of SQ22536 and indomethacin, the corresponding biological blockers, showed no significant hypotensive effect on the aortic rings (Supplementary Figs. 5 and 6). In short, like H₂S and NO, the mechanisms of the FA-

induced vasorelaxant effects are complex and might involve multiple pathways or ion exchangers (Catterall, 1998; Li et al., 2011, 2008).

In the previous study, Zhang et al. examined the involvement of endothelial cells in the FA-induced vasodilatory effect, using FA to treat rat aortic rings with intact endothelium or without endothelium. A significant augmentation of FA-induced vasorelaxation was found in aortas with an intact endothelium, when compared to aortas with endothelial cells stripped. This seems to imply that the endothelium may play a vital role in the FA induced process of vasodilatation (Zhang et al., 2018). Numerous studies have indicated that many physiological processes like vascular modeling, angiogenesis and vascular tone are regulated by endothelial cells via the function of releasing NO by eNOS, one type of NOS constitutively expressed in the endothelium (Bai et al., 2016; Li et al., 2010; Vanhoutte et al., 2016; Yam et al., 2018). This study showed that FA, at higher concentrations (0.64 mM–2.56 mM), was able to up-regulate the NO/cGMP signaling pathway. By increasing eNOS and NO levels, sGC is activated followed by the conversion of GTP to cGMP, and thus downstream physiological processes could be regulated (Mota et al., 2015; Poulos, 2006).

Protein expression of BK_{Ca} and K_{ATP} channels, was enhanced when FA exerted vasodilatory effects. K⁺ channels play a significant role in cellular signaling processes including the maintenance of the membrane potential of VSMCs to regulate contractile tone (Francis et al., 2010; Santos et al., 2017; Shieh et al., 2000). It has been reported that K_{ATP} channels, which are broadly expressed in the vasculature, can finely regulate vascular smooth muscle (Yamamoto et al., 2015). They are tetrameric ion channel complexes assembled by two different subunits, an inward-rectifying K⁺ channel 6.x family (Kir6.x) pore-forming subunit and a modulatory sulfonylurea receptor (SUR.x) subunit of the ATP-binding cassette (ABC) protein superfamily (Uttio et al., 2017). In our study, we mainly focused on the effect of FA on the expression of Kir6.x, including Kir6.1 and Kir6.2, in rat aortas. Results showed that the protein content of Kir6.1 and Kir6.2 in the rat aortas treated with FA (0.64 mM–2.56 mM) was higher than that in the control group, and surprisingly, the highest was found in the group treated with 0.64 mM FA. BK_{Ca} channels, comprising a pore-forming α -subunit plus two accessory β -subunits (encoded by several genes, with the $\beta 1$ -subunit being the only one that has been identified in the smooth muscle), are responsible for the diverse Ca²⁺ signals, and could decrease contractility indirectly by membrane hyperpolarization (Toro et al., 1998; Zhang et al., 2015, 2014). We found that the expression of both the α -subunit and β -subunit of the BK_{Ca} channels also increased significantly with FA treatment. Among several protein kinases (other than Ca²⁺ and voltage) that could modulate BK_{Ca} channels, cGMP-dependent protein kinase (PKG) is one that can activate it. Based on our findings, the elevation of cGMP by FA turned out to be an effective way to increase PKG levels, which could in turn activate BK_{Ca} channels to relax the blood vessels.

To investigate the effect of FA at low concentrations (0.08 mM to 0.32 mM) on the rat aortas, we conducted a second round of experiments. Taken together, 0.64 mM FA induced the maximum expression of Kir6.1, BK_{Ca} α and BK_{Ca} β subunits in the smooth muscle of rat aortas, while the strongest expression of Kir6.2 for K_{ATP} was induced by 0.32 mM FA (Supplementary Fig. 7a, b). Such a bi-aphasic dose-response, manifesting as a low dose stimulation and a high dose inhibition, is known as hormesis, characterized by an inverted

U-shape (Mattson, 2008). An array of studies revealed an hormetic-like dose response in various cell lines and organs, very similar to the response trend seen in our study. For instance, the human ovarian cancer cell line viability was promoted by a low dose of Cadmium, but viability decreased with high dose exposure. Another study found that human lymphocyte DNA synthesis also showed an inverted U-shape following treatment with Arsenic (Calabrese and Baldwin, 2001).

