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## Ferulic acid-4-O-sulfate rather than ferulic acid relaxes arteries and lowers blood pressure in mice

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### Running title

Vasorelaxant activity of ferulic acid-4-O-sulfate

### Chemical compounds studied

Ferulic acid (PubChem CID: 445858), ferulic acid-4-O-sulfate (PubChem CID: 6305574)

## Abstract

Consumption of foods rich in ferulic acid (FA) such as wholegrain cereals, or FA precursors such as chlorogenic acids in coffee, is inversely correlated with risk of cardiovascular disease and type 2 diabetes. As a result of digestion and phase II metabolism in the gut and liver, FA is converted predominantly into ferulic acid-4-O-sulfate (FA-sul), an abundant plasma metabolite. Although FA-sul may be the main metabolite, very little has been reported regarding its bioactivities. We have therefore compared the *ex vivo* vasorelaxing effect of FA and FA-sul ( $10^{-7}$  -  $3 \cdot 10^{-5}$  M) on isolated mouse arteries mounted in tissue myographs. FA-sul, but not FA, elicited a concentration-dependent vasorelaxation of saphenous and femoral arteries and aortae. The FA-sul mediated vasorelaxation was blunted by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a soluble guanylate cyclase (sGC) inhibitor. The role of sGC was confirmed in femoral arteries isolated from sGC $\alpha_1$ <sup>(-/-)</sup> knockout mice. Furthermore, 4-aminopyridine, a specific inhibitor of voltage-dependent potassium channels, significantly decreased FA-sul mediated effects. In anesthetized mice, intravenous injection of FA-sul decreased mean arterial pressure, whereas FA had no effect, confirming the results obtained *ex vivo*. FA-sul is probably one of the major metabolites accounting for the blood pressure-lowering effects associated with FA consumption.

## Key words

Ferulic acid, Ferulic acid-4-O-sulfate, mouse, vasorelaxation, blood pressure

## Abbreviations

4-AP: 4-aminopyridine, Ach: Acetylcholine, Ca<sup>2+</sup>/CaM: Ca<sup>2+</sup>/calmodulin, cGK: cGMP dependent protein kinase, cGMP: Cyclic guanosine monophosphate, COX: Cyclooxygenase, FA: Ferulic acid, FA-glu:FA-4-O-glucuronide, FA-sul: Ferulic acid-4-O-sulfate, KRB: Krebs-Ringer-Bicarbonate, K<sub>v</sub>: Voltage-dependent K<sup>+</sup>-channel, L-Arg: L-arginine, L-Cit: L-citrulline, L-NAME: N<sup>6</sup>-nitro-L-arginine methyl ester, MAP: mean arterial pressure, NO: Nitric oxide, NOS: Nitric oxide synthase, ODQ: 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, PDE: Phosphodiesterase, Phe: Phenylephrine, sGC:

soluble guanylate cyclase, SNP: Sodium Nitroprusside, TEA: Tetraethyl ammoniumchloride, VOCC: Voltage-operated Ca<sup>2+</sup>-channels, VSMC: Vascular smooth muscle cell.

## 1: Introduction

Polyphenols are considered to be beneficial to health, as their consumption has been inversely related to development of cancer, diabetes and cardiovascular diseases [1,2]. The hydroxycinnamic acid ferulic acid (FA) is found in rice bran, citrus fruits, banana, beetroot, spinach, cabbage, broccoli, tomatoes, cereals and whole grain products [3–5]. In these foods, FA is esterified to cell wall polysaccharides, limiting its absorption in the small intestine. In addition, coffee is a major source of FA in the form of chlorogenic acid esters formed between quinic acid and trans-cinnamic acids such as FA, *p*-coumaric acid and caffeic acid [6–8]. Upon arrival in the colon, esterase activity of the resident microflora releases FA [5], followed by conjugation to ferulic acid-4-*O*-sulfate (FA-sul) and ferulic acid-4-*O*-glucuronide (FA-glu) during uptake in the intestinal epithelium and passage through the liver [9]. Maximal total plasma FA concentrations can reach 11  $\mu$ M in rats consuming chow containing 1.5 g/kg FA for 9 days [10] and between ~290 and 800 nM in human volunteers upon coffee consumption [11–13]. In volunteers consuming tomatoes containing 21-44 mg FA, 4-5% was recovered in urine as unmodified FA, increasing to 11-25% when conjugated FA was included [9], comparable to 2-20% of the excreted FA present as glucuronide or sulfate conjugates after consumption of *Pinus maritima* extract [14]. When FA was perfused into the rat small intestine, free FA was the major form present in the mesenteric vein (60%), but in aorta, 75% of the FA was conjugated [15], while only conjugated FA was found in plasma and bile [16]. Peak plasma concentrations of FA-sul upon coffee consumption reached between  $76 \pm 9$  and  $226 \pm 113$  nM [8,17,18]. In urine, FA-sul was the predominant form of FA, followed by FA-glu and FA aglycone [11].

FA consumption reduces the risk of diabetes and cardiovascular diseases, improves lipid metabolism [4,19,20] and decreases blood pressure in rat models of hypertension [21]. The *ex vivo*

vasorelaxant activity of FA is more pronounced in aortae isolated from hypertensive compared to normotensive rats [22–24]. However, the biological effects and their underlying mechanisms predicted by such *ex vivo* experiments with FA may differ from those observed *in vivo*, because of the metabolic transformation of the parent compound in the liver and during absorption in the gut epithelium. In this study, we have investigated the vasorelaxing effect of both the food-derived aglycone FA and the circulating conjugate FA-sul using mouse saphenous artery, femoral artery and aorta. In addition the influence of FA-sul and FA on blood pressure was measured in anesthetized mice.

## 2: Materials and methods

### 2.1: Animals

Male Swiss mice were obtained from Janvier (Saint-Berthevin, France), and soluble guanylate cyclase (sGC) alpha 1 subunit knockout (sGC $\alpha_1^{(-/-)}$ ) mice and their control mice sGC $\alpha_1^{(+/+)}$  with a 129SvJ background [25] were obtained from the department of Biomedical Molecular Biology (VIB, Ghent, Belgium). The animals were kept in an acclimatised room and food and water were provided ad libitum. All animals were treated in accordance to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health. This study was approved by the local ethical committee for animal experiments.

