

Inhibition of As(III) and Hg(II) caused aortic hypercontraction by eugenol, linalool and carvone

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Submitted October 15, 2014; accepted in final form December 8, 2014

Abstract

Acute and chronic exposure to arsenic and mercury is known to produce vasoconstriction. There is, however, no clarity concerning the pathways leading to this increased contraction. In this study we elicit and compare maximum contractility of rat aortas under resting conditions in the presence of arsenic and mercury, and delineate pathways mediating this effect. Phenylephrine (PE) induced hypercontraction of 37% and 32% were obtained when isolated aortic segments were exposed to 25 μ M As(III) and 6 nM Hg(II), respectively. Isometric contraction measurements in presence of apocynin, verapamil and sodium nitroprusside indicates that the major causes of increased contraction are reactive oxygen species (ROS) and depletion of nitric oxide (NO). Calcium influx plays a minor role in arsenic and mercury caused hypercontraction. In unexposed aorta, eugenol causes relaxation by inhibiting ROS and elevating NO, linalool by blocking voltage dependent calcium channel (VDCC) and elevating NO, and carvone by blocking calcium influx through VDCC. Since the arsenic and mercury hypercontraction is mediated by increased ROS and depleted NO, we hypothesize that molecules which neutralize ROS or elevate NO will be better ameliorators. In line with this argument, we found eugenol to be the best ameliorator of arsenic and mercury hypercontraction followed by linalool and carvone.

Key words: As(III), Hg(II), eugenol, carvone, linalool

Introduction

Arsenic and mercury are considered high-risk environmental pollutants impacting human health. Chronic exposure to these metals is associated with a wide range of illnesses including cancer, hyperkeratosis, diabetes, and cardiovascular diseases (1). Cardiovascular effects of arsenic and mercury toxicity include hypertension, atherosclerosis, peripheral vascular disorders and vasoconstriction (2, 3, 4). At low and high mercury

concentrations in pre-contracted aorta, relaxation and hypercontraction, respectively, has been reported (5). Oxidative stress and endothelial dysfunction are consistently observed in arsenic and mercury exposed aortic smooth muscle, but emerging evidence suggests that these factors also have a causal role in calcium influx leading to vasoconstriction (6, 7). Reactive oxygen species (ROS) may directly alter vascular function or cause changes in vascular tone by altering nitric oxide (NO) bioavailability or signalling (8). ROS-producing entities involved in increased vascular oxidative stress, observed during hypertension, include NADPH oxidase, xanthine oxidase, the mitochondrial respiratory chain and uncoupled endothelial nitric oxide synthase (NOS) (9). Molecules which can quench ROS, inhibit calcium influx or increase availability of NO, will therefore be relaxant and can potentially ameliorate deleterious effects of As(III) and Hg(II) on smooth muscle.

Various essential oils are rich in eugenol, linalool and carvone, which are known to possess strong antioxidant, anti-inflammatory and anti-spasmodic properties. These active principles are reported to show relaxation effect on the cardiovascular system (10, 11, 12). The relaxant effects of eugenol and linalool on both the aorta and trachea has been shown in our previous work (13, 14). Additionally, the calcium-dependent and calcium-independent inhibitory effect of eugenol has also been reported on various smooth muscle tissues (10, 15). Linalool, a monoterpene, is known to be a vasorelaxant in cardiovascular systems (16). Its mechanism of action is still not clear, but a recent report shows a calcium antagonist effect of linalool with NO playing a partial role in mediating this relaxant effect (11). Carvone has been shown to cause vasorelaxation in various systems, its effect on tracheal chains and ileum smooth muscle has been attributed to its calcium antagonist activity (17, 18).

The effect of exposure of the aorta to arsenic and mercury, its underlying mechanism and the possible ameliorative effects of eugenol, linalool and carvone on As(III) and Hg(II) exposed aortic segments is largely unknown. In this study we elicit and compare maximum contractility of aortic segments under resting conditions with that of arsenic and mercury exposed aortas and have attempted to delineate pathways mediating this effect by employing various inhibitors. The relaxant and ameliorative effect of eugenol, (+)-linalool and (–)-carvone are investigated.