The expression of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$, two subunits comprising L-type Ca^{2+} channels in vascular SMCs, were down-regulated by FA. LTCC is a kind of voltage-gated Ca^{2+} channel, composed of $\alpha1$ subunit isoforms ($\alpha1S$, $\alpha1C$, $\alpha1D$, $\alpha1F$) and accessory subunits, including $\alpha2-6$ and β subunits (Catterall, 1998). Among these subunits, $\alpha1S$ and $\alpha1F$ express only in skeletal muscle and the retina, while $\alpha1C$ ($\text{Ca}_v1.2$) and $\alpha1D$ ($\text{Ca}_v1.3$) subunits are widely expressed in electrically excitable cells in the cardiovascular system (Alexandra et al., 2001; Platzer et al., 2000). Ca^{2+} influx via LTCC, causing membrane depolarization, regulates blood pressure and mediates the development of hypertension (Michiels et al., 2014). The fact that the levels of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ subunits in vascular SMCs decrease with increasing FA concentrations, implies the possibility that FA might have an inhibitory effect on the opening of LTCC, and thereby blocking the Ca^{2+} influx so that vasorelaxation is potentiated and enhanced. In addition, our data were consistent with data from previous studies showing that LTCC, formed by $\text{Ca}_v1.3$ subunits, are much less abundant than those formed by $\text{Ca}_v1.2$ in most cases (Hell et al., 1993).

Zhang et al. systematically showed that FA might induce vasorelaxation of rat aortas via the regulation of the NO-cGMP signaling pathway and ion channels, including the L-type Ca^{2+} channel and the BK_{Ca} channel (Zhang et al., 2018). In the present study, we not only monitored the vasoactive effects of FA on rat aortic rings at one specific time point, instead, we measured at three time points (2 min, 6 min, 10 min) to investigate the progress of FA-induced vasorelaxation. Hence, it was revealed that the regulation manner of FA on the vascular tone was not only dose-dependent, but also time-dependent. Our results for ion channel expression measured by immunohistochemistry and indicated by immunofluorescent assay are largely parallel to the results that Zhang et al. obtained using a western blot. It is indeed a strong demonstration, in our case applying two different methods, that the work we both did is consistent and credible. In addition, both the effect of FA on BK_{Ca} channel and K_{ATP} channel expression shown in this work, further indicates the regulatory role of FA on potassium channel expression. By using indomethacin and SQ22536 in vitro, we found that FA-induced vasorelaxation had nothing to do with cAMP and prostaglandin, which again suggests that the vasodilatation induced by FA may not be mediated by these compounds. Last but not least, the expression of K^+ channels in our study exhibit a biphasic dose-response, which can be considered to be an hormesis, being characterized by an inverted U-shape (Mattson, 2008)."

5. Conclusion

In conclusion, this study has shown that FA might induce vasorelaxation via the up-regulation of the NO/cGMP pathway, and the modulation of ion channels, such as the activation of the K_{ATP} and BK_{Ca} channels and the inhibition of the LTCC of vascular

smooth muscle in rat aortas. To our knowledge, few studies have focused on the vasorelaxation of endogenous FA. Importantly, we post that endogenous FA appears to act as a gaseous vasorelaxant factor, which is preliminary but ponderable to explore the possible pathological mechanisms involved in cardiovascular effects induced by FA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

Dr. Zhang (Institute of Environmental Medicine and Toxicology, Institute of Environmental Science, Shanxi University) has supported this work.

Funding

This research was supported by the National Key Research and Development Program of China (2017YFC0702700), the National Natural Science Foundation of China (21577045) and self-determined research funds of CCNU from the colleges' basic research and operation of MOE (CCNU18JCXK07).

