CHARLES UNIVERSITY

FACULTY OF PHARMACY IN HRADEC KRALOVE

Effects of isoflavonoids and their metabolites on vascular smooth muscles *in vitro* and *in vivo*

(Účinky isoflavonoidů a jejich metabolitů na hladký cévní sval *in vitro* a *in vivo* studie)

DISSERTATION THESIS

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STATEMENT OF AUTHORSHIP

I hereby declare that this thesis is my original authorial work, which I developed independently under the guidance of my supervisor Prof. Přemysl Mladěnka and my consultant Assoc. Prof. Jana Pourová. All literature and other sources from which I drew information during processing are quoted in the list of used literature and duly cited in the work. The work has not been used to obtain another or the same degree.

In Hradec Králové, Thomas Migkos, MSc.

Date: …….. …….

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ABSTRACT

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Title of dissertation thesis: Effects of isoflavonoids and their metabolites on vascular smooth muscles *in vitro* and *in vivo*

The dietary intake of flavonoids seems to be inversely related to cardiovascular mortality, in particular on coronary artery disease. The consumption of isoflavonoids, one class of flavonoids, has been increasing in the general population, especially due to the use of food supplements and a variety of isoflavonoid-rich foods. Although their bioavailability is low, they undergo extensive gastrointestinal metabolism by human bacteria, leading to smaller metabolites with a much higher degree of bioavailability. However, detailed studies on the impact of individual pure isoflavonoids on vascular system were mostly missing and much less was known for the effect of their colonic metabolites in this field. In the present study sixteen isoflavonoids, four metabolites and the racemic mixture of one of them were initially screened *ex vivo* for their vasorelaxant properties on rat aortas. The most potent of them, biochanin A, glycitein, O-desmethylangolensin (O-DMA), *S*-equol and *R,S*-equol were further tested for the mechanism of action on porcine coronary arteries. All abovementioned compounds induced an endothelium independent relaxation of the coronary vasculature *ex vivo,* with EC_{50} ranged from 5.5 to 17 μM. Biochanin A, *S*-equol and *R,S*-equol, but not glycitein and O-DMA, were able to block the vasoconstriction caused by KCl, $CaCl_2$, serotonin and U46619 in a concentration-

dependent manner. Another series of experiments suggested that the major mechanism of action of biochanin A was the inhibition of L-type calcium channels and this was further confirmed by experiments using human aortic and coronary smooth muscle cells, loaded with a calcium indicating fluorescent dye. Biochanin A in relatively small concentrations (2-4 μM) also interfered with the cGMP, but not cAMP, pathway in isolated coronary arteries. Moreover, O-DMA, *S*-equol and *R,S*-equol dilated smaller resistant mesenteric arteries *ex vivo*, while the more abundant human metabolite, O-DMA, decreased *in vivo* arterial blood pressure in spontaneously hypertensive rats, without impacting the heart function. Similarly to biochanin A, O-DMA blocked the calcium influx in human aortic smooth muscle cells, as well. These results indicate that several isoflavonoids, in particular biochanin A, and their metabolites are able to have vasodilatory effects in micromolar concentrations which is of potential clinical interest for the management of some cardiovascular pathologies.

ABSTRAKT (v češtině)

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Katedra farmakologie a toxikologie

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Název dizertační práce: Účinky isoflavonoidů a jejich metabolitů na hladký cévní sval, *in vitro* a *in vivo* studie

Příjem flavonoidů potravou je pravděpodobně nepřímo úměrný úmrtnosti na kardiovaskulární onemocnění, zejména na ischemickou chorobu srdeční. Spotřeba isoflavonoidů, které představují jeden podtyp flavonoidů, v populaci obecně roste a to zejména díky konzumaci řady potravin bohatých na isoflavonoidy a používání potravních doplňků. Přestože je biodostupnost isoflavonoidů obecně nízká, tyto látky jsou rozsáhle metabolizovány střevní mikroflórou v trávicím traktu za vzniku malých metabolitů s výrazně vyšší biodostupností. Detailní studie zaměřená na vliv parentních isoflavonoidů na vaskulární systém dosud neexistovala a ještě méně bylo známo o vaskulárních účincích příslušných střevních metabolitů. V předkládané práci byla nejprve u šestnácti isoflavonoidů, jejich čtyřech metabolitů a racemické směsi jednoho metabolitu otestována za *ex vivo* podmínek schopnost dilatovat potkaní aortu. U pěti nejúčinnějších látek, biochaninu A, glyciteinu, O-desmethylangolensinu (O-DMA), *S*-ekvolu a *R,S*-ekvolu, byly následně detailně studovány možné mechanismy účinku na prasečí věnčité tepně *ex vivo*. Všechny zkoumané látky navodily na endotelu nezávislou relaxaci koronární tepny s EC_{50} v rozsahu od 5,5 do 17 μM. Biochanin A, *S*-ekvol a *R,S*-ekvol, ale ne glycitein nebo O-DMA, blokovaly vasokonstrikci navozenou KCl, $CaCl_2$, serotoninem nebo U46619 a jejich účinek byl závislý na použité koncentraci. V další sérii pokusů se ukázalo, že hlavním mechanismem účinku biochaninu A byla inhibice L-

typu vápníkových kanálů, což bylo dále potvrzeno v experimentech na lidských aortálních a koronárních hladkosvalových buňkách za použití vápník-fluorescenční sondy. Biochanin A navíc v relativně malých koncentracích (2- 4 μM) také ovlivnil cGMP signální kaskádu v isolovaných věnčitých tepnách, ale ne cAMP kaskádu. Kromě toho byly O-DMA, *S*-ekvol a *R,S*-ekvol schopny dilatovat i malé odporové mesenterické arterie potkana *ex vivo*, a O-DMA, což je z nich nejběžnější lidský metabolit, signifikantně snížil arteriální krevní tlak v *in vivo* modelu u spontánně hypertenzních potkanů, aniž by ovlivnil srdeční funkci. Podobně jako biochanin A, také O-DMA zablokoval vstup vápníku do lidských aortálních hladkosvalových buněk. Z těchto výsledků vyplývá, že několik isoflavonoidů, zejména biochanin A, a jejich metabolity jsou schopny v mikromolárních koncentracích působit vasodilatačně. To naznačuje možný potenciál pro klinické využití těchto látek v léčbě některých kardiovaskulárních chorob.

LIST OF ABBREVIATIONS

AoVSMC, human aortic smooth muscle cells;

CASMC, human coronary artery smooth muscle cells;

DMSO, dimethylsulfoxide;

DPBS, Dulbecco's phosphate buffered saline;

EDTA, ethylenediaminetetraacetic acid;

EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid;

ER-β, estrogen receptor β;

FBS, fetal bovine serum;

Fura-2 AM, Fura-2 acetoxymethyl ester;

HBSS, Hank's balanced salt solution;

NE, norepinephrine bitartrate;

O-DMA, O-desmethylangolensin;

ODQ, [1H-[1,2,4]oxadiazolo-[4, 3-a]quinoxalin-1-one];

SHR, spontaneously hypertensive rat(s);

SmBM, smooth muscle cell basal medium;

HPPA, 2-(4-hydroxyphenyl) propionic acid;

5-HT, serotonin.

1. INTRODUCTION

Pharmacotherapy has contributed to a marked improvement in the treatment of cardiovascular diseases, which have, however, remained the leading cause of mortality and morbidity worldwide in the 21st century (Choi et al., 2014; Joseph et al., 2017). Among them, coronary artery disease and stroke are, according to the World Health Organization, the major culprits of mortality (WHO, 2018). In addition, cardiovascular fatal outcomes might follow other diseases, as was the case of COVID pandemic (Zheng et al., 2020). These epidemiological data indicate the necessity of developing innovative health and drug policies focused on both the treatment and prevention of cardiovascular disorders. Vascular function is one of the considered main targets.