Methods

Animals

Male Wistar rats, weighing 300–500 g, (from Project No. 883) as approved by the Institutional Animal Ethical Committee (IAEC, no. 004/2013), Jamia Millia Islamia University (New Delhi, India) were used in this study. They were kept under conditions of constant temperature (27 ± 2 °C) with a standard light/dark cycle (12/12 h), and free access to food and water. All animals were cared for the compliance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institute of Health (National Institute of Health, US).

Solutions and drugs

Eugenol, (+)-linalool, (–)-carvone (Sigma, purity 98.0%) were first dissolved in a small volume of dimethyl sulfoxide (DMSO, Sigma Chemicals Co., St Louis, MO, USA) and the total volume made up with distilled water. Final vehicle concentration to which tissue was exposed was always less than 0.05%. At this concentration DMSO did not influence phenylephrine (PE)-induced contraction (19). PE, acetylcholine (ACh), sodium nitroprusside (SNP), apocynin, verapamil, N^G-nitro-L-arginine methyl ester (L-NAME), arsenic (As(NO₂)₃) and mercury (HgCl₂) were procured from Sigma Chemicals, St. Louis, USA. Sodium chloride, potassium

chloride, magnesium sulphate, dextrose, calcium chloride, potassium dihydrogen phosphate and sodium bicarbonate obtained from Merck (India) were used for preparation of Krebs buffer with composition (in mM): 120 NaCl; 25 NaHCO₃; 1.2 MgSO₄; 1.2 KH₂PO₄; 4.72 KCl, 2.5; CaCl₂ and 11 C₆H₁₂O₆.

Measurement of aortic contractile activity

Rats were anesthetized with chloroform and sacrificed by cervical dislocation (20). Thoracic aortae were removed and immersed in Krebs medium at room temperature. After removing adhering fat and connective tissue, each aorta was cut transversally into cylindrical ring-like segments (4–5 mm) with care to avoid any damage to the endothelium. Rings were mounted in 15 mL organ baths containing Krebs medium continuously bubbled with 95% O₂ and 5% CO₂ at 37 °C. All experiments were performed after an equilibration period of 60 minutes with bathing medium renewed every 15 minutes which ruled out trauma or any other extraneous effect. Endothelium intact aortic rings were stretched with a passive tension of 2.0 g. Tension was recorded using an isometric force transducer (MLT0420, AD Instruments, Australia) connected to a PC-based Data acquisition system from AD Instruments (PL3508 PowerLab 8/35). Control contractions were induced at 1 μM PE as we get maximum response at this concentration (Fig. 1), this was in good agreement with other reports (21). Each aortic preparation was challenged at the beginning of the experiment with 1 μM of ACh, and if the vasorelaxant response to ACh was greater than 50% of PE-induced contraction, the aortic segment was considered to possess an intact endothelium.

Four series of experiments were performed as follows.

1) Series 1

This series of experiments were carried out to assess the acute effects of 40 minutes incubation of aortic segments with 10 μM, 25 μM, 50 μM arsenic and 6 nM, 0.5 μM, 1 μM mercury on PE-induced contractions of the endothelium-intact aortic rings.

2) Series 2

In this series of experiments, investigation on the possible mechanisms involved in arsenic and mercury induced hypercontraction was performed by pre-incubating the aortic segments with apocynin (100 μM), verapamil (100 μM) or SNP (1 μM) for 40 minutes followed by exposure to either As(III) or Hg(II) for another 40 minutes. The relaxant effect of apocynin (100 μM), verapamil (1 μM) or SNP (1 μM) alone was determined by incubating non-contracted rings for 80 minutes before eliciting a contractile response.

3) Series 3

This series of experiments were aimed to examine the inhibitory effects of exposure of the aortic rings for 40 minutes to eugenol, linalool and carvone at 100 μM each, on the contractile responses to PE (1 μM) in unexposed or As(III)/Hg(II) exposed aortic preparations with intact endothelium.

4) Series 4

In this series of experiments, the relaxation pathways for each of eugenol, linalool and carvone were investigated. Experiments were performed on endothelium containing aortic preparations incubated for 40 minutes with apocynin (100 μM), verapamil (1 μM) or L-NAME (30 μM) followed by another 40 minutes incubation of tissues with 100 μM of either eugenol or linalool or carvone.