### 2.2: Tissue preparation and mounting

On the day of the experiment, animals (age 8-13 weeks) were sacrificed by cervical dislocation. Saphenous and femoral arteries from both legs and thoracic aorta were carefully removed and placed in Krebs-Ringer-Bicarbonate (KRB) solution (composition as follows (mM): NaCl, 135; KCl, 5; NaHCO<sub>3</sub>, 20; glucose, 10; CaCl<sub>2</sub>, 2.5; MgSO<sub>4</sub>, 1.3; KH<sub>2</sub>PO<sub>4</sub>, 1.2 and EDTA, 0.026 in H<sub>2</sub>O) bubbled with carbogen gas (95% O<sub>2</sub> and 5% CO<sub>2</sub>). Arteries were cleaned of surrounding connective and fat tissues, cut into segments and mounted in a wire myograph for isometric tension recording. Tissues were allowed to equilibrate for 30 minutes in KRB solution (37°C) bubbled with carbogen gas whilst replacing the KRB solution every 10 minutes. After equilibration,

isolated femoral and saphenous arteries were set to their normalised internal diameter by progressive stretching. The passive wall tension, based on internal diameter measurements obtained during progressive stretching, was used to adjust the arterial diameter to 90% of the diameter the vessel would have under a transmural pressure of 100 mmHg [26]. Aortic rings were stretched gradually until a stable preload of 0.5 g was obtained.

### 2.3: Experimental protocol for isometric tension recordings

After the equilibration period (30 min) at the optimized resting tension, femoral and saphenous arteries were repeatedly activated by treatment with KRB solution containing 120 mM K<sup>+</sup> and 5 μM phenylephrine (Phe), after which tissues were rinsed with KRB solution until a stable baseline tension was reached. Aortas were contracted once with KRB solution containing 120 mM K<sup>+</sup> and 1 μM Phe for 15 min, after which the organ baths were rinsed until a stable baseline tension was reached. In sGCα<sub>1</sub><sup>(-/-)</sup> mice femoral arteries, the efficiency of sGC was tested by cumulative concentration-response curves (10<sup>-9</sup> - 10<sup>-4</sup> M) of sodium nitroprusside (SNP), a nitric oxide (NO)-donor. In knockout mice, the response toward SNP was decreased compared to the effect in wild type mice, confirming effective knockout of sGC. To evaluate the presence of functional endothelium, arteries were exposed to 10 μM acetylcholine (Ach). Only arteries containing functional endothelium, as indicated by sudden relaxation upon Ach treatment, were used. Fully functional vessels were rinsed with KRB solution and baseline tension was maintained for 30 min whilst refreshing the KRB solution every 5 minutes. Arteries were contracted with Phe for 30 min (femoral/saphenous) or 1h (aorta) until a stable contraction was reached, after which cumulative concentration-response (1.10<sup>-7</sup> - 3.10<sup>-5</sup> M) curves for FA and FA-sul were established, exposing the tissues in 10 min intervals. FA-sul (90%) was synthesized by Dr. Nikolai U. Kraut (University of Leeds, Leeds, UK). Polyphenol stocks were prepared in a concentration of 10<sup>-2</sup> M in 10% DMSO in distilled water. Appropriate dilutions of DMSO were used as control and vehicle concentrations in the organ baths never exceeded 0.1%.

### 2.4: Mechanistic investigation

By use of specific modulators added 20 min prior to the final contraction with Phe, the mechanism of FA-sul mediated vasorelaxations was investigated. The NO synthase blocker  $N^G$ -nitro-L-arginine methyl ester (L-NAME) (100  $\mu$ M), cyclooxygenase (COX) inhibitor indomethacin (10  $\mu$ M), sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10  $\mu$ M), non-specific  $K^+$ -channel blocker tetraethyl ammoniumchloride (TEA) (3 mM), small conductance  $K_{Ca}$ -channel inhibitor apamin (500 nM), voltage-dependent  $K^+$ -channel ( $K_v$ ) inhibitor 4-aminopyridine (4-AP) (3 mM), inward rectifying  $K^+$ -channel inhibitor  $Ba^{2+}$  (10  $\mu$ M) and the ATP sensitive  $K^+$ -channel inhibitor glibenclamide (3  $\mu$ M) were used in this study. Glibenclamide was dissolved in DMSO; TEA, apamin, 4-AP,  $BaCl_2$ , L-NAME in distilled water and indomethacin and ODQ in ethanol. Incubation with KRB solution containing 120 mM  $K^+$  and 3 mM TEA resulted in significantly higher Phe-induced contraction levels. Endothelium was removed by gently rubbing the intimal surface with a stainless steel wire (aorta and femoral artery) or a hair (saphenous artery). Complete removal of the endothelium was verified by the lack of response toward Ach.

### 2.5: *In vivo study*

Swiss mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and placed on a heated blanket to maintain their body temperature at 37°C. A PE-10 tube filled with heparinized saline (25 U/mL) was inserted in the carotid artery and connected to a pressure transducer to measure the mean arterial pressure (MAP). The effect FA and FA-sul (standardized injection volume of 10  $\mu$ L) on MAP was determined after injection through another PE-10 tube introduced to the jugular vein. Ferulic acid was dosed at 11.42  $\mu$ g/kg (dose 1), and 114.2  $\mu$ g/kg (dose 2). For FA-sul, doses were 16.13 and 161.3  $\mu$ g/kg respectively. Appropriate dilutions of vehicle (DMSO) served as controls. Treatments order (DMSO, FA, FA-sul) were randomized and tested sequentially in the same animal, with a total of 4 biological replicates. Between each dose, the cannulas were flushed four times with heparinized saline to remove residual agent.

### 2.6: *Data analysis and statistics*

Data are represented as mean values  $\pm$  S.E.M..  $n$  represents the number of independent preparations in organ bath experiments or the number of mice used for *in vivo* experiments. For isometric tension measurements, data is expressed relative to the contraction level induced by Phe in the same preparation before cumulative addition of the compounds. *In vivo*, the MAP was determined prior to the injection of FA or FA-sul, and at the lowest point obtained after injection. These values were used to calculate the  $\Delta$  MAP.

Normality of data was checked and appropriate statistical tests (Student's t-test or Mann-Whitney U test) were used accordingly. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ .

### 3: Results

#### 3.1: Response to FA and FA-sul *ex vivo*

We compared the vasorelaxing properties of FA and FA-sul in mouse aorta, femoral and saphenous arteries. Within the concentration range tested, FA elicited no significant response. FA-sul, however, caused significant concentration-dependent relaxations in all three tissues. The observed  $E_{\max}$  was  $64.2 \pm 4.0\%$  in saphenous artery,  $89.8 \pm 4.5\%$  in femoral artery and  $70.7 \pm 3.5\%$  in aorta whereas in vehicle controls, tension decreased by  $32.8 \pm 7.4\%$ ,  $14.5 \pm 13.5\%$  and  $15.9 \pm 10.5\%$  respectively (Fig. 1). Immediate relaxations were observed upon application of a higher concentration of FA-sul (Fig. 2).

To exclude toxicity of FA-sul, tissues were rinsed after FA-sul treatment, maintained at basal resting tension for 20 minutes and contracted with Phe. The successful contraction excluded vasorelaxations due to tissue damage inflicted by FA-sul in our experimental setup (data not shown).

#### 3.3: Response to FA and FA-sul *in vivo*

In Swiss mice, the basal MAP was  $114.1 \pm 1.7$  mmHg. Immediately after intravenous injection of FA-sul, the MAP decreased significantly by  $9.6 \pm 1.2$  (dose 1) and  $34.9 \pm 3.2$  mm Hg (dose 2) (Fig. 3 A). Intravenous injection of FA did not decrease MAP significantly compared to vehicle controls (Fig. 3 A). Representative traces of FA (dose 2) and FA-sul (dose 1 and 2) are presented in Fig. 3

B-D, showing that after a sudden decrease upon administration of FA-sul, the MAP restores to the original level.

### 3.2: Vasorelaxing mechanism

Because previous studies partly attribute FA-mediated vasorelaxation to the endothelium [22,23], we investigated the involvement of the endothelium in isolated aorta, femoral and saphenous arteries by mechanic removal and by incubation with L-NAME and indomethacin (Fig. 4). FA-sul induced relaxation was not attenuated by these treatments, thereby excluding the endothelium as an active participant in FA-sul-mediated vasorelaxation. Endothelium denudation and L-NAME treatment further amplified vasorelaxation upon FA-sul treatment, although the effect was only significant in aorta and femoral arteries respectively (Fig. 4 A,B,C,G,H,I).

Even though the endothelium was not involved, a direct effect of FA-sul on sGC could not be ruled out and was further investigated by incubation with the sGC inhibitor ODQ (Fig. 5 A for femoral artery, data for aorta and saphenous artery not shown). ODQ strongly decreased FA-sul-induced relaxations, indicating involvement of sGC in the three arteries. The vasorelaxing effect of FA-sul was less pronounced in femoral arteries isolated from sGC $\alpha_1$ <sup>(-/-)</sup> mice compared to control mice, confirming sGC as a mediator of FA-sul dependent vasorelaxations (Fig. 5 B).

While big arteries like aorta play a role in conducting the blood and damping pulse pressure, smaller arteries contribute more to arterial resistance and hence blood pressure regulation [27]. Consequentially, femoral or saphenous arteries provide a more relevant model for *ex vivo* tension measurements compared to large diameter arteries such as aorta. Further mechanistic investigations were therefore conducted only on femoral arteries where the FA-sul mediated relaxations were found to be maximal. Vasorelaxation pathways involving K<sup>+</sup>-channel opening were investigated by exposing femoral arteries to a high concentration of K<sup>+</sup> (Fig. 6 A) or K<sup>+</sup>-channel blockers (Fig. 6 B-F). Decreased FA-sul-induced vasorelaxation in the presence of K<sup>+</sup> and TEA confirmed the involvement of K<sup>+</sup> channels and more specifically K<sub>v</sub>-channels, as indicated by

the decreased response in the presence of 4-AP (Fig. 6 A,B,F). Ba<sup>2+</sup>, apamin and glibenclamide did not affect FA-sul-induced relaxations (Fig. 6 C,D,E).

#### 4: Discussion

We have shown for the first time that the phenolic acid conjugate FA-sul, as present in circulation following consumption of phenolic-rich food, is more active as an *ex vivo* vasorelaxant and *in vivo* blood pressure lowering compound compared to the parent aglycone FA. Since FA is a major phenolic present in human diet, the daily intake can exceed 100 mg [28], supporting high circulating FA-sul concentrations. Indeed, phase II conjugates constitute a significant portion of the bioavailable FA present in plasma [8,9,14,15,17,18] and urine [11,18] and could contribute to beneficial health effects of FA-rich foods. Although glucuronidation and sulfation generally decrease biological activity [29], we showed that sulfation of FA enhanced its bioactivity, a unique finding similar to the K<sup>+</sup>-channel-dependent vasodilatory activity of the circulating metabolite minoxidil sulfate, derived from the vasodilator drug minoxidil [30].

To our knowledge, no other studies have determined cardiovascular health benefits of FA-sul *in vitro*, *ex vivo* or *in vivo*. Biological effects of FA on the other hand have been described. Ferulic acid at higher concentrations exhibits vasorelaxant properties in isolated rat arteries [22–24] and *in vivo* [21,24,31] in an endothelium-dependent fashion attributed to stimulation of NO production or antioxidant effects preventing NO degradation. A direct effect on vascular smooth muscle is not ruled out, although involvement of sGC is not confirmed [22,31,32]. The effects of FA are most prominent in pathophysiological conditions [21–23,31] using high concentrations of 10 μM to 30 mM [22–24,32], which could explain the discrepancy compared to the results obtained in the current study where a normotensive model and lower concentrations were used.

By using concentrations slightly exceeding those currently reported in plasma (226 nM [17]), the potential of FA-sul as a blood pressure lowering compound and *ex vivo* vasorelaxant was demonstrated. The vasorelaxing mechanism of FA-sul could be investigated in more detail in isolated mouse arteries.