Flavonoids are naturally occurring polyphenols that are abundant in the human diet and their intake is inversely related to cardiovascular mortality according to epidemiological studies (Kim & Je, 2017; Liu et al., 2017). In addition to their well-known antioxidant activity, they influence the cardiovascular function in multiple ways, demonstrating a pleiotropic pharmacological profile, which include interaction with several enzymes as well as antiplatelet and vasodilatory effects (Mladenka et al., 2010). Nevertheless, flavonoids have poor oral bioavailability (Chen et al., 2022) and hence rather their metabolites can be responsible for the observed cardiovascular effects. In particular, small phenolic compounds formed by human gastrointestinal microflora have been attracting raising degree of interest of the scientific community. This interest is mainly driven by increasing evidence that these metabolites can be at least partly responsible for the biological effects of several parent polyphenolic compounds. In addition, current studies have shown that small phenolic compounds can be absorbed more extensively than the parent compounds. Moreover, they have been shown to possess various positive effects on the organism (e.g. anti-inflammatory, antiplatelet and vasodilatory) (Applova et al., 2019; Larrosa et al., 2009; Najmanova et al., 2016; Pourova et al., 2018).

Flavonoids are secondary plant metabolites and comprise a large family of structurally similar compounds sharing the same structural core and differing in substitution patterns (Figure 1).

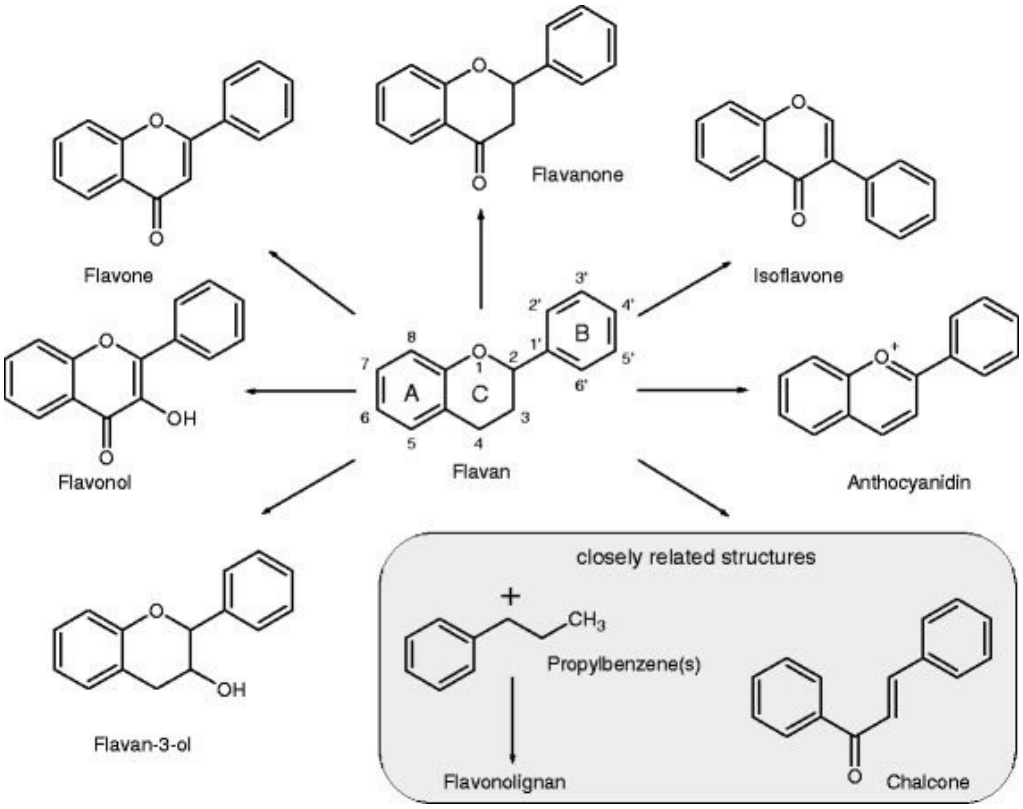
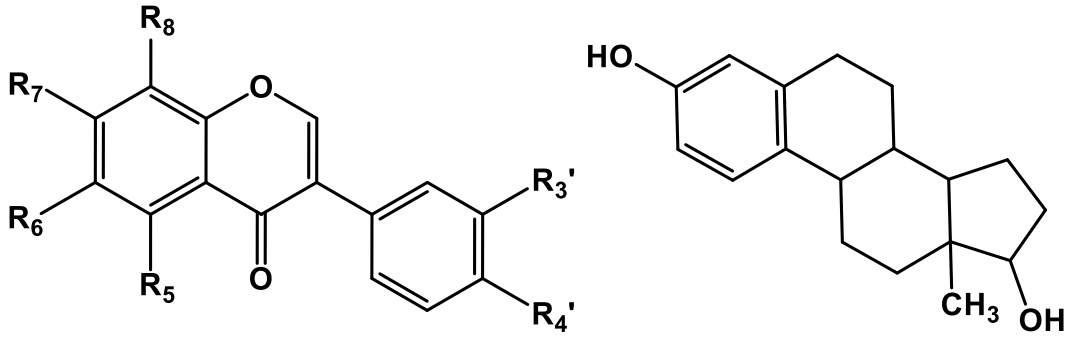


Figure 1: Chemical structure of flavonoids and related compounds. The figure was taken from Busch et al. (2015).

Isoflavonoids (mostly isoflavones, Figure 1) are structurally similar to endogenous oestrogens (Figure 3-3) and were repeatedly shown to be able to bind to their receptors (Kostelac, Rechkemmer, & Briviba, 2003; Mueller et al., 2004). Therefore, they are characterized as phytoestrogens. The term “phytoestrogen” comes from the combination of the Greek words “phyto”, which means plant, and “oestrogen”, that stands for a hormone that influences the female fertility in vertebrates (Krizova et al., 2019). Actually, along with lignans, isoflavonoids constitute the main class of phytoestrogens.



isoflavonoids estradiol

Figure 3: The structural similarity between isoflavonoids and estradiol.

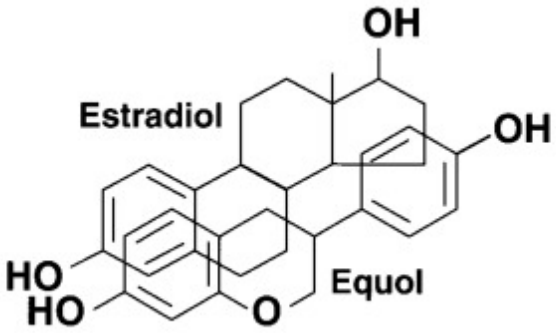


Figure 2: Comparison of the planar spatial arrangement of the isoflavone metabolite S-equol with that of estradiol (Setchell & Cassidy, 1999).

1.1. Natural and dietary isoflavonoid occurrence

The occurrence of isoflavoids, in particular in soybeans, became firstly known more than 90 years ago in 1931 (Setchell et al., 2001). However, it was during 1940s, when their biological role had been taken into consideration, due to fertility impairment observed in sheep grazing in Australian pastures, where *Trifolium subterraneum* (subterranean clover) was prevalent. The so called "clover disease", was manifested by symptoms of diverse reproduction disorders. Young immature animals showed signs of oestrus, ewes were not able to become pregnant, and those that were pregnant often aborted. Other findings included increased

incidence of uterine abnormalities, endometriosis, abnormal development of the mammary gland or abnormal lactation, uterine prolapse, or uterine dystocia. Males were also affected, as lower sperm count and motility were observed in rams. The principal responsible compound was suggested to be the isoflavone formononetin, present in the plant (Krizova et al., 2019; Setchell et al., 2001).

The main natural sources of isoflavonoids are legumes and herbs, such as soybean (*Glycine max*), red clover (*Trifolium pretense*), white clover (*Trifolium pratense*), mentioned subterranean clover *(Trifolium subterraneum*), liquorice (*Glycyrrhiza glabra*), milkvetch (*Astragalus sp.*), alfalfa (*Medicago sativa*) sprouts, kudzu (*Pueraria sp.), Prunus* and *Ononis* species (Choi et al., 2014; Duncan et al., 2003; Krizova et al., 2019; Li et al., 2018; Reinli & Block, 1996; Setchell et al., 2001; Tseng et al., 2016; Yeung et al., 2006). Isoflavonoid intake in humans has been also increasing due to the use of a variety of dairy products, processed food and food supplements, derived from the aforementioned plants (Krizova et al., 2011; Socas-Rodriguez et al., 2017). Soybeans are the richest food source of isoflavones (Duncan, Phipps, & Kurzer, 2003; Franke, Lai, & Halm, 2014). Thus, isoflavonoid intake was assumed to be higher in Asian population (25–40 mg/day) in the past (Atkinson, Frankenfeld, & Lampe, 2005; Duncan, Phipps, & Kurzer, 2003), but currently it can be considerable in European and Western populations as well. The likely reason is the discussed extensive use of food supplements and the increasing popularity of soy products (Otun et al., 2019).