The duration of the incubation in various experiments and controls in respect of As/Hg, eugenol, linalool and carvone was always 40 minutes. Incubation time for molecules employed as inhibitors i.e. apocynin, verapamil, SNP and L-NAME was always 80 minutes (both in experiments and controls).

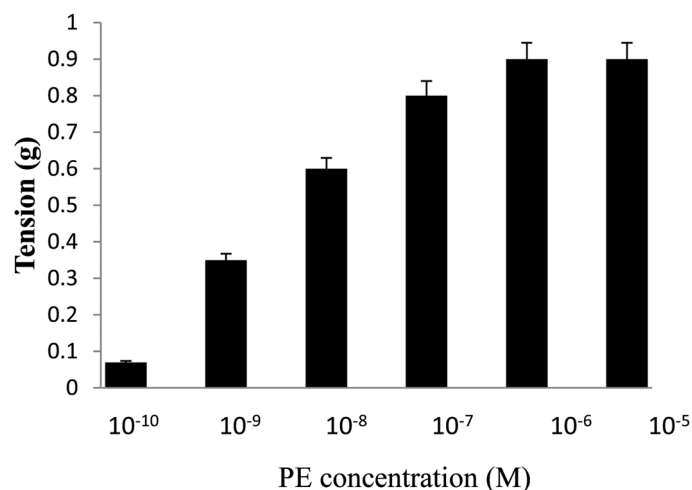


Fig. 1. Concentration response curve for PE (10^{-10} – 10^{-5} M) on ring segments of the rat aorta. The data represents the mean \pm S.E.M. ($n=5$); n : number of tissues.

Arsenic and mercury incubation did not lead to any significant change in the applied passive tension of 2.0 g. Of all the other tested molecules viz: apocynin, verapamil, L-NAME, SNP, eugenol, linalool and carvone, only SNP incubation lead to a decrease in the resting tension. This was reset to the passive tension of 2.0 g before eliciting contraction by PE. SNP caused relaxation has also been reported by other investigators (7).

Statistical analysis

Each response was tested on 5 thoracic aortic rings taken from different rats. Data is expressed as the mean \pm standard error of the mean (S.E.M.). The significance ($P \leq 0.05$) of the results was assessed by means of unpaired Students *t*-test, and one-way analysis of variance (ANOVA) followed by Duncan's multiple range test.

Results

The normal concentration of arsenic in human plasma is 2.4 $\mu\text{g/L}$ which can increase up to 38 $\mu\text{g/L}$ in chronic arsenic exposure (22). Plasma arsenic concentration could vary from 2.2 μM to 44.0 μM in some acute exposures. Arsenic toxicity is found to be more severe in humans than in other species (23). The concentration of HgCl_2 that is toxic for humans is about 1.5 $\mu\text{g/L}$ of blood i.e. 7 nM (7). Unlike other studies where the effect of Hg(II) was seen on pre-contracted aortic segments, in this study we have compared the maximum contractile response of aortic segments that have been pre-incubated with As(III) and Hg(II) in the relevant concentration range (5). Optimum incubation time was found to be 40 minutes. Fig. 2 shows contractile response of aortic rings that have been incubated with various concentrations of As(III) and Hg(II) for 40 minutes. Increased PE-induced contractility in the case of As(III) , compared to unexposed aortic segments, at 10 μM , 25 μM and 50 μM , was $13.0 \pm 7.1\%$, $37.0 \pm 1.5\%$ and $31.0 \pm 2.0\%$, respectively. As the optimum response was observed at 25 μM As(III) , further experiments were performed at that concentration using $\text{As(NO}_2)_3$. Similar working concentrations have been employed by other investigators (24). The increase in contraction following incubation with Hg(II) as HgCl_2 was $32.0 \pm 3.8\%$, $23.0 \pm 2.8\%$ and $26.0 \pm 1.1\%$ at Hg(II) concentrations of 6 nM, 0.5 μM and 1 μM , respectively. As the optimal response was seen at 6.0 nM, further studies were performed at 6 nM to ensure healthy tissue and linearity in contractile response. Similar concentration ranges for Hg(II) have