FA-sul-induced vasorelaxation was not endothelium nor NO-dependent, as indicated by the unchanged relaxation in endothelium denuded arteries or upon inhibition of NOS and COX by L-NAME and indomethacin. However, in the absence of NO, FA-sul induced relaxations were stimulated, and the effect reached significance in endothelium denuded aorta and L-NAME-treated femoral arteries. Consequentially, the potency of FA-sul was increased in the absence of NO, suggesting a role for FA-sul as a competitor to NO for its molecular target, sGC. Direct interactions between FA-sul and sGC were therefore investigated by incubation with ODQ, an sGC inhibitor that oxidizes the sGC heme, abolishing its NO sensitivity [33,34]. FA-sul-mediated relaxation was attenuated by ODQ, and the involvement of sGC in FA-sul-mediated vasodilation in Swiss mice was further supported by the decreased response in sGC $\alpha_1$ <sup>(-/-)</sup> mice femoral arteries. Catalytically active sGC is a heterodimeric haemoprotein consisting of  $\alpha_1/\beta_1$  or  $\alpha_2/\beta_1$  subunits, of which the  $\beta_1$  subunit contains the prosthetic haem group [35–37]. Although the decreased response in sGC $\alpha_1$  knockout mice indicates that sGC is involved, the residual activity of sGC $\alpha_2\beta_1$ , whose enzymatic activity is substantial and unaffected in sGC $\alpha_1$ <sup>(-/-)</sup> mice [25], may explain the incomplete loss of tension in sGC $\alpha_1$ <sup>(-/-)</sup> mice compared to wild-type animals. These observations indicate that FA-sul directly activates or stimulates sGC, enhancing the generation of the second messenger cyclic guanosine monophosphate (cGMP) [25,34].

Drugs that activate or stimulate sGC have been developed to treat pathologies associated with impaired sGC signalling. Haem-dependent sGC stimulators such as YC-1, BAY 41-2272, BAY 41-8543, CFM 1571 and A-350619 synergistically increase sGC activity by allosteric binding in the presence of submaximal concentrations of NO [38,39]. In our experiments, the role of FA-sul as a sGC stimulator is doubtful because FA-sul caused stronger vasodilation in the absence of NO compared to in the presence of NO, as observed in endothelium denuded arteries or in the

presence of L-NAME. This suggests that FA-sul does not act as a sGC stimulator.

sGC activators such as cinaciguat (BAY 58-2667) and HMR-1766 can increase sGC activity even in the absence of NO and a functional haem group in sGC [34,37]. This is of great interest since sGC activators are still effective when sGC has become unresponsive due to haem oxidation by increased oxidative stress, as is the case in diabetes, arterial hypertension, atherosclerosis and obesity [35,39]. Since oxidation of the ferrous haem cofactor by O<sub>2</sub> [34] results in almost complete abrogation of the effects of FA-sul, and the potency of FA-sul does not increase when the haem-moiety is oxidized, as is the case for most sGC activators [40], haem-independent sGC activator activity of FA-sul is unlikely. Our data therefore indicate that FA-sul causes a haem-dependent activation of sGC, but further mechanistic studies are required to confirm this hypothesis.

As a consequence of their major role in blood pressure regulation [41,42] and participation in downstream sGC signaling [42–44], K<sup>+</sup>-channels could be involved in the response to FA-sul.

Nonspecific K<sup>+</sup>-channel blockade by TEA and 120 mM K<sup>+</sup> resulted in decreased response to FA-sul, thus confirming that FA-sul acts through K<sup>+</sup>-channel opening. By using inhibitors targeting specific K<sup>+</sup>-channel subtypes, we observed a decreased response in the presence of 4-AP, supporting K<sub>v</sub>-channel gating as a response to FA-sul. Since sGC inhibition was very effective in our setup and the K<sub>v</sub>-channels are activated in the downstream signaling cascade of sGC in rat arteries [45,46], indirect effects of FA-sul downstream of sGC were possible although direct activation of K<sup>+</sup>-channels by FA-sul could not be excluded. For example, minoxidil sulfate possibly acts by induction of K<sup>+</sup>-channel mediated activity through increased intracellular Ca<sup>2+</sup>, cGMP-dependent protein kinase (cGK) mediated enhancement of K<sub>Ca</sub><sup>+</sup>-channels sensitivity to Ca<sup>2+</sup> [47] or direct sulfation of a protein subunit in K<sup>+</sup>-channels [48]. Similar mechanisms could be involved in the FA-sul mediated response, but they were not investigated in this study.

The magnitude of inhibition by TEA and K<sup>+</sup> exceeded that of 4-AP, suggesting that other potassium channels were involved. However, inhibition of inward rectifying, ATP-sensitive K<sup>+</sup>-channels and

small conductance  $K_{Ca}$ -channels by  $Ba^{2+}$ , glibenclamide and apamin was unsuccessful in our experiments, excluding them as potential mediators of FA-sul dependent vasorelaxations.

Taken together, these data indicate that FA-sul enhances sGC activity, resulting in the production of cGMP and activation of its associated signaling pathways.  $K_v$ -channel opening as a consequence of FA-sul treatment results in  $K^+$  efflux, hyperpolarization and inhibition of voltage-operated  $Ca^{2+}$ -channels, decreased intracellular  $Ca^{2+}$  followed by vasorelaxation [32,42]. This pathway and the proposed role of FA-sul is summarized in Fig. 7.

In conclusion, we have for the first time compared the vasorelaxing potential of FA and FA-sul in three types of isolated mouse arteries and *in vivo* in anesthetized mice and identified FA-sul as a potent vasorelaxing and blood pressure-lowering compound. Our data demonstrate that sGC and  $K^+$ -channels such as voltage-gated  $K^+$ -channels are involved in the FA-sul-dependent vasorelaxation. Therefore, phase II conjugated polyphenols such as FA-sul could be very relevant as mediators of the health benefits associated with consumption of FA-rich foods such as coffee or wholegrain products.

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## Conflict of interest

GW has recently received other research funding from Florida Department of Citrus, USA and conducted consultancy for Nutrilite, USA and Suntory, UK.