The major isoflavones present in soy foods include genistein and daidzein and, to a lesser extent, glycitein. They occur in soy in the form of glycosides, i.e. conjugated to sugar moieties. Principal compounds are genistin, daidzin and glycitin. Although soybeans are the predominant food source of isoflavonoids, variations in their content occur because of genetic differences of miscellaneous varieties, environmental conditions (location and harvesting year) and maturity (Atkinson, Frankenfeld, & Lampe, 2005; Reinli & Block, 1996). Daidzein and

glycitein are also present in beer, whereas biochanin A in bourbon whisky (Reinli & Block, 1996). The latter is found in high concentrations in clover, cabbage and alfalfa sprouts. It should be also mentioned that it is a precursor of genistein while another common plant isoflavone formononetin is a precursor of daidzein (Duncan, Phipps, & Kurzer, 2003; Reinli & Block, 1996). Interestingly, new isoflavonoids continue to be discovered, during the last years, such as 6-methoxy-5,7,8,4'-tetrahydroxyisoflavone and 4'-methoxy-5,6-dihydroxyisoflavone-7-O-β-D-glucopyranoside from the rhizomes of *Belamcanda chinensis* and others (Song, 2007; Wang et al., 2019).

Most of the aforementioned plant species are traditionally used in Chinese medicine, among others, for the treatment of cardiovascular diseases (Yeung et al., 2006).

1.2. Clinical practice

During the last decades, products containing isoflavonoids, have been commercially available as food supplements and they became popular among women for the treatment of postmenopausal symptoms. Most of them consist of red clover extracts, being rich in biochanin A, genistein, daidzein and formononetin. It should be emphasized that there is huge variability in the content of isoflavonoids in these food supplements. Producer-independent chemical analyses of different red clover food supplements reported the content of the aforementioned isoflovonoids between 1 and 26 mg per tablet and the total isoflavonoid content up to 44 mg per tablet (Booth et al., 2006; Howes et al., 2002). It is also worth mentioning, that the suggested dosage schemes often include the intake of two tablets per day (Booth et al., 2006). There is also evidence that many of the commercial supplements contain lower levels of isoflavones than those claimed by the manufacturer, or even the amount of phytoestrogens is negligible (Setchell et al., 2001). Another commercial product of interest is the soy-based feeding formula

for infants who are intolerant to cow's-milk-based formula (Duncan, Phipps, & Kurzer, 2003). Significantly increased plasma concentrations of genistein and daidzein were detected in infants fed with soy-based formula, than in those fed with cow-milk formula or breast milk (Setchell et al., 1997).

1.3. Pharmacokinetics

A large number of pharmacokinetic studies have shown that pure isoflavonoids and isoflavone extracts are rapidly and efficiently absorbed after oral administration (Duncan, Phipps, & Kurzer, 2003; Howes et al., 2002; Setchell et al., 2001; Setchell et al., 2003). However, this might not be fully applicable for the dietary supplements, which depending on factors such as the starting material, complexity and isoflavonoid concentration, can considerably vary in their pharmacokinetics (Duncan et al., 2003).

Intestinal bacteria are the major factors in the process of absorption, including presystemic metabolism of the isoflavonoids. The steps involved in the absorption and metabolism of major isoflavones are illustrated below (Figures 4 - 6). Following oral ingestion, glucosidases, which are produced by intestinal bacteria, hydrolyze the glycosidic isoflavones to their corresponding aglycones, producing the mentioned genistein, daidzein but also others including glycitein and tectorigenin (Duncan, Phipps, & Kurzer, 2003). Simultaneously, demethylation of the methylated isoflavonoids can occur. This was documented for formononetin and biochanin A and resulted in the formation of the demethylated structures of daidzein and genistein, respectively (Howes et al., 2002; Setchell et al., 2001). Although the demethylation of glycitein to daidzein is also possible, it has been observed to be less pronounced (Setchell, 2001).

Before absorption occurs, however, more specific biotransformations of the isoflavone aglycones may take place by intestinal bacteria. Specifically, daidzein is transformed to *S*-equol (7-hydroxy-3(4'hydroxyphenyl)-chroman) and/or O-desmethylangolensin (O-DMA), through the intermediate metabolites dihydrodaidzein and *cis/trans* 4-hydroxy-equol (syn. tetrahydrodaidzein, cis/trans-isoflavan-4-ol; Figure 4), also existing in the form of dehydroequol; all of which may also be absorbed (Duncan, Phipps, & Kurzer, 2003; Jackman, Woodman, & Sobey, 2007).

Equol was firstly isolated from equine urine of pregnant mares in 1932, hence its name derived (Krizova et al., 2019; Setchell & Clerici, 2010a). Since equol was soon found also in the urine of stallions and non-pregnant mares, the original hypothesis of the connection between equol and high estrogen concentration in the pregnant organism was disproved. Seasonality of equol occurrence in the equine urine (declining in autumn and not detected in winter) also contributed to the discovery of its dietary source. Now it is known, that equol in blood and urine from these animals comes mostly from formononetin, one of the main isoflavonoids of clover species (Krizova et al., 2019). Fifty years later, equol was also identified in human blood and urine, as a metabolite of the soybean isoflavones daidzin and daidzein (Krizova et al., 2019; Setchell & Clerici, 2010a). Furthermore, it is present in bovine milk, in concentrations ranging from 5 to 30 μg/L (0.02-0.134 μM) and this should be considered as another important dietary source of the metabolite (Antignac et al., 2003).

Due to the chiral center, equol can occur in the form of two isomers (*R*- and *S*-equol), but intestinal bacteria are enantiospecific in synthesizing exclusively the *S*-(-)equol enantiomer (Setchell & Clerici, 2010a). *S*-equol and O-DMA have been detected in a variety of body fluids, including blood, urine, feces, prostatic fluid and breast tissue (Atkinson, Frankenfeld, & Lampe, 2005).

Since the biotransformation of daidzein is performed by specific bacteria, their presence or absence split the population into equol and/or O-DMA producers and non-producers, respectively. Furthermore, it seems that more than one bacterial species are involved, such as human *Lactococcus* strain 20-92, *Eggerthella* sp. strain Julong 732, *Bifidobacterium* sp. and *Slackia* sp. (Kim et al., 2009; Shimada et al., 2012), *Lactobacillus* sp., *Streptococcus* sp. and Gram-positive bacteria do03 in rats (Deng et al., 2022), which can further vary in different individuals (Atkinson et al., 2005). Numerous observational and crossover studies indicate that the amount of equol producers ranges from 30 to 50% in general population (Atkinson, Frankenfeld, & Lampe, 2005; Franke, Lai, & Halm, 2014; Jackman, Woodman, & Sobey, 2007; Rowland et al., 2000), while 80 to 90% of the population produce O-DMA (Atkinson, Frankenfeld, & Lampe, 2005). The ability to harbor equol producing microbiota might be limited to 30–35% for omnivorous individuals and but presented up to 60% of vegetarians and Asians (Franke, Lai, & Halm, 2014).

In contrast to past hypotheses (Jackman, Woodman, & Sobey, 2007), a person's stable ability to produce equol over time is debatable (Franke, Lai, & Halm, 2014). Thus, part of the population can be also characterized as inconsistent producers (Franke, Lai, & Halm, 2014). Interestingly changes in equol-producing status were not associated with antibiotic use, which logically can disrupt the ecological balance of resident gut bacterial (Franke, Lai, & Halm, 2014).