As and Hg caused aortic hypercontraction

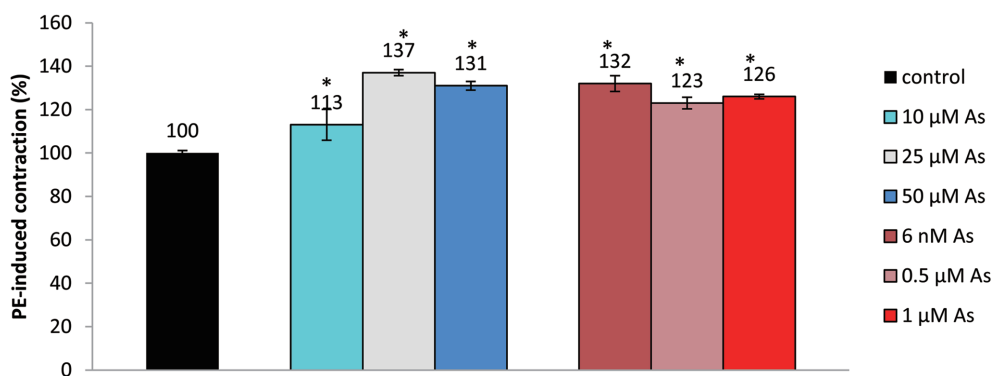


Fig. 2. Effect of various concentrations of arsenic and mercury on ring segments of the rat aorta. Values represent the mean \pm S.E.M. ($n=5$); $*P\leq 0.05$; indicates a significant difference in PE-induced contraction with respect to control (ANOVA) followed by Duncan's multiple range test; n : number of tissues.

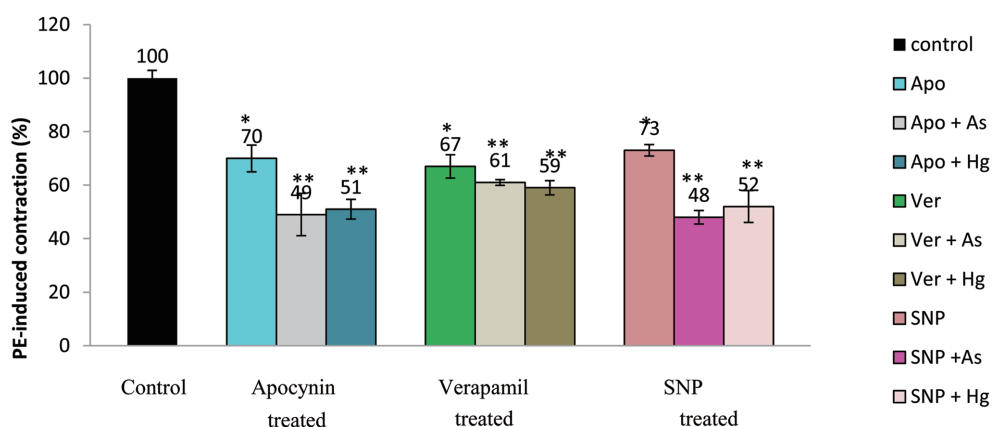


Fig. 3. Effect of apocynin, verapamil and SNP on metal unexposed and metal exposed aortic rings. Values represent the mean \pm S.E.M. ($n=5$); $**P\leq 0.05$; represents significant difference between effects of apocynin, verapamil, SNP on pollutant exposed and unexposed rings (unpaired Students t - test), $*P\leq 0.05$; indicates a significant difference in PE-induced contraction with respect to control (ANOVA) followed by Duncan's multiple range test; n : number of tissues.

been shown to be effective by other workers (7). In the absence of agonist, arsenic 25 μ M or mercury (6 nM) incubation did not lead to any significant tension change from the applied passive tension of 2.0 g.