References

- Alexandra K, Reimer D, Huber I, Grabner M, Glossmann H, Engel J, Striessnig J, 2001 $\alpha 1D$ (Cav1.3) Subunits can form L-type Ca^{2+} channels activating at negative voltages. *J. Biol. Chem.* 276, 22100–22106.
- Aydin Aydın S, Canpınar H, Ünde er Ü, Güç D, Çolako lu M, Kars A, Ba aran N, 2013 Assessment of immunotoxicity and genotoxicity in workers exposed to low concentrations of formaldehyde. *Arch. Toxicol.* 87, 145–153. [PubMed: 23100157]
- Bai F, Pang XF, Zhang LH, Wang NP, McKallip RJ, Garner RE, Zhao ZQ, 2016 Angiotensin II AT1 receptor alters ACE2 activity, eNOS expression and CD44hyaluronan interaction in rats with hypertension and myocardial fibrosis. *Life Sci.* 153, 141–152. [PubMed: 27085217]
- Bucci M, Papapetropoulos A, Vellecco V, Zhou Z, Zaid A, Giannogonas P, Cantalupo A, Dhayade S, Karalis KP, Wang R, 2012 cGMP-dependent protein kinase contributes to hydrogen sulfide-stimulated vasorelaxation. *PLoS One* 7, e53319.
- Calabrese EJ, Baldwin LA, 2001 Hormesis: U-shaped dose responses and their centrality in toxicology. *Trends Pharmacol. Sci.* 22, 285–291. [PubMed: 11395156]
- Catterall WA, 1998 Structure and function of neuronal Ca^{2+} channels and their role in neurotransmitter release. *Cell Calcium* 24, 307–323. [PubMed: 10091001]
- Francis SH, Busch JF, Corbin JD, 2010 cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. *Pharmacol. Rev.* 62, 525–563. [PubMed: 20716671]
- Furchgott RF, Jothianandan D, 1991 Endothelium-dependent and -independent vasodilatation involving cyclic GMP: relaxation induced by nitric oxide, carbon monoxide and light. *Blood Vessels* 28, 52–61. [PubMed: 1848126]
- Furchgott RF, Zawadzki JV, 1980 The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholin. *Nature* 288, 373–376. [PubMed: 6253831]
- Gerin F, Erman H, Erboga M, Sener U, Yilmaz A, Seyhan H, Gurel A, 2016 The effects of ferulic acid against oxidative stress and inflammation in formaldehydeinduced hepatotoxicity. *Inflammation* 39, 1377–1386. [PubMed: 27235018]
- Hell JW, Westenbroek RE, Warmer C, Ahljianian MK, Prystay W, Gilbert MM, Snutch TP, Catterall WA, 1993 Identification and differential subcellular localization of the neuronal class C and class D L-type calcium channel $\alpha 1$ subunits. *J. Cell Biol.* 123, 949–962. [PubMed: 8227151]
- IARC, 2006 Formaldehyde, 2-Butoxyethanol and 1-tert-Butoxypropan-2-ol. Monographs on the Evaluation of Carcinogenic Risks to Humans. World Health Organization, Geneva, Switzerland.