Within both equol and O-DMA producers, the isoflavonoid structure is predominantly catabolized to *S*-equol (Atkinson, Frankenfeld, & Lampe, 2005). When daidzein was used as substrate, 70 % was biotransformed to *S*-equol and only 5-20 % to O-DMA (Jackman et al., 2007). O-DMA can be further degraded by intestinal microbiota into other smaller phenolic substances, namely 2-(4-hydroxyphenyl)propionic acid (HPPA) and resorcinol (Braune & Blaut, 2011; Frankenfeld, 2011) (Figure 4). HPPA, along with 4-ethylphenol (syn. *p*-

ethylphenol) and 1,3,5-trihydroxybenzene, also belongs to the catabolites of genistein and hence of its precursors genistin and biochanin A (Figure 4) (Coldham et al., 2002; Duncan, Phipps, & Kurzer, 2003; Park et al., 2006; Paul et al., 2017).

Following absorption, isoflavones undergo extremely efficient first-pass intestinal and/or hepatic conjugation with glucuronic or sulfuric acids, to produce forms detectable in higher quantities in biological fluids (Jackman, Woodman, & Sobey, 2007; Setchell et al., 2001). Similarly to endogenous steroids, they undergo enterohepatic circulation whereby they are deconjugated in the intestine and re-absorbed or excreted in the feces. The plasma levels of unconjugated forms, at least for daidzein and genistein, are very low (Setchell et al., 2001). Also other metabolic processes are possible. Noteworthily, daidzein can be metabolized to 8-hydroxy-daidzein in the liver (Jackman, Woodman, & Sobey, 2007).

It takes at least 6–8 h for equol to appear in substantial amounts in plasma after ingestion of isoflavones. This observation is consistent with its formation being of distal or colonic origin (Setchell, 2001). Additionally, equol has a longer plasma half-life than its parent compound, daidzein (Jackman, Woodman, & Sobey, 2007). When ingested themselves, *S*-equol and *R*-equol can reach peak plasma concentrations within 1-2 h (delayed to up to 3 h when co-administration with food occurs). Both undergo phase II metabolism by conjugation with glucuronic acid or to a minor extent with sulfuric acid. *S*-equol circulates in plasma also in free form but it is excreted predominantly as the 7-glucuronide conjugate in urine (Setchell & Clerici, 2010b).

Studies in humans demonstrate that serum and urinary concentrations of isoflavones increase in accordance with the amount consumed, indicating that absorption occurs in a dose-dependent manner (Duncan et al., 2003). It should be also mentioned, that isoflavones reach plasma concentrations, that exceed by several orders of magnitude the amounts of endogenous estrogens (Setchell, 1998). These levels of circulating isoflavones may extend up to a 22000-

fold higher concentrations than estradiol. This was observed in infants fed with soy-based formula (Duncan, Phipps, & Kurzer, 2003). Concretely, significantly increased daidzein (1.16 μM) and genistein (2.53 μM) values were measured in infant plasma, in comparison to those fed cow-milk formulas (8.1 nM and 11.6 nM, respectively) or breast-milk (5.86 nM and 10.2 nM, respectively). Additionally, equol was present in plasma of the infants consuming cow-milk formula (16.9 nM), while O-DMA and dihydrodaidzein were not detected (Setchell et al., 1997).

Red clover isoflavones are able to inhibit the cytochrome P450 system, in particular CYP1A1, CYP1B1 and CYP2C9 isoforms, which may cause increased plasma levels of those drugs that are metabolized through these pathways (Booth et al., 2006). Concerning biochanin A, it exerts effects mainly on the CYP1A2 and CYP3A4 isozymes (Kopecna-Zapletalova et al., 2017; Yu et al., 2019). Daidzein and genistein are inhibitors of the CYP2C9 and CYP3A4 isoforms and equol (studied as racemate, *R,S*-equol) of the CYP3A4, as well. However, the *in vitro* tested inhibitory concentrations often exceed by far the isoflavonoid levels achievable by common oral administration (Kopecna-Zapletalova et al., 2017). Hence, while an interaction between isoflavonoids and drugs metabolized by cytochrome P450, such as anticoagulants, could exist (Yu et al., 2019), its practical importance is likely low (Kopecna-Zapletalova et al., 2017).

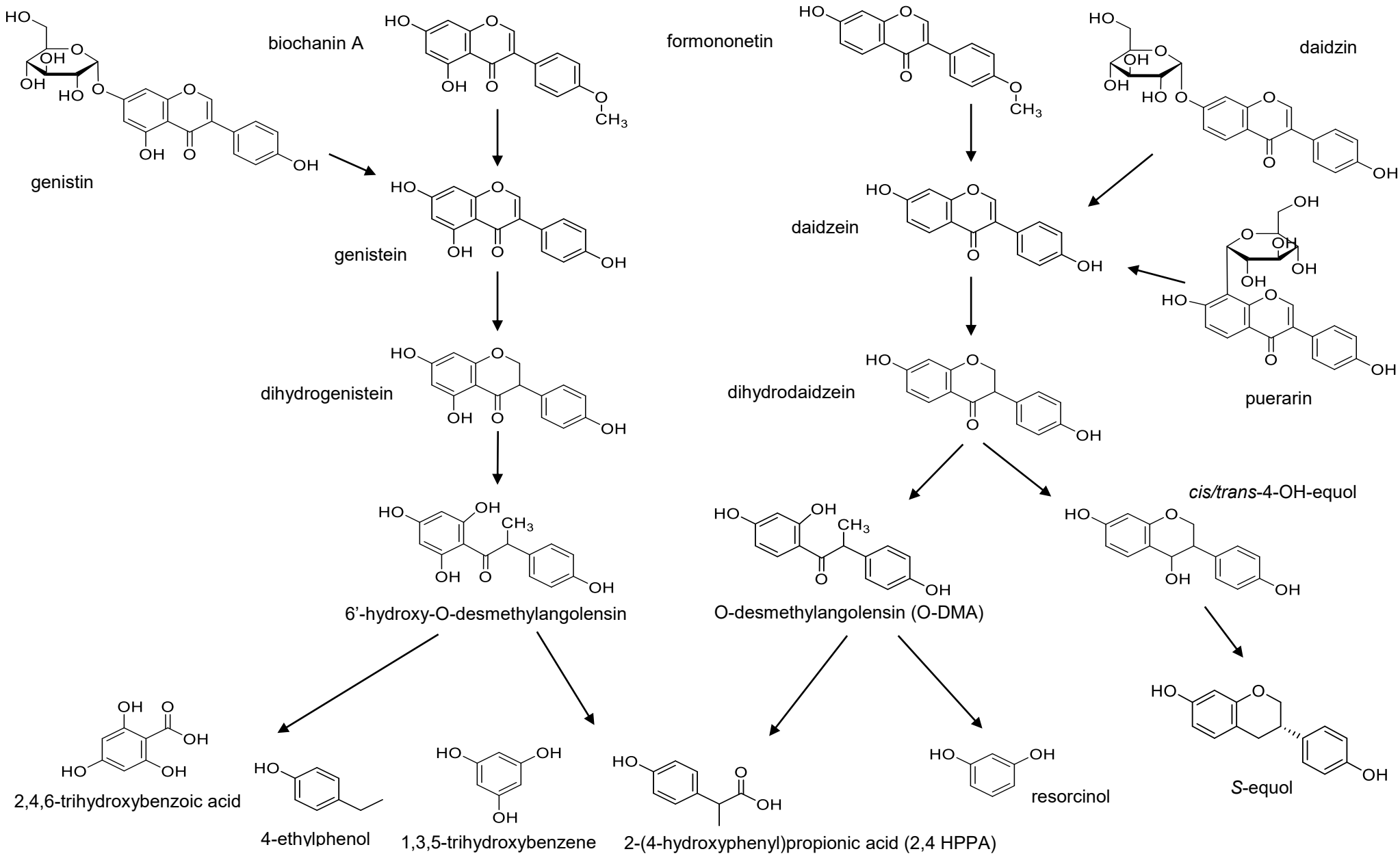


Figure 4: The main intestinal metabolic pathways of genistein, daidzein and structurally related isoflavonoids.