Fig. 3. shows the effects of apocynin, verapamil and SNP on PE-induced contractions for pollutant unexposed and As(III) or Hg(II) incubated aorta. Apocynin and verapamil themselves did not significantly affect the resting tension. SNP, however, caused relaxation of aortic segments from the applied passive tension of 2.0 g, which was reset to 2.0 g before eliciting contraction by PE. Apocynin, a naturally occurring acetophenone widely used as an antioxidant, caused $30.0 \pm 5.0\%$ relaxation in unexposed endothelium-intact aortic rings as compared to control rings. PE-induced contractile response was reduced to $33.0 \pm 4.5\%$ and $27.0 \pm 2.2\%$ in presence of verapamil (L-type calcium channel blocker) and SNP (NO donor), respectively. Pre-incubation of aorta with apocynin (100 μ M) followed by pollutant exposure reduced the contractile response by $51.0 \pm 7.9\%$ and $49.0 \pm 3.7\%$ in the case of arsenic and mercury, respectively. With verapamil and SNP, this decrease in contraction was $39.0 \pm 1.2\%$ and $52.0 \pm 2.8\%$ for As(III) and $41.0 \pm 2.6\%$ and $48.0 \pm 6.0\%$ for Hg(II)-exposed

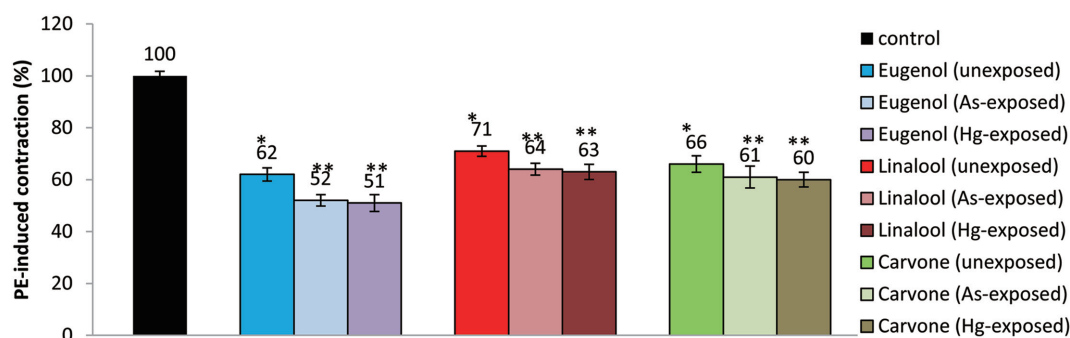


Fig. 4. Effect of eugenol (100 μ M), linalool (100 μ M) and carvone (100 μ M) on the contraction induced by PE in endothelium-intact thoracic aortic rings. Results are presented as the mean \pm S.E.M. ($n=5$); ** $P \leq 0.05$ compares a significant variation in contractions shown by these molecules in both unexposed and exposed ring segments of the rat aorta (unpaired Students t -test); * $P \leq 0.05$ shows significant difference of PE-induced contractions shown by eugenol, linalool and carvone versus control (ANOVA) followed by Duncan's multiple range test; n : number of tissues.

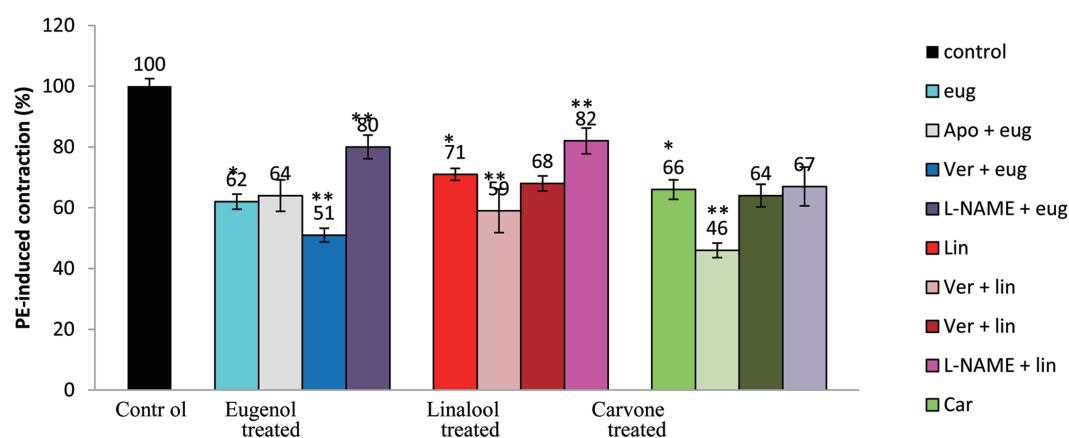


Fig. 5. Effect of eugenol, linalool and carvone and in both the absence and presence of apocynin (100 μ M), verapamil (1 μ M) and L-NAME (30 μ M) in pollutant unexposed ring segments of the rat aorta. Values represent the mean \pm S.E.M. ($n=5$); ** $P \leq 0.05$ represents significant contraction variation by these plant derivatives in the presence versus the absence of inhibitors (unpaired Students t -test), * $P \leq 0.05$ shows a significant difference in PE-induced contractions by eugenol, linalool and carvone versus control (ANOVA) followed by Duncan's multiple range test; n : number of tissues.

tissue.