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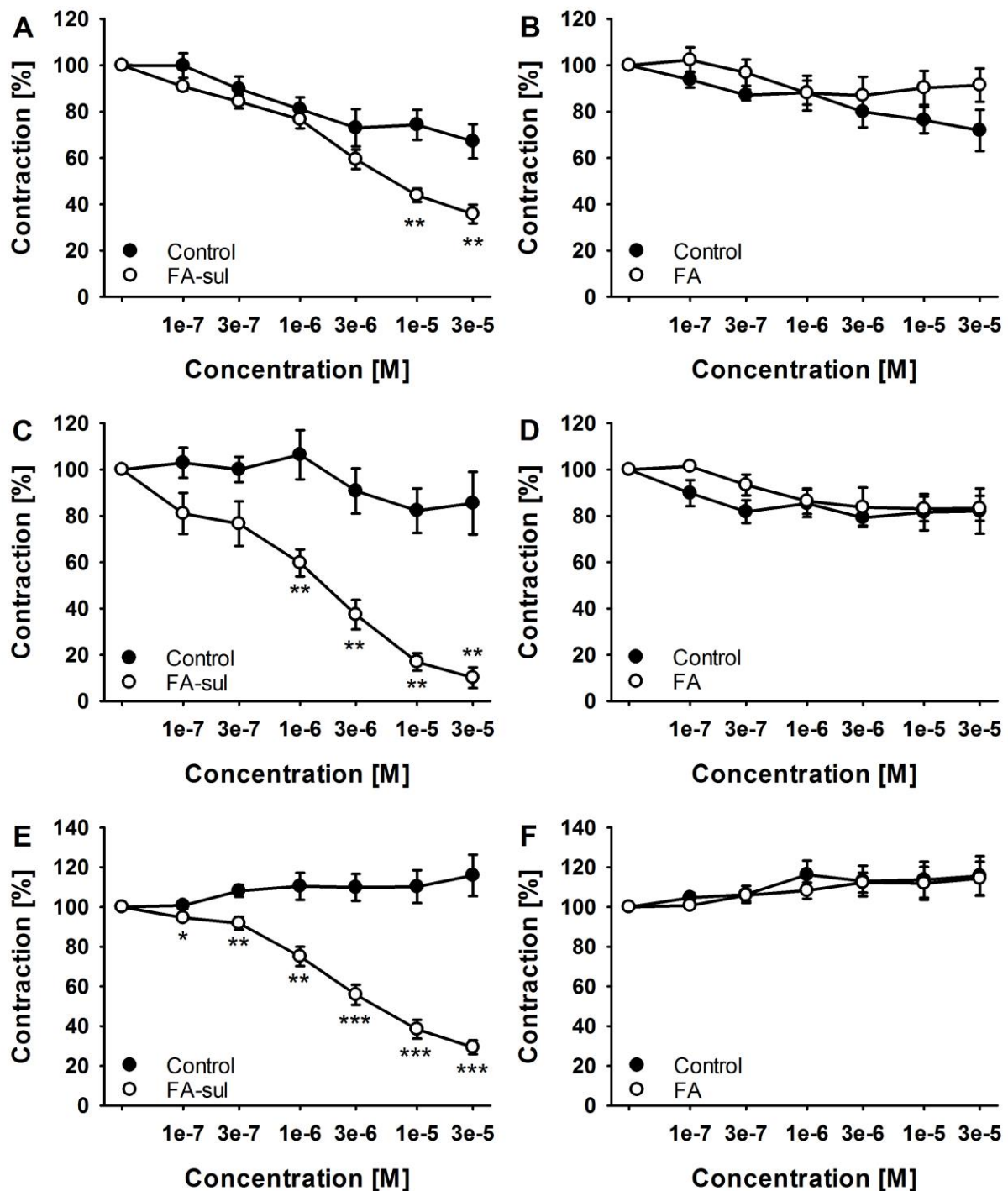


Figure 1: Vasorelaxing effects of FA-sul (left) and FA (right) (○) on isolated Swiss mouse saphenous artery (A,B), femoral artery (C,D) and aorta (E,F), compared to their solvent controls (●). Contraction (y-axis) is expressed relative to the Phe-induced contraction in the same preparation before cumulative addition of the compounds. Cumulative concentration-response curves were established between  $1 \cdot 10^{-7}$  and  $3 \cdot 10^{-5}$  M (x-axis) using 10 minute intervals.  $n=5$ . \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ .

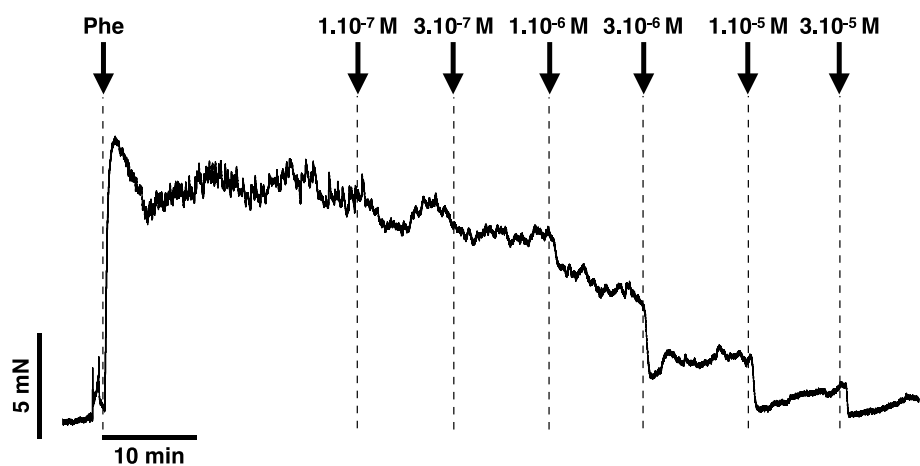


Figure 2. Original representative trace of the response to increasing concentrations of FA-sul in isolated Swiss mouse femoral artery contracted with 5  $\mu$ M Phe. A higher concentration of FA-sul is added every 10 min, as indicated by the arrows.