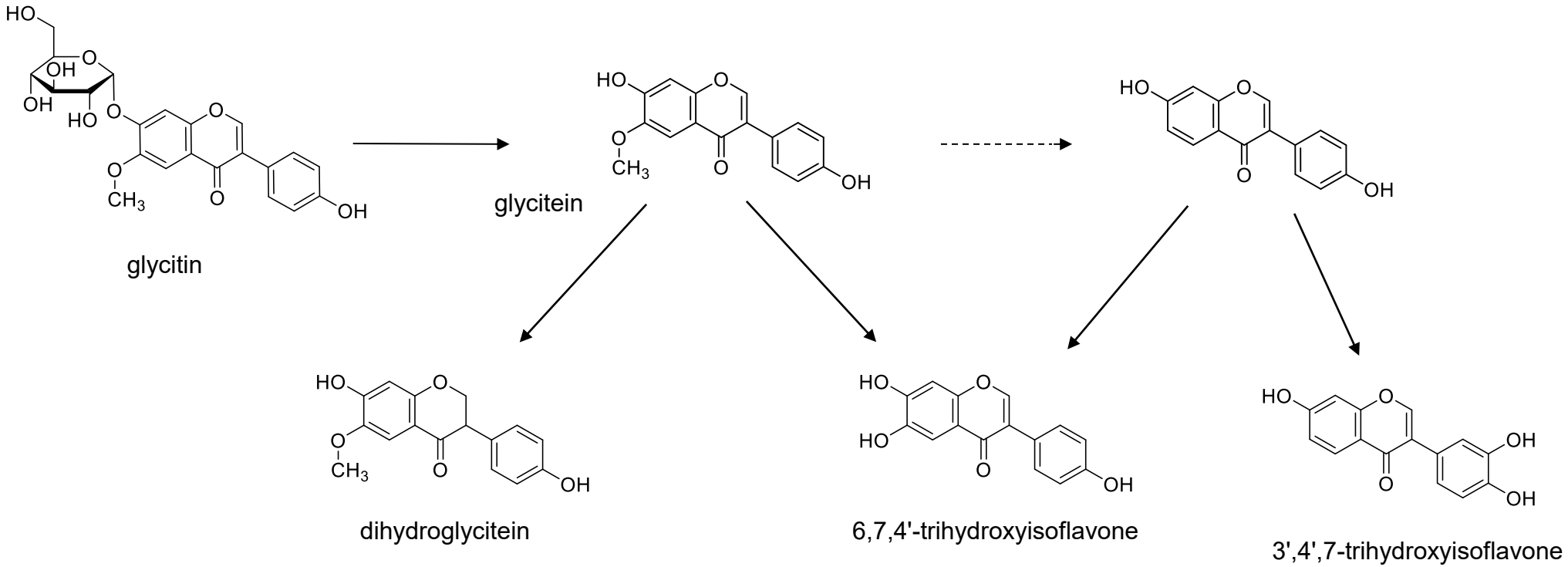


Figure 5: The metabolic fate of glycitin and it aglycone glycitein

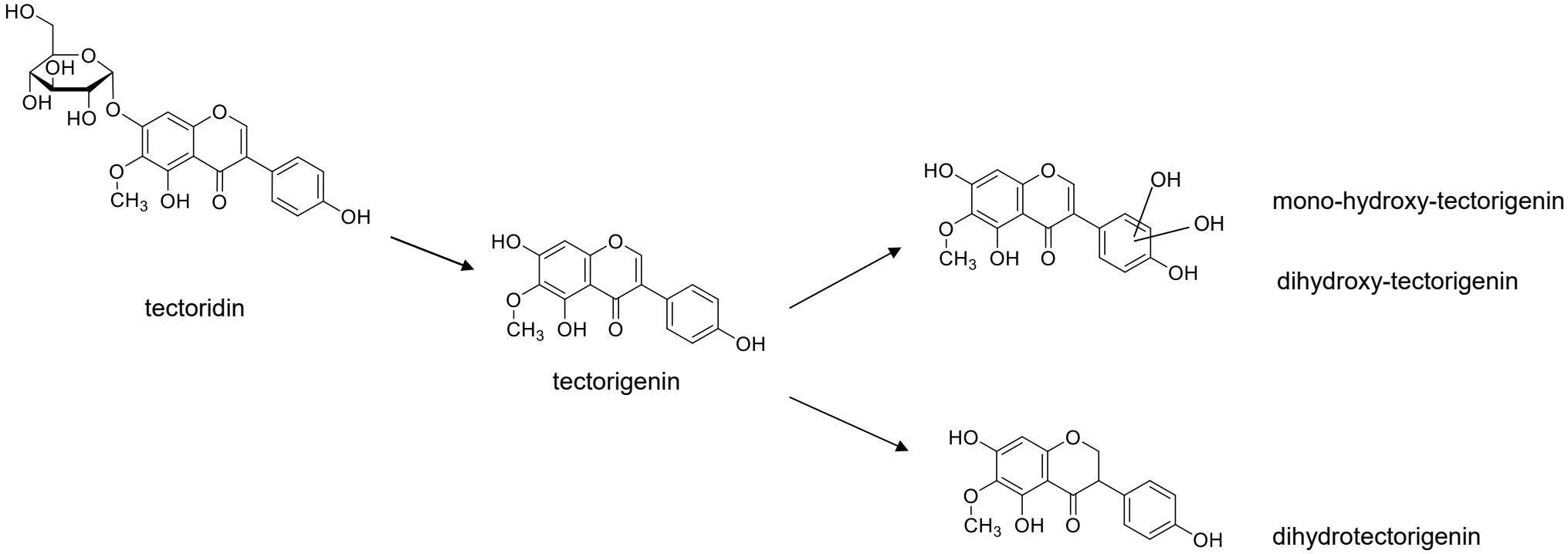


Figure 6: The bacterial biotransformation of tectoridin and its aglycon tectorigenin.

1.4. Epidemiological studies

There is a number of available epidemiological data, concerning the effects of isoflavonoids, isoflavonoid plant extracts, isoflavone rich diet and food supplements on human health. These studies are mainly focusing in the oestrogenic / anti-oestrogenic action of the compounds, and thus on their potentially protective role in breast and prostate cancer, osteoporosis, menopausal symptoms and cardiovascular diseases (Atkinson, Frankenfeld, & Lampe, 2005). Clinical studies investigating the association of higher dietary isoflavones intake to a decline in the risk of gastric cancer have been also conducted. Discouragingly, a meta-analysis of the evidence indicates no positive impact (You et al., 2018).

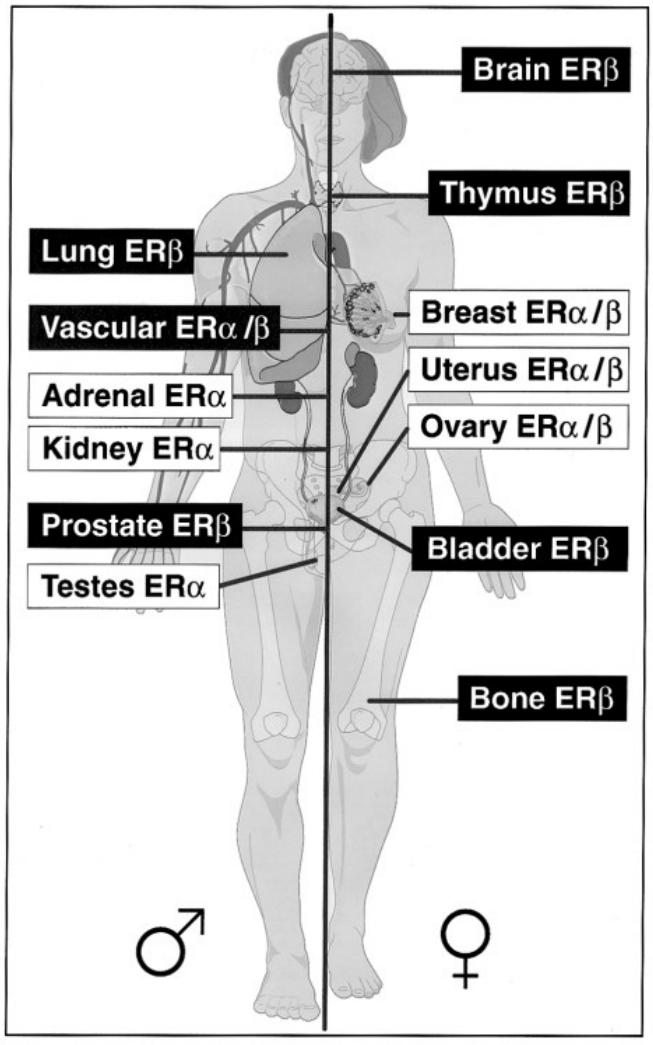


Figure 7: Diagram illustrating the anatomical distribution of estrogen receptors α (ERα) and β (ERβ), in men and women. Taken from Setchell & Cassidy (1999).