Fig. 4. shows the inhibitory effects of eugenol, linalool and carvone on the contractions induced by PE in rat aortic rings in unexposed and As(III) or Hg(II) exposed preparations. For pollutant unexposed rings optimal responses for eugenol, linalool and carvone was seen following 40 minutes incubation, so this duration was used for all experiments with these molecules. Eugenol, linalool and carvone alone did not cause any change in the applied passive tension in the absence of PE. Eugenol caused $38.0 \pm 2.5\%$, $48.0 \pm 2.2\%$ and $49.0 \pm 3.3\%$ inhibition of contraction in metal unexposed, As(III) exposed and Hg(II) exposed aortic rings, respectively. Similar inhibition of contraction caused by linalool was $29.0 \pm 2.0\%$, $36.0 \pm 2.3\%$ and $37.0 \pm 2.9\%$ for unexposed, As(III) exposed and Hg(II) exposed tissue. Carvone showed a relaxant effect of $34.0 \pm 3.2\%$ in unexposed; $39.0 \pm 4.2\%$ in arsenic exposed; and $40.0 \pm 2.9\%$ in mercury exposed aortic segments, respectively.

The effects of various inhibitors on the contraction elicited by aortic rings incubated with eugenol, linalool or carvone are illustrated in Fig. 5. The saturating effect producing concentration of apocynin (100 μ M)

caused relaxations of $36 \pm 5.2\%$, $41.0 \pm 7.2\%$ and $54.0 \pm 2.5\%$ with eugenol, linalool and carvone, respectively. In the presence of verapamil ($1 \mu\text{M}$), eugenol, linalool and carvone showed $49.0 \pm 2.3\%$, $32.0 \pm 4.9\%$ and $36.0 \pm 1.5\%$ inhibition of contraction, respectively. L-NAME ($30 \mu\text{M}$); a potent inhibitor of NO, caused a decrease in the contractile response of eugenol, linalool and carvone by $20.0 \pm 3.99\%$, $18.0 \pm 4.2\%$ and $33.0 \pm 6.4\%$, respectively. In an earlier study, we have shown eugenol to be a more effective relaxant in the presence of indomethacin, to be less effective in the presence of L-NAME, while there was no change in relaxation in the presence of apocynin (13). Co-incubation of eugenol with verapamil caused more relaxation than eugenol alone. Linalool and carvone were more effective relaxants in the presence of apocynin, whereas the presence of verapamil did not alter the aortic contractility in respect of either linalool or carvone. L-NAME decreased the effectiveness of linalool as a relaxant but made no difference to the relaxant capacity of carvone.

Discussion

In this study we have shown that in isolated aortic segments under resting conditions when exposed to arsenic and mercury, there is an increase in isometric tension measured via agonist stimulation in an organ bath. Incubation in the presence of $25 \mu\text{M}$ arsenic and 6 nM mercury did not have any effect on the applied passive tension (2.0 g) in the absence of agonist, but caused hypercontraction of 37% and 32% after PE-induction. Under similar *in vitro* conditions, arsenic and mercury also cause hypercontraction of tracheal smooth muscle (unpublished results). These results are in line with the existing literature suggesting aortic vasoconstriction with arsenic (4). Mercury has also been reported to enhance contraction responses in aortic segments (7). A biphasic effect of mercury has recently been reported, with vasorelaxation at lower concentrations and vasoconstriction at higher concentrations in pre-contracted aortic segments (5). We did not observe biphasic responses in this study, possibly because we measured the tension after 40 minutes of Hg(II) incubation of the resting aortic segments which may lead to generation of ROS.