- IARC, 2012 Monographs on the Evaluation of Carcinogenic Risks to Humans-formaldehyde. World Health Organization, Geneva, Switzerland 100, Part F, pp. 401–436.
- Katsnelson BA, Degtyareva TD, Privalova LI, Minigaliyeva IA, Slyshkina TV, Ryzhov VV, Beresneva OY, 2013 Attenuation of subchronic formaldehyde inhalation toxicity with oral administration of glutamate, glycine and methionine. *Toxicol. Lett.* 220, 181–186. [PubMed: 23660335]
- Li L, Whiteman M, Guan YY, Neo KL, Cheng Y, Lee SW, Zhao Y, Baskar R, Tan CH, Moore PK, 2008 Characterization of a novel, water-soluble hydrogen sulfide-releasing molecule (GYY4137): new insights into the biology of hydrogen sulfide. *Circulation* 117, 2351–2360. [PubMed: 18443240]
- Li J, Li RJ, Meng ZQ, 2010 Sulfur dioxide upregulates the aortic nitric oxide pathway in rats. *Eur. J. Pharmacol.* 645, 143–150. [PubMed: 20674563]
- Li L, Rose P, Moore PK, 2011 Hydrogen sulfide and cell signaling. *Annu. Rev. Pharmacol.* 51, 169–187.
- Lin Z, Luo W, Li H, Zhang Y, 2005 The effect of endogenous formaldehyde on the rat aorta endothelial cells. *Toxicol. Lett.* 159, 134–143. [PubMed: 15961263]
- Mattson MP, 2008 Hormesis defined. *Ageing Res. Rev.* 7, 1–7. [PubMed: 18162444]
- Michiels CF, Van Hove CE, Martinet W, De Meyer GRY, Franssen P, 2014 L-type Ca^{2+} channel blockers inhibit the window contraction of mouse aorta segments with high affinity. *Eur. J. Pharmacol.* 738, 170–178. [PubMed: 24886884]
- Mota F, Gane P, Hampden-Smith K, Allerston CK, Garthwaite J, Selwood DL, 2015 A new small molecule inhibitor of soluble guanylate cyclase. *Bioorg. Med. Chem.* 23, 5303–5310. [PubMed: 26264842]
- Mustafa AK, Gadalla MM, Snyder SH, 2009 Signaling by gasotransmitters. *Sci. Signal.* 2 re2.
- Platzer J, Platzer J, Engel J, Schrott-Fischer A, Stephan K, Bova S, Chen H, Zheng H, Striessnig J, 2000 Congenital deafness and sinoatrial node dysfunction in mice lacking class D L-type Ca^{2+} channels. *Cell* 102, 89–97. [PubMed: 10929716]
- Pontel L, Rosado IV, Burgos-Barragan G, Garaycochea JI, Yu R, Arends MJ, Chandrasekaran G, Broecker V, Wei W, Liu L, Swenberg JA, Crossan GP, Patel KJ, 2015 Endogenous formaldehyde is a hematopoietic stem cell genotoxin and metabolic carcinogen. *Mol. Cell* 60, 177–188. [PubMed: 26412304]
- Poulos TL, 2006 Soluble guanylate cyclase. *Curr. Opin. Struct. Biol.* 16, 736–743. [PubMed: 17015012]
- Ramos CO, Nardeli CR, Campos KK, Pena KB, Machado DF, Bandeira AC, de Paula Costa G, Talvani A, Bezerra FS, 2017 The exposure to formaldehyde causes renal dysfunction, inflammation and redox imbalance in rats. *Exp. Toxicol. Pathol.* 69, 367–372. [PubMed: 28336174]
- Santos SS, Jesus RLC, Simões LO, Vasconcelos WP, Medeiros IA, Veras RC, Casais-E-Silva LL, Silva DF, 2017 NO production and potassium channels activation induced by *Crotalus durissus cascavella* underlie mesenteric artery relaxation. *Toxicon* 133, 10–17. [PubMed: 28428070]
- Schachter D, 2007 L-Glutamine *in vitro* regulates rat aortic glutamate content and modulates nitric oxide formation and contractility responses. *Am. J. Physiol., Cell Physiol.* 293, C142–C151. [PubMed: 17329397]
- Shieh CC, Coghlan M, Sullivan JP, Gopalakrishnan M, 2000 Potassium channels: molecular defects, diseases, and therapeutic opportunities. *Pharmacol. Rev.* 52, 557–594. [PubMed: 11121510]
- Sobrinho A, Vallejo S, Novella S, Lázaro-Franco M, Mompeón A, Bueno-Betí C, Walthter T, Sánchez-Ferrer C, Peiró C, Hermenegildo C, 2017 Mas receptor is involved in the estrogen-receptor induced nitric oxide-dependent vasorelaxation. *Biochem. Pharmacol.* 129, 67–72. [PubMed: 28131844]
- Takeshita D, Nakajima-Takenaka C, Shimizu J, Hattori H, Nakashima T, Kikuta A, Matsuyoshi H, Takaki M, 2009 Effects of formaldehyde on cardiovascular system in *in situ* rat hearts. *Basic Clin. Pharmacol.* 105, 271–280.
- Tani T, 1981 Relaxation of vascular smooth muscle induced by formaldehyde (author's transl). *Nihon Yakurigaku Zasshi* 77, 221–230. [PubMed: 7239363]
- Toro L, Wallner M, Pratap M, Tanala Y, 1998 Maxi- K_{Ca} , a unique member of the voltage-gated K channel superfamily. *News Physiol. Sci.* 13, 112–117. [PubMed: 11390773]