1.4.1. Breast cancer

As non-steroidal phytoestrogens, isoflavonoids bind to the estrogen receptors (preferentially to the receptor β, Figure 7), they might exert hormonal or anti-hormonal effects relevant to the risk of hormone-dependent disease and/or appear as a suitable dietary alternative to hormone replacement therapy (Duncan, Phipps, & Kurzer,

2003; Setchell, 1998). Observational studies suggested inverse associations between breast cancer risk and soy consumption, but they have not been fully consistent (Duncan, Phipps, & Kurzer, 2003). Additionally, a large cohort study confirmed the negative relationship between all-cause mortality and dietary intake of isoflavones, but mainly in women who were diagnosed with estrogen receptor-negative / progesterone receptor-negative tumors (Zhang et al., 2017). In accordance, *in vitro* studies underline also estrogen receptor-independent mechanisms, by which phytoestrogens may protect against breast cancer risk. Isoflavones are also able to inhibit enzymes involved in the synthesis of steroid hormones, including aromatase and 17β-hydroxysteroid dehydrogenase. A long term exposure to isoflavonoids might be of importance, as consumption of isoflavone-rich soy foods in adolescence is related to lower breast cancer risk in adult life (Duncan et al., 2003).

1.4.2. Prostate cancer

Despite the hypothesis that isoflavone-rich soy-containing diet is the reason of the lower prostate cancer incidence, in Asian *versus* Western men, and the first supportive epidemiological data, subsequent studies showed large evidence inconsistency (Duncan, Phipps, & Kurzer, 2003). Furthermore, a large-scale meta-analysis did not find a strong evidence that prediagnostic circulating concentrations of isoflavones are negatively associated with prostate cancer risk, although populations with high isoflavone intakes have to be yet studied (Perez-Cornago et al., 2018). Alike to the breast cancer, the potential protective role of isoflavonoids might not exclusively be related to estrogen receptor. Isoflavones can, by the above-mentioned inhibition of sex hormone synthesis (aromatase and 17β-hydroxysteroid dehydrogenase) but also by inhibition of 5α-reductase, induce the apoptosis in prostate cancer cells (Duncan et al., 2003).

1.4.3. Osteoporosis–menopausal symptoms

Some interventional studies have found positive effects of soy and soy isoflavones on body mass density and osteoblastic activity, in peri- and post-menopausal women. Moreover, a synthetic isoflavone, ipriflavone (Figure 8), effectively reduces bone loss in post-menopausal women. In peri- and post-menopausal women, cross-sectional and prospective studies suggest that soy consumption is significantly negatively correlated with the number of hot flushes, while interventional studies provided debatable data about the role of soy, isoflavone extracts and red clover derived supplements (Booth et al., 2006; Duncan, Phipps, & Kurzer, 2003).

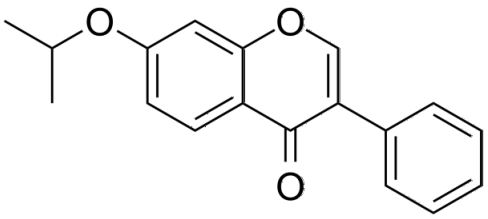


Figure 8: The chemical structure of ipriflavone.

1.4.4. Cardiovascular diseases

The lower incidence of cardiovascular disorders in east Asian populations, compared to those from Western countries, has also been attributed to the higher consumption of isoflavonoids, particularly soy isoflavones (Torregrosa et al., 2003). There is evidence that soy protein reduces blood lipids, which when elevated represent a cardiovascular risk factor. It is, however, not clear whether this is due to the isoflavones, which are present in the protein matrix (Duncan, Phipps, & Kurzer, 2003). Moreover, red clover extract food supplements, rich in isoflavonoids, such as biochanin A, increased HDL plasma levels and decreased triglycerides, in a couple of interventional studies (Booth et al., 2006). In particular, isoflavonoids appear to be related to a lower incidence of myocardial and cerebral infarctions (Kokubo et al., 2007), to

reduced arterial stiffness (Pase, Grima, & Sarris, 2011) and to arterial blood pressure normalization (Sagara et al., 2004). Also an improvement in systemic arterial compliance in peri- and post-menopausal women was reported (Duncan et al., 2003).

Despite these encouraging epidemiological observations, most of the conducted human studies have investigated the effect of isoflavone-rich diets, plant extracts and mixtures of isoflavonoids (Kokubo et al., 2007; Pase, Grima, & Sarris, 2011; Sagara et al., 2004). The data on the effects of individual purified isoflavonoids themselves are hence largely missing, with some exceptions (Teede et al., 2003). Additionally, *ex vivo* and animal vascular studies are limited due to relatively small number of tested compounds (Sun et al., 2007; Torregrosa et al., 2003; Tseng et al., 2016; Wu et al., 2010; Zhao et al., 2012), and even less information is available in respect to coronary vessels (Figtree et al., 2000; Lee et al., 2004; Lee & Man, 2003).

1.5. Pharmacodynamic effects of the isoflavonoid metabolites

Several studies have demonstrated that, similarly to its biological precursors, isoflavones, equol also exhibits strong estrogenic activity. Indeed, *S*-equol is able to inhibit osteoclast formation and prevent bone loss (Wu et al., 2007), while vasomotor menopausal symptoms were ameliorated in women supplemented by, or in those naturally producing, *S*-equol (Aso, 2010). Further effects, associated with *S*- and *R,S*-equol, include their direct radical-scavenging activity and antioxidant capacity, which results from catalase and superoxide dismutase activation, appeared to be more pronounced compared to daidzein and other isoflavones (Choi, 2009; Choi & Kim, 2014; Rimbach et al., 2003; Rufer & Kulling, 2006). *R,S*-equol may also positively influence prediabetic or diabetic states through 1) upregulation of peroxisome proliferator-activated receptor (PPAR) γ-mediated transcriptional activity *in vitro* (Cho et al., 2010), and 2) decrease in leptin levels in females capable of producing the

substance (Sakane et al., 2014). The inhibition of excessive nitric oxide and prostaglandin E_2 production, as well as the suppression of cyclooxygenase-2 (COX-2) gene expression by equol, is in accord with its anti-inflammatory action (Blay et al., 2010; Lau & Leung, 2006). We recently proved that both *R,S*-equol and *S*-equol are able to strongly block platelet aggregation, as well; occasionally as potently as the standard drug acetylsalicylic acid (Migkos et al., 2019).

Nevertheless O-DMA is produced by the intestinal microflora of most humans, its health impacts have not been adequately evaluated, in comparison to equol. O-DMA also exhibits antiplatelet capacity, but in a smaller extent than equol (Migkos et al., 2019) and, similarly to isoflavonoids, both O-DMA and equol are capable of binding to estrogen receptors. *S*-equol is an enantiomer that has selective affinity for the estrogen receptor β (Setchell & Clerici, 2010a) and so does O-DMA as well. However, they possess a weaker transcriptional activity than estradiol, and thus O-DMA as well as equol, can act as estrogen agonists or antagonists, depending on the endogenous estrogen concentration (Hwang et al., 2006). A number of studies have shown that O-DMA possesses antioxidant properties (including both direct radical scavenging and interference with enzymes) which exceeded daidzein in some assays (Choi & Kim, 2014; Rimbach et al., 2003; Rufer & Kulling, 2006). An antiproliferative effect of O-DMA in certain cancer cell lines has also been observed (Frankenfeld, 2011).

Evidence on possible biological effects of other isoflavonoid-specific metabolites, like 4-ethyphenol and 2-(4-hydroxyphenyl)propionic acid, is rare, despite a few exceptions. 4-Ethylphenol was found 1) to enhance interleukin-4 production in activated T cells (Park et al., 2006), 2) to have a partial estrogenic effect on corpus luteum (Woclawek-Potocka et al., 2006) and recently, 3) to possess a pronounced direct radical scavenging-effect as well (H. Li et al., 2018). 4-Ethylphenol can inhibit platelets in mM concentrations due to non-specific effect on plasma membrane (Kitagawa et al., 1990; Tsuchiya, 2001), while in μM concentrations it is

able to block platelet aggregation induced by arachidonic acid and collagen, with an efficacies comparable to those of the clinically used acetylsalicylic acid (Migkos et al., 2019).