The excitation-relaxation mechanism of smooth muscle is regulated by changes in the intracellular calcium concentration (25), vasodilation by endothelium released nitric oxide (26), and excessive production of ROS (27). In this study, each of these pathways were inhibited to gain insight into the hypercontraction mechanisms of As(III) and Hg(II). In unexposed rings, magnitude of the maximum inhibition of contraction (or relaxation) caused by apocynin, verapamil, and SNP was almost identical, indicating that all major pathways contribute almost equally to the measured contraction. The decreased contractile responses shown by the arsenic and mercury exposed aortic rings in the presence of apocynin and SNP was greater as compared to unexposed aortic segments. This difference was 21% and 25% for As(III), and 19% and 21% for Hg(II). The difference in relaxation given by verapamil for unexposed and As(III) or Hg(II) exposed aorta was only 6% and 8% , respectively. The significantly higher effectiveness of apocynin and SNP in the case of pollutant hypercontracted aortic segments indicates that ROS generation and NO depletion play major roles in causing hypercontraction. This seems to be in agreement with other studies which indicate that exposure to arsenic and mercury cause overproduction of ROS resulting in oxidative stress with subsequent damage to endothelium which leads to reduced NO bioavailability in the vascular system (7, 28). In the presence of verapamil, the small difference in contraction suggests that calcium influx plays a minor role in hypercontraction of smooth muscle tissues in response to acute exposure to either arsenic or mercury.

The fact that plant-derived agents, eugenol, linalool and carvone, possess powerful anti-oxidant and calcium antagonist activity, highlights the importance of understanding their effects on As(III) and Hg(II) caused hypercontraction and the underlying mechanisms. Pre-incubation of aortic segments with eugenol, linalool or

carvone could effectively reduce PE-induced contraction. Similar relaxant actions by these active compounds have been shown by others investigators in various smooth muscle tissues (11, 12, 15). Eugenol has been reported to inhibit PE-induced contraction by the same magnitude in both the absence and presence of apocynin, suggesting that it acts by inhibiting ROS (13). Increased inhibition of contraction seen when verapamil was co-incubated with eugenol points to different site of action of these two molecules. We have also observed similar antioxidant effects of eugenol in the tracheal system (14). These results are in line with those suggesting that eugenol exerts cardiopreventive effects through its antioxidant properties (29), while a calcium antagonist action of eugenol has also been observed, but at higher concentrations (10). The magnitude of relaxation shown by carvone or linalool in the presence of verapamil was unchanged, but these molecules when co-incubated with apocynin lead to significantly increased relaxation. This indicates that carvone and linalool cause relaxation via calcium channel blockage. These observations are consistent with reports suggesting that both linalool and carvone may act as calcium antagonists (11, 18). L-NAME, known to inactivate NOS (30), caused the relaxation elicited by eugenol and linalool to decrease, while that of carvone remained unchanged. This indicates that the vasorelaxation caused by eugenol and linalool is NO-dependent whereas the vasorelaxing actions of carvone are NO-independent. The results obtained in this study are supported by reports that both eugenol and linalool induce *in vivo* antispasmodic effects which are endothelium released nitric oxide dependent whereas carvone acts by NO-independent mechanisms in rat isolated stomach preparations (11, 31, 32).

To summarize, in rat aortas eugenol causes relaxation by inhibiting ROS and elevating NO, linalool inhibits contraction by blocking L-type voltage dependent calcium channels (VDCC) and elevating NO. Carvone causes relaxation by blocking calcium influx through VDCC. As(III) and Hg(II) caused hypercontraction is mediated by increased ROS and depletion of NO. Based on this we hypothesize that molecules which neutralize ROS or elevate NO will be ameliorators of arsenic and mercury caused vasoconstriction. In line with this we find eugenol, which neutralizes ROS and elevates NO, to be best ameliorator followed by linalool which only elevates NO in this system. Carvone with its major effect only of VDCC blocking comes out as poor ameliorator of As(III) and Hg(II) caused hypercontraction of aorta.

Acknowledgements

This work was supported by the funding from UGC (SAP), DRS-I (F.3-20/2011) to Prof. SFB and Prof. LAK.

Conflict of interest

The authors declare no conflict of interest.

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