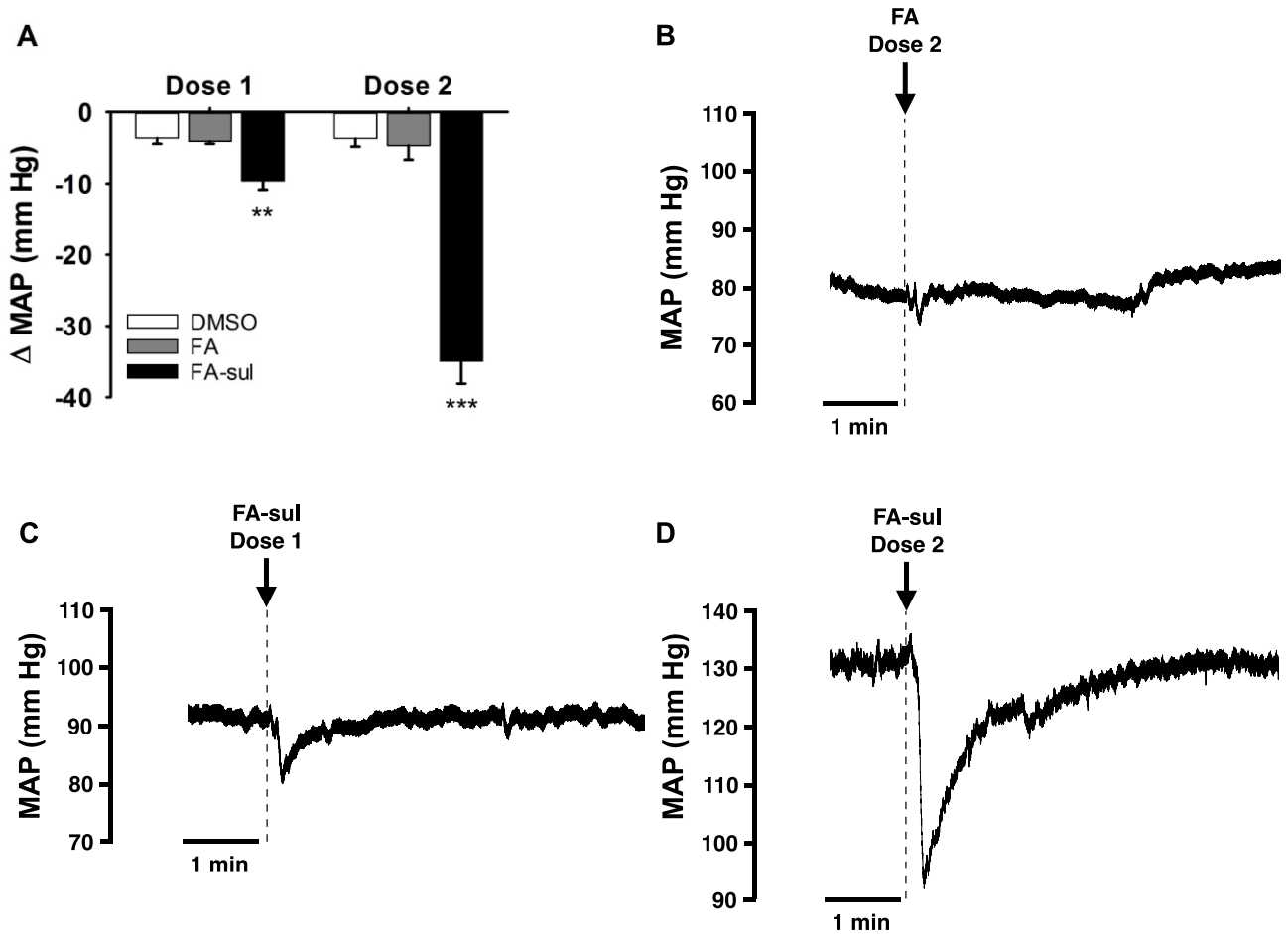


Figure 3: Effects of FA and FA-sul on blood pressure in anesthetized Swiss mice.

A: changes in mean arterial pressure (MAP,  $\Delta$  MAP = MAP<sub>after</sub> - MAP<sub>before</sub>) upon intravenous administration of 10  $\mu$ L DMSO (vehicle control, white), FA (grey) or FA-sul (black). Bars represent averages and S.E.M., n=4. \*\*: p<0.01, \*\*\*: p<0.001 compared to vehicle controls. Original tracings obtained upon intravenous injection of FA (dose 2, B) and FA-sul (dose 1, C and dose 2, D) show the change in MAP in Swiss mice.