2. AIM OF THE WORK

The primary aim of this work was to investigate and compare the effect of sixteen pure isoflavones, most of which were tested for the first time, as well as their colonic metabolites on vascular smooth muscles at both *in vitro* and *in vivo* level.

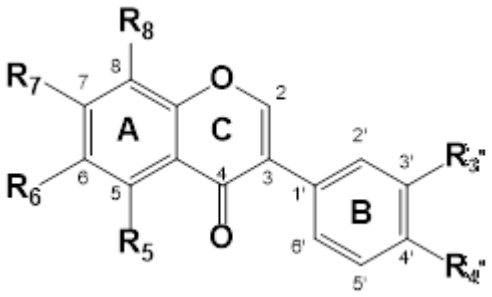
The secondary aim was to define the mechanism(s) of such effects.

3. MATERIALS AND METHODS

3.1. Tested compounds

Calycosin (purity: 99%), cladrin (98%) and isoformononetin (99%) were purchased from Phytolab (Vestenbergsgreuth, Germany). Biochanin A (≥99%), daidzin (≥99%), formononetin (≥99%), genistin (≥99%), glycitein (≥95%), glycitin (≥95%), ononin (≥99%), prunetin (≥95%), puerarin (≥99%) were purchased from Extrasynthese (Lyon, France) whereas daidzein (≥98%), genistein (≥98%), tectoridin (≥98%), tectorigenin (≥98%), (2RS)−2-(4-hydroxyphenyl)propionic acid (HPPA, ≥95%) and 4-ethylphenol (99%) from Sigma Aldrich (Prague, Czech Republic). *R,S*-equol (98%) and *S*-equol (97%) were purchased from Toronto Research Chemicals (Toronto, Canada). O-desmethylangolensin (O-DMA, ≥99%) was synthesized at the Department of Organic and Bioorganic Chemistry, Faculty of Pharmacy in Hradec Králové, Czech Republic (Migkos et al., 2019).

Table 1: The chemical structures of the sixteen tested isoflavonoids.



	Isoflavonoid:	R_5	R_6	R_7	R_8	$R_{3'}$	$R_{4'}$
aglycones	**daidzein**	H	H	**OH**	H	H	**OH**
	genistein	**OH**	H	**OH**	H	H	**OH**
	biochanin A	**OH**	H	**OH**	H	H	**O-CH_3**
	glycitein	**H**	**O-CH_3**	**OH**	H	H	**OH**
	isoformononetin	H	H	**O-CH_3**	H	H	**OH**
	formononetin	H	H	**OH**	H	H	**O-CH_3**
	calycosin	H	H	**OH**	H	**OH**	**O-CH_3**
	tectorigenin	**OH**	**O-CH_3**	**OH**	H	H	**OH**
	cladrin	H	H	**OH**	H	**O-CH_3**	**O-CH_3**
	prunetin	**OH**	H	**O-CH_3**	H	H	**OH**
glycosides	**daidzin**	H	H	**O-Glc**	H	H	**OH**
	genistin	**OH**	H	**O-Glc**	H	H	**OH**
	glycitin	H	**O-CH_3**	**O-Glc**	H	H	**OH**
	ononin	H	H	**O-Glc**	H	H	**O-CH_3**
	tectoridin	**OH**	**O-CH_3**	**O-Glc**	H	H	**OH**
	puerarin	H	H	**OH**	**Glc**	H	**OH**

Glc stands for glucose.

3.2. Other chemicals

Urethane, norepinephrine bitartrate (NE), sodium nitroprusside, acetylcholine, bradykinin acetate salt, nifedipine, indomethacin, serotonin (5-HT), forskolin, ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), Dulbecco's phosphate buffered saline without calcium and magnesium (DPBS), trypsin, pluronic F-127, Tween 80, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), $MgCl_2.6H_2O$, NaH_2PO_4, KCl and $CaCl_2.2H_2O$ were purchased from Sigma-Aldrich, [1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one] (ODQ) from Enzo Life Sciences (Lyon, France), U46619 from Cayman Chemical Company (Ann Arbor, MI, USA) and Bay K8644 from Axon Medchem BV (Groningen, Netherlands). NaCl, $NaHCO_3$ and D-glucose were provided by PENTA s.r.o (Prague, Czech Republic), saline by Baxter Czech spol. s.r.o. (Prague, Czech Republic), $MgSO_4.7H_2O$ by Erba Lachema s.r.o. (Brno, Czech Republic) and KH_2PO_4 by Dr. Kulich Pharma s.r.o. (Hradec Kralove, Czech Republic).

Smooth muscle cell basal medium (SmBM), fetal bovine serum (FBS), human epidermal growth factor (hEGF), human fibroblastic gowth factor (hFGF), insulin, gentamicin/amphotericin B were purchased from Lonza Australia Pty. Ltd. (Sydney, Australia), fura-2 acetoxymethyl ester (Fura-2 AM) from Abcam (Cambridge, UK).

A Krebs solution with a pH of 7.4 had the following composition in mM: NaCl 119, KCl 4.7, $CaCl_2.2H_2O$ 1.25, KH_2PO_4 1.18, $MgSO_4.7H_2O$ 1.17, $NaHCO_3$ 25, D-glucose 11.

The Hank's balanced salt solution (HBSS) used had the following composition in mM: NaCl 137, KCl 5.4, $CaCl_2.2H_2O$ 2, KH_2PO_4 0.44, $MgCl_2.6H_2O$ 0.5, $MgSO_4.7H_2O$ 0.4, NaH_2PO_4 0.34, $NaHCO_3$ 4.2, HEPES 10, D-glucose 5.5. The pH of the solution was adjusted to 7.4 by NaOH.

3.3. Experiments on rat aortas

The experiments were carried out on male Wistar:Han rats obtained from Velaz (Czech Republic)/ Charles River (Germany). The animals were maintained at 23-25 °C, under a 12-h dark/light cycle. The rats were provided a standard diet and tap water ad libitum. The study (reg. No. MSMT-7041/2014-10) was approved by the Ministry of Education, Youth and Sports and conformed to The Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8th edition, revised 2011, ISBN-13: 978-0-309-15400-0).