- Tulpule K, Dringen R, 2013. Formaldehyde in brain: an overlooked player in neurodegeneration? *J. Neurochem.* 127, 7–21 2013. [PubMed: 23800365]
- Uttio RC, Dosa PI, Fautsch MP, 2017 ATP sensitive potassium channel openers: a new class of ocular hypotensive agents. *Environ. Eye Res.* 158, 85–93.
- Vanhoutte PM, Zhao YZ, Xu AM, Leung SW, 2016 Thirty years of saying NO: sources, fate, actions, and misfortunes of the endothelium-derived vasodilator mediator. *Circ. Res.* 119, 375–396. [PubMed: 27390338]
- Wang R, Szabo C, Ichinose F, Ahmed A, Whiteman M, Papapetropoulos A, 2015 The role of H₂S bioavailability in endothelial dysfunction. *Trends Pharmacol. Sci.* 36, 568–578. [PubMed: 26071118]
- Yam MF, Chu ST, Ruan SB, 2018 Vasorelaxant effect of sinensetin via the NO/sGC/ cGMP pathway and potassium and calcium channels. *Hypertens. Res.* 41, 787–797. [PubMed: 30111856]
- Yamamoto T, Takahara K, Inai T, Node K, Teramoto N, 2015 Molecular analysis of ATP-sensitive K (+) channel subunits expressed in mouse portal vein. *Vascul. Pharmacol.* 75, 29–39. [PubMed: 26163942]
- Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, Meng Q, Mustafa AK, Mu W, Zhang A, Snyder SH, Wang R, 2008 H₂S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine- γ -lyase. *Science* 322, 587–590. [PubMed: 18948540]
- Yu PH, Deng YL, 1998 Endogenous formaldehyde as a potential factor of vulnerability of atherosclerosis: involvement of semicarbazide-sensitive amine oxidasemediated methylamine turnover. *Atherosclerosis* 140, 357–363. [PubMed: 9862279]
- Zhang FW, Du J, Tang C, 2010a Endogenous formaldehyde and cardiovascular diseases. *Prog. Psychobiol. Physiol. Psychol.* 41, 17–21.
- Zhang LP, Tang XJ, Rothman N, Vermeulen R, Ji ZY, Min S, Qiu CY, Guo WH, Liu SW, Reiss R, Freeman LB, Ge YC, Hubbard AE, Hua M, Blair A, Galvan N, Ruan XL, Alter BP, Xin KX, Li SH, Moore LE, Kim SK, Xie YX, Hayes RB, Azuma M, Hauptmann M, Xiong J, Stewart P, Li LY, Rappaport SM, Huang HLFJF Jr, Smith MT, Lan Q, 2010b Occupational exposure to formaldehyde, hematotoxicity, and leukemia-specific chromosome changes in cultured myeloid progenitor cells. *Cancer Epidemiol. Biomark.* 19, 80–88.
- Zhang QX, Tian JJ, Bai YL, Lei XD, Li M, Yang ZH, Meng ZQ, 2014 Effects of gaseous sulfur dioxide and its derivatives on the expression of K_{ATP}, BK_{Ca} and L-Ca²⁺ channels in rat aortas in vitro. *Eur. J. Pharmacol.* 742, 31–41. [PubMed: 25192964]
- Zhang QX, Bai YL, Tian JJ, Lei XD, Li M, Yang ZH, Meng ZQ, 2015 Effects of sodium metabisulfite on the expression of BK_{Ca}, K_{ATP}, and L-Ca²⁺ channels in rat aortas in vivo, and in vitro. *J. Hazard. Mater.* 284, 151–162. [PubMed: 25463229]
- Zhang QX, Tian PR, Zhai MM, Lei XD, Yang ZH, Liu Y, Liu MT, Huang H, Zhang XR, Yang X, Zhao Y, Meng ZQ, 2018 Formaldehyde regulates vascular tensions through nitric oxide-cGMP signaling pathway and ion channels. *Chemosphere* 193, 60–73. [PubMed: 29126066]
- Zhao W, Zhang J, Lu Y, Wang R, 2001 The vasorelaxant effect of H₂S as a novel endogenous gaseous K_{ATP} channel opener. *EMBO J.* 20, 6008–6016. [PubMed: 11689441]

a **b** **Fig. 1.**

Vasorelaxant effect of FA on rat aortic rings. (a) Vasodilatation of FA (0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56 mM) on rat aortic rings pre-contracted by 10^{-6} M NE at different time ($n = 6$). (b) H&E staining of rat aortic rings exposed to saline and FA, respectively. Panel: (b1) saline (control group), (b2) 0.04 mM FA, (b3) 0.08 mM FA, (b4) 0.16 mM FA, (b5) 0.32 mM FA, (b6) 0.64 mM FA, (b7) 1.28 mM FA, (b8) 2.56 mM FA. TA: tunica adventitia; TM: tunica media; TI: tunica intima. Yellow arrows: the sites where middle elastic plates have been loose, broken, or disappearing. All scale bars represent 50 μm . *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$, compared with control group.