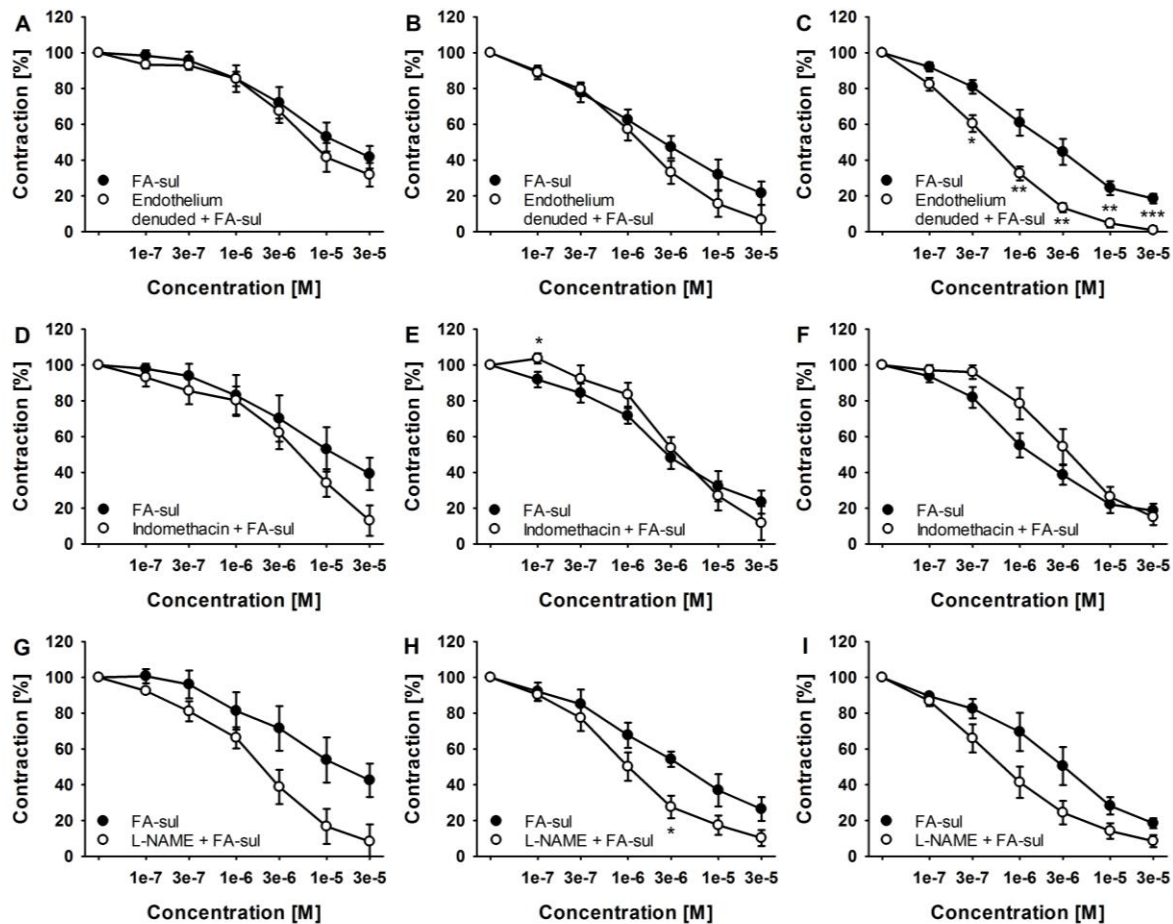


Figure 4: Endothelium dependency of FA-sul induced vasorelaxation in isolated Swiss mouse saphenous arteries (A,D,G), femoral arteries (B,E,H) and aorta (C,F,I). Vasorelaxing effects of FA-sul in untreated (●) or endothelium denuded (A,B,C), indomethacin treated (D,E,F) or L-NAME treated (G,H,I) (○) arteries are compared. Contraction (y-axis) is expressed relative to the Phe-induced contraction in the same preparation before cumulative addition of the compounds. Cumulative concentration-response curves were established between  $1 \cdot 10^{-7}$  and  $3 \cdot 10^{-5}$  M (x-axis) using 10 min intervals.  $n=3$  (L-NAME saphenous),  $n=4$  (indomethacin saphenous, indomethacin aorta, L-NAME aorta)  $n=6$  (L-NAME femoral, endothelium denuded saphenous, endothelium denuded aorta) or  $n=7$  (endothelium denuded femoral, indomethacin femoral). \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$

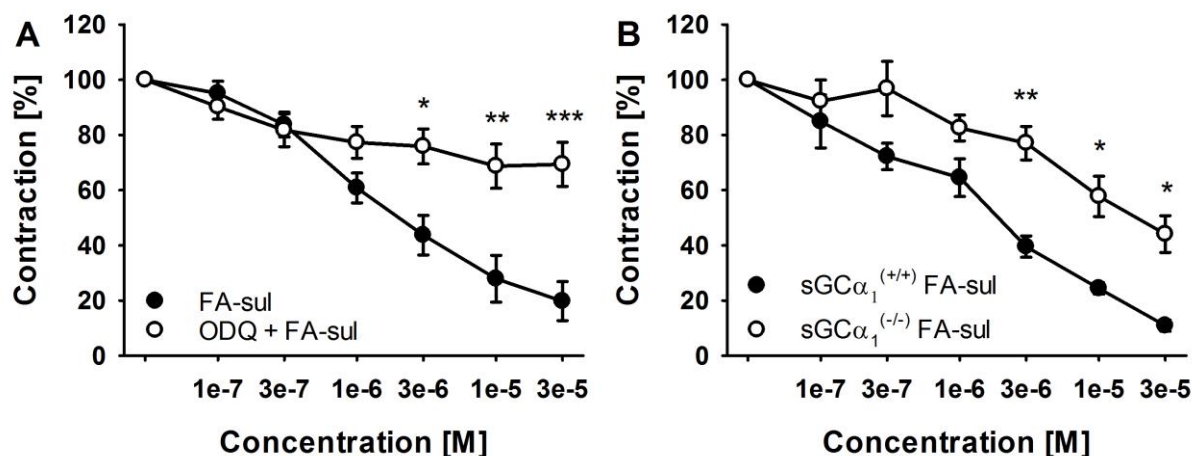


Figure 5: sGC dependency of FA-sul induced vasorelaxation in isolated femoral artery of Swiss (A) and sGCα<sub>1</sub><sup>(-/-)</sup> mice (B). Vasorelaxing effects of FA-sul (●) are compared to vasorelaxing activity of FA-sul in the presence of ODQ (A) in Swiss mice (○). Vasorelaxing effects in sGCα<sub>1</sub><sup>(+/+)</sup> (●) are compared to vasorelaxing activity in sGCα<sub>1</sub><sup>(-/-)</sup> mice (○) in the presence of FA-sul (B). Contraction (y-axis) is expressed relative to the Phe-induced contraction in the same preparation before cumulative addition of the compounds. Cumulative concentration-response curves were established between 1.10<sup>-7</sup> and 3.10<sup>-5</sup> M (x-axis) using 10 minute intervals. n=4, \*: p<0.05, \*\*: p<0.01, \*\*\*: p<0.001.