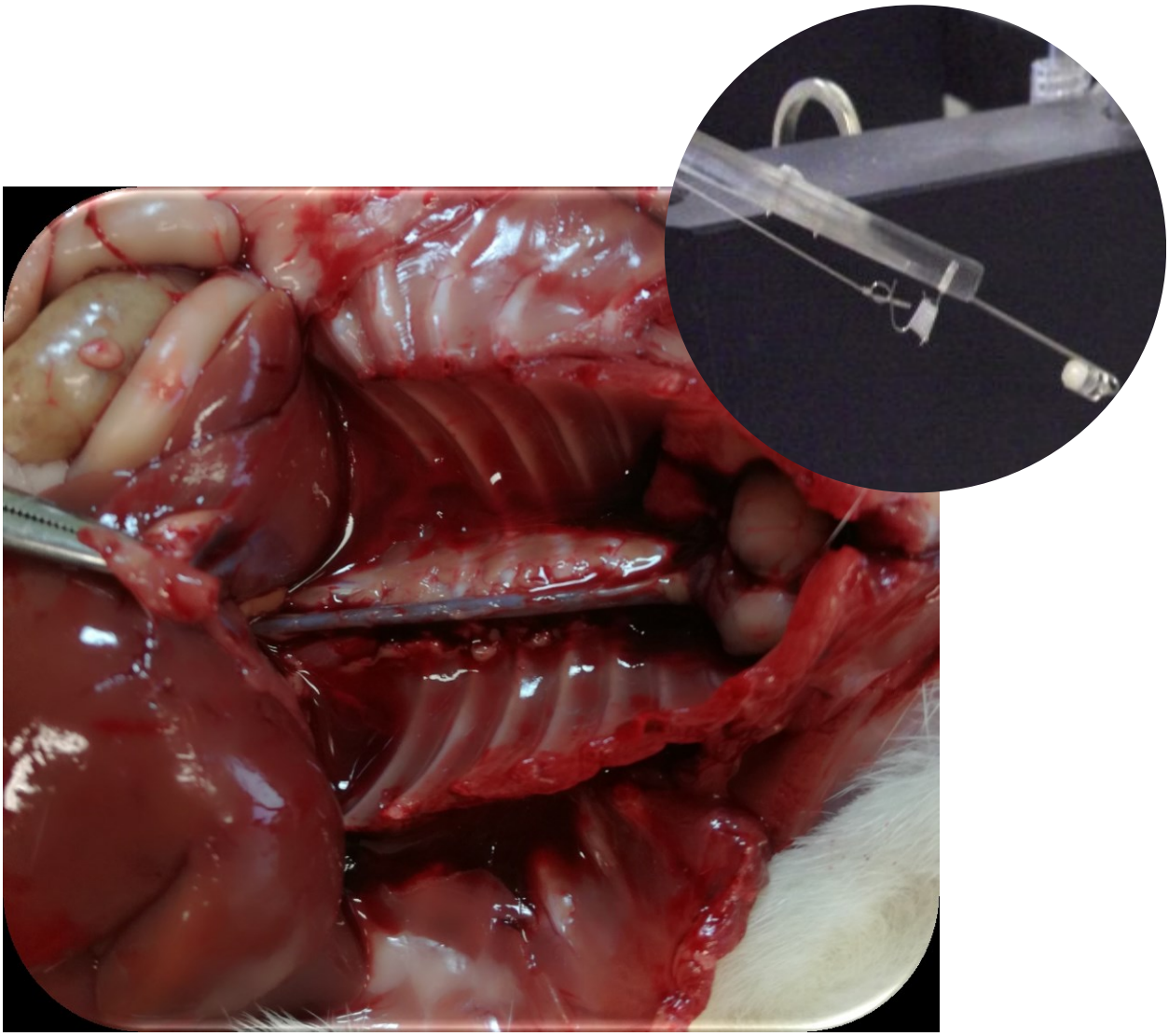


Figure 9: Rat thoracic aorta.

The rats were anesthetized by urethane 1.2 g/kg (i.p.) and euthanized by exsanguination. The thoracic aorta (Figure 9) was excised and cleaned of connective tissue and blood and then cut into ca. 3 mm long rings. When needed for mechanistic studies, the endothelial layer was mechanically disrupted by gently rubbing the luminal surface with dental floss. The rings were maintained in tissue baths with the Krebs solution oxygenated by 95% O_2, 5% CO_2 and kept at 37°C. Each aortic ring was hung between two stainless-steel wire hooks, one of them rigidly attached to the end of a fixed support rod and the second one connected to a force-displacement transducer and a computer (Figure 10). The changes in tension were recorded by S.P.E.L. Advanced kymograph Software (Experimetria Ltd., Hungary). This arrangement allowed the measurement of both contraction and relaxation. The rings were equilibrated at a tension of 2 g for 45 min and washed with the Krebs solution every 10 min. After equilibration, the tissue baths were filled with 5 mL of the Krebs solution. To assess the vasorelaxant potency, the aortic

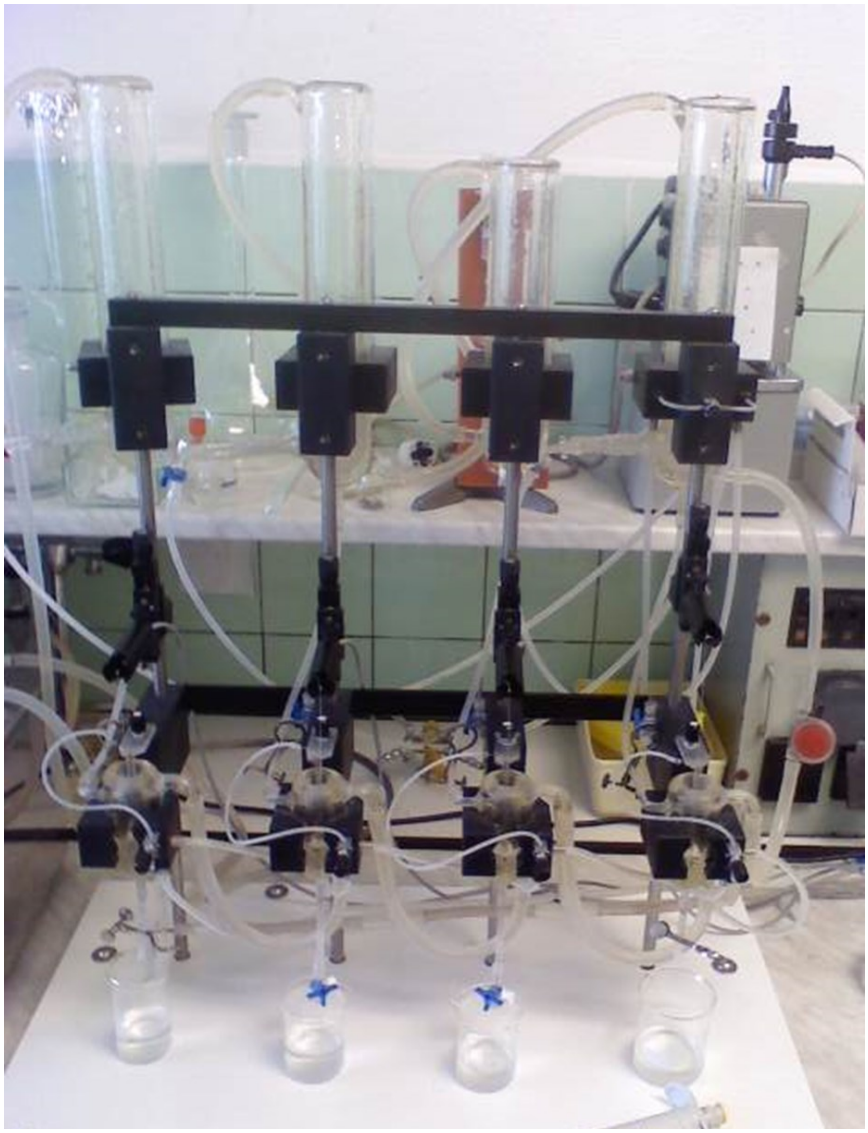


Figure 10: Apparatus used for the isolated rat aortic rings.

rings were contracted by NE (10 μM). When the response was stabilized (approx. after 45 min), acetylcholine (10 μM) was added to confirm either an intact or denuded endothelium. The tissue baths were then washed, filled with the Krebs solution and NE (10 μM) addition was repeated. After stabilization (approx. after 45 min), the tested isoflavonoids or metabolites were added to the bath cumulatively in final concentrations ranging from 100 nM to 1 mM.

The isoflavonoids and metabolites were initially dissolved in DMSO and then diluted by the Krebs solution. The same solvent, e.g. with increasing concentrations of DMSO, was cumulatively added to some aortic rings as the negative control in each experiment. The final concentration of DMSO in the bath was generally not higher than 0.1% in the case of very efficient compounds, while when additional experiments were needed for the determination of EC_{50}, it was increased up to 2 % at maximum. In experiments with isoflavonoids having lower activity, additional experiments for the determination of EC_{50} were performed with a concentration of DMSO up to 1–2 % due to their low water solubility. At the end of each experiment, the addition of sodium nitroprusside (10 μM) provoked relaxation of the aortic rings, which was considered maximal (100%) relaxation. This concentration was calculated using the vasorelaxant curve of sodium nitroprusside repeatedly obtained in our pre-experiments.

3.4. Experiments on rat mesenteric arteries

Similarly to aortas, mesenteric arteries (Figure 11) were isolated from the Wistar:Han rats (Velaz) and cut into 2 mm length rings. The endothelium was then removed and the rings were mounted under a tension of 1 g onto the Small Vessel Wire myograph system (Experimetria Ltd., Hungary) filled with the Krebs solution and maintained under the same conditions as described above. Acetylcholine (10 μM) was used to confirm the absence of endothelium and subsequently the arteries were contracted by NE (10 μM). After stabilization

of the contraction selected metabolites were cumulatively added (in concentrations ranging from 100 nM to 1 mM). As mentioned above, maximal relaxation was induced using sodium nitroprusside (10 μM) at the end of each experiment.