Fig. 2.

The effect of FA on the NO/cGMP pathway in the rat aortas. (a, b) Inhibitory effects of L-NAME and MB on the vasorelaxation of the FA groups (0.64 mM–2.56 mM) ($n = 6$). The aortic rings were incubated with L-NAME (100 μM) and MB (10 μM) for about 10 min before the contraction caused by NE (10^{-6} M). (c) Immunohistochemical staining of eNOS in rat aortic rings exposed to FA in the presence of L-arg ($n = 7$). Panel: (c1) negative control group, (c2) saline (control group), (c3) L-arg, (c4) L-arg + 0.64 FA, (c5) L-arg + 1.28 F A, (c6) L-arg + 2.56 FA. All scale bars represent 50 μm. (d) The average optical density of immunohistochemistry for eNOS ($n = 7$). (e, f, g, h) NO, sGC, cGMP and PKG production in

isolated rat aortic hemi-segments treated with FA and L-arg and incubated for 2 h (e: n = 5, f: n = 6, g: n = 5, h: n = 5). *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$, compared with the control group; #: $p < 0.05$, ##: $p < 0.01$, ###: $p < 0.001$, compared with the L-arg group.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 3.

Effects of high concentrations of FA on subunit expression of BK_{Ca} channels in rat aortas. (a) Inhibitory effect of TEA on the FA (0.64 mM–2.56 mM) (n = 6) induced vasorelaxation. The aortic rings were pre-incubated with TEA (10 mM) for about 10 min before the contraction caused by NE (10⁻⁶ M). (b, c) Immunohistochemical staining and the average optical density of the BK_{Ca} α subunits in rat aortic rings (n = 6). Panel: (b1) negative control group, (b2) saline (control group), (b3) 0.64 FA, (b4) 1.28 FA, (b5) 2.56 F A. All scale bars represent 50 μm. (d) Immunofluorescence of BK_{Ca} α subunits in rat aortic rings. DAPI

(blue): nuclei; fluorescein isothiocyanate (FITC, green): BK_{Ca} α subunit. All scale bars represent 50 μ m. (e, f) Immunohistochemical staining and the average optical density of BK_{Ca} β subunits in rat aortic rings (n =5–6). Panel: (e1) negative control group, (e2) saline (control group), (e3) 0.64 F A, (e4) 1.28 F A, (e5) 2.56 FA. All scale bars represent 50 μ m. (g) Immunofluorescence of BK_{Ca} β subunits in rat aortic rings. DAPI (blue): nuclei; FITC (green): BK_{Ca} β subunit. All scale bars represent 50 μ m. *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$, compared with the control group, #: $p < 0.05$, ##: $p < 0.01$, ###: $p < 0.001$, compared with the 0.64 FA group.

Fig. 4.

Effects of high concentrations of FA on subunit expression of K_{ATP} channels in rat aortas. (a) Inhibitory effect of glibenclamide on the vasorelaxation of FA (0.64 mM–2.56 mM) ($n = 6$). The aortic rings were pre-incubated with glibenclamide (10 μ M) for about 10 min before the contraction caused by NE (10^{-6} M). (b, c) Immunohistochemical staining, and the average optical density of Kir6.1 subunit in rat aortic rings ($n = 6$). Panel: (b1) negative control group, (b2) saline (control group), (b3) 0.64 FA, (b4) 1.28 FA, (b5) 2.56 F A. All scale bars represent 50 μ m. (d) Immunofluorescence of Kir6.1 subunit in rat aortic rings.