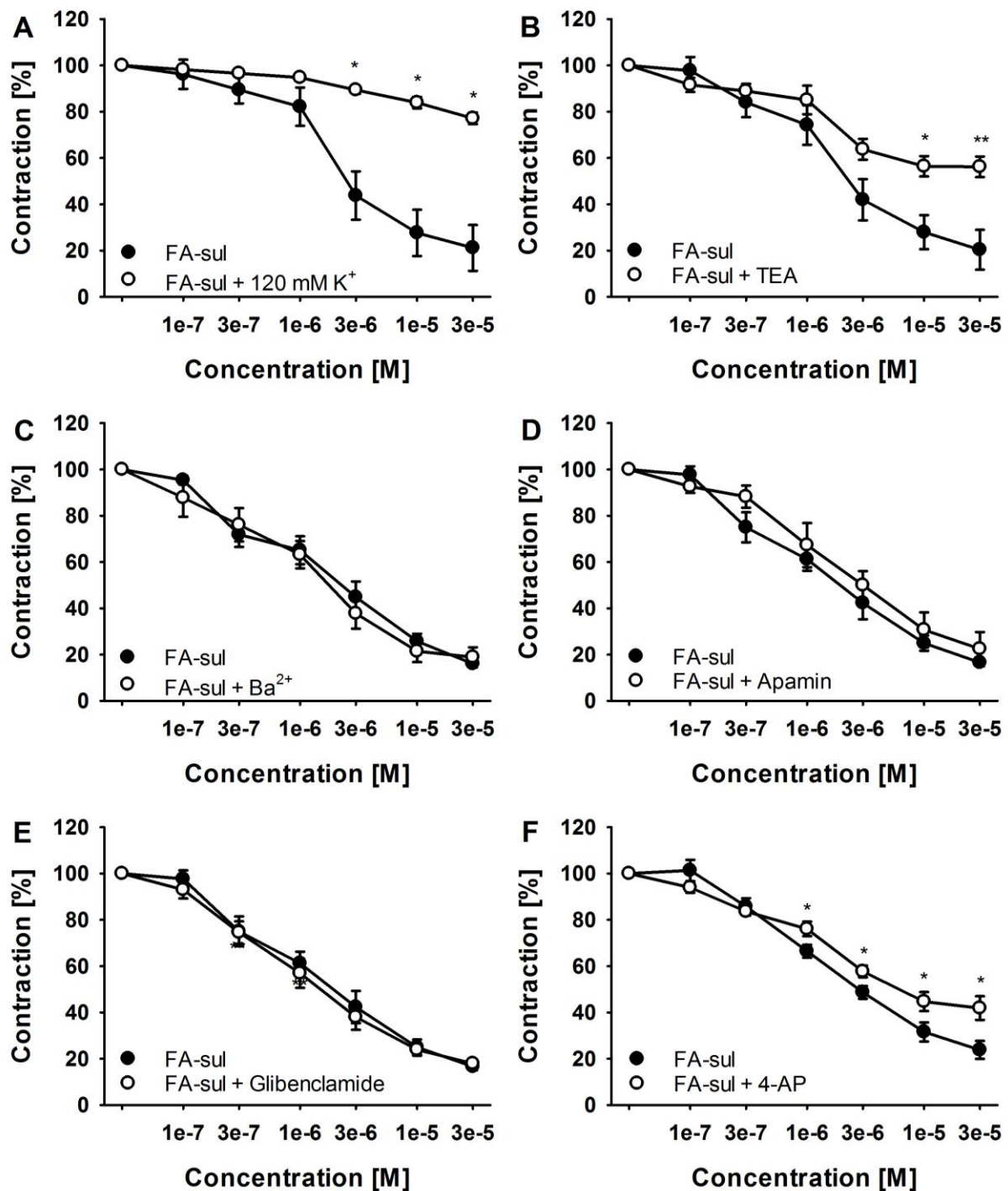


Figure 6: Potassium channel dependency of FA-sul induced vasorelaxation in isolated Swiss mouse femoral arteries. Vasorelaxing effects of FA-sul (●) are compared to vasorelaxing activity of FA-sul in the presence of different K<sup>+</sup> channel inhibitors (○): K<sup>+</sup> (A), TEA (B), Ba<sup>2+</sup> (C), apamin (D), glibenclamide (E) and 4-AP (F). Contraction (y-axis) is expressed relative to the Phe-induced contraction in the same preparation before cumulative addition of the compounds. Cumulative concentration-response curves were

established between  $1 \cdot 10^{-7}$  and  $3 \cdot 10^{-5}$  M (x-axis) using 10 min intervals. n=4 ( $\text{Ba}^{2+}$ , apamin, glibenclamide), n=5 (120 mM  $\text{K}^+$ ) or n=6 (TEA, 4-AP). \*:  $p < 0.05$ , \*\*:  $p < 0.01$ .

ACCEPTED MANUSCRIPT

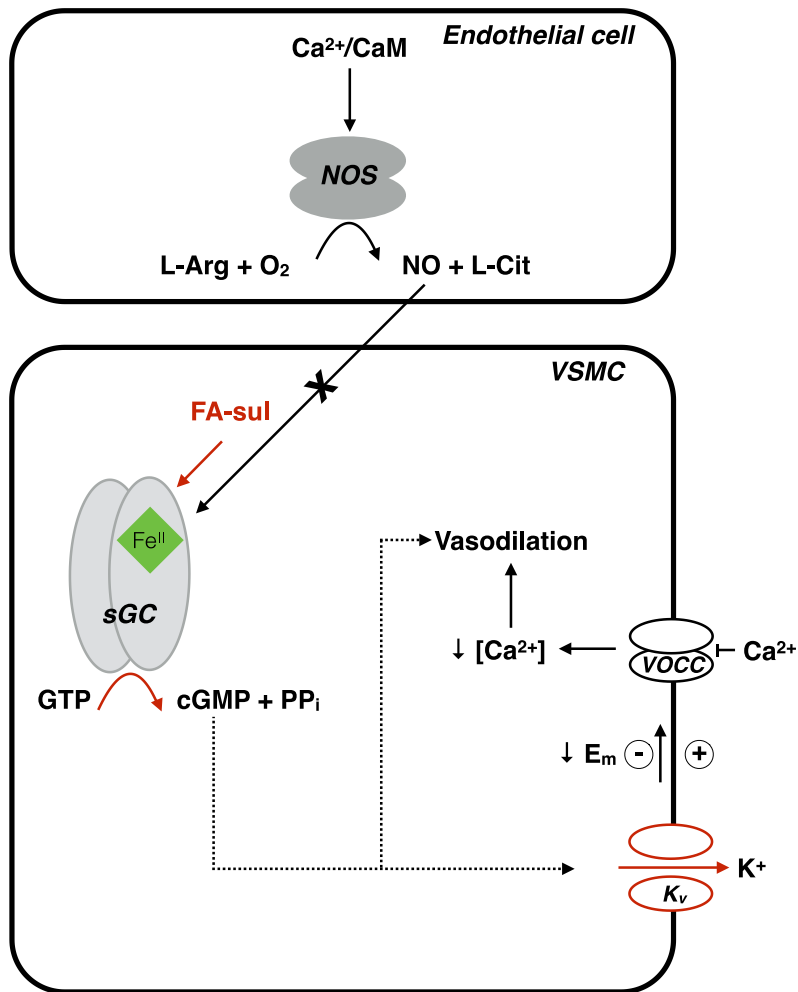


Figure 7: Proposed mechanism of vasodilation induced by FA-sul in vascular smooth muscle cells (VSMC). Targets of FA-sul are indicated in red. In the endothelial cell, a  $\text{Ca}^{2+}/\text{Calmodulin}$  ( $\text{Ca}^{2+}/\text{CaM}$ ) complex binds NOS, which oxidizes L-arginine (L-Arg) to L-citrulline (L-Cit), resulting in the release of NO which diffuses out of the endothelial cell, and binds the  $\text{Fe}^{\text{II}}$  haem of sGC. This stimulates the conversion of GTP to cGMP and consequential vasodilation. FA-sul activates sGC even in the absence of NO. Ferulic acid-sulfate mediated opening of  $\text{K}_v$ -channels results in efflux of  $\text{K}^+$ , hyperpolarization of the cell membrane and decreased influx of  $\text{Ca}^{2+}$  through voltage operated  $\text{Ca}^{2+}$ -channels (VOCC) and subsequent vasodilation.