DAPI (blue): nuclei; FITC (green): Kir6.1 subunit. All scale bars represent 50 μm . (e, f) Immunohistochemical staining and the average optical density of Kir6.2 subunits in rat aortic rings ($n = 5-6$). Panel: (e1) negative control group, (e2) saline (control group), (e3) 0.64 F A, (e4) 1.28 F A, (e5) 2.56 F A. All scale bars represent 50 μm . (g) Immunofluorescence of Kir6.2 subunits in rat aortic rings. DAPI (blue): nuclei; FITC (green): Kir6.2 subunit. All scale bars represent 50 μm . *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$, compared with the control group, #: $p < 0.05$, ##: $p < 0.01$, ###: $p < 0.001$, compared with the 0.64 FA group.

Fig. 5.

Effects of high concentrations of FA on subunit expression of L-type Ca^{2+} channels in rat aortas. (a) Inhibitory effect of nifedipine on the vasorelaxation of FA (0.64 mM–2.56 mM) ($n = 6$). The aortic rings were pre-incubated with nifedipine (1 μM) for about 10 min before the contraction induced by NE (10^{-6} M). (b, c) Immunohistochemical staining and the average optical density of $\text{Ca}_v\alpha_{1C}$ subunits in rat aortic rings ($n=6$). Panel: (b1) negative control group, (b2) saline (control group), (b3) 0.64 FA, (b4) 1.28 FA, (b5) 2.56 FA. All scale bars represent 50 μm . (d) Immunofluorescence of $\text{Ca}_v1.2$ subunits in rat aortic rings.

DAPI (blue): nuclei; FITC (green): Ca_v1.2 subunit. All scale bars represent 50 μ m. (e, f) Immunohistochemical staining and the average optical density of Ca_v1.3 subunits in rat aortic rings (n = 7). Panel: (e1) negative control group, (e2) saline (control group), (e3) 0.64 F A, (e4) 1.28 F A, (e5) 2.56 FA. All scale bars represent 50 μ m. (g) Immunofluorescence of Ca_v1.3 subunits in rat aortic rings. DAPI (blue): nuclei; FITC (green): Ca_v1.3 subunit. All scale bars represent 50 μ m. *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$, compared with the control group, #: $p < 0.05$, ##: $p < 0.01$, ###: $p < 0.001$, compared with the 0.64 FA group.

Fig. 6.

The effect of low concentrations of FA on the BK_{Ca}, K_{ATP} and L-type Ca²⁺ channel expression in rat aortas. (a, b) Immunohistochemical staining and the average optical density of BK_{Ca} α and subunits in rat aortic rings (n = 5–6). Panel: (a1) negative control group, (a2) saline (control group), (a3) 0.64 FA, (a4) 1.28 FA, (a5) 2.56 FA. All scale bars represent 50 μm. (c, d) Immunohistochemical staining and the average optical density of BK_{Ca} β subunits in rat aortic rings (n = 5–6). Panel: (c1) negative control group, (c2) saline (control group), (c3) 0.64 FA, (c4) 1.28 F A, (c5) 2.56 FA. All scale bars represent 50 μm. (e, f) Immunohistochemical staining and the average optical density of Kir6.1 subunit in rat aortic rings (n = 5–6). Panel: (e1) negative control group, (e2) saline (control group), (e3) 0.64 FA, (e4) 1.28 F A, (e5) 2.56 F A. All scale bars represent 50 μm. (g, h) Immunohistochemical staining and the average optical density of Kir6.2 subunits in rat aortic rings (n = 5–6). Panel: (g1) negative control group, (g2) saline (control group), (g3) 0.64 F A, (g4) 1.28 F A, (g5) 2.56 F A. All scale bars represent 50 μm. (i, j) Immunohistochemical staining and the

average optical density of $\text{Ca}_v1.2$ subunits in rat aortic rings ($n=5-6$). Panel: (i1) negative control group, (i2) saline (control group), (i3) 0.64 FA, (i4) 1.28 FA, (i5) 2.56 FA. All scale bars represent 50 μm . (k, l) Immunohistochemical staining and the average optical density of $\text{Ca}_v1.3$ subunits in rat aortic rings ($n=6$). Panel: (k1) negative control group, (k2) saline (control group), (k3) 0.64 F A, (k4) 1.28 F A, (k5) 2.56 FA ($n=5-6$). All scale bars represent 50 μm . *: $P < 0.05$; **: $P < 0.01$, ***: $P < 0.001$, compared with the control group, #: $p < 0.05$, ##: $p < 0.01$, ###: $p < 0.001$, compared with the 0.08 FA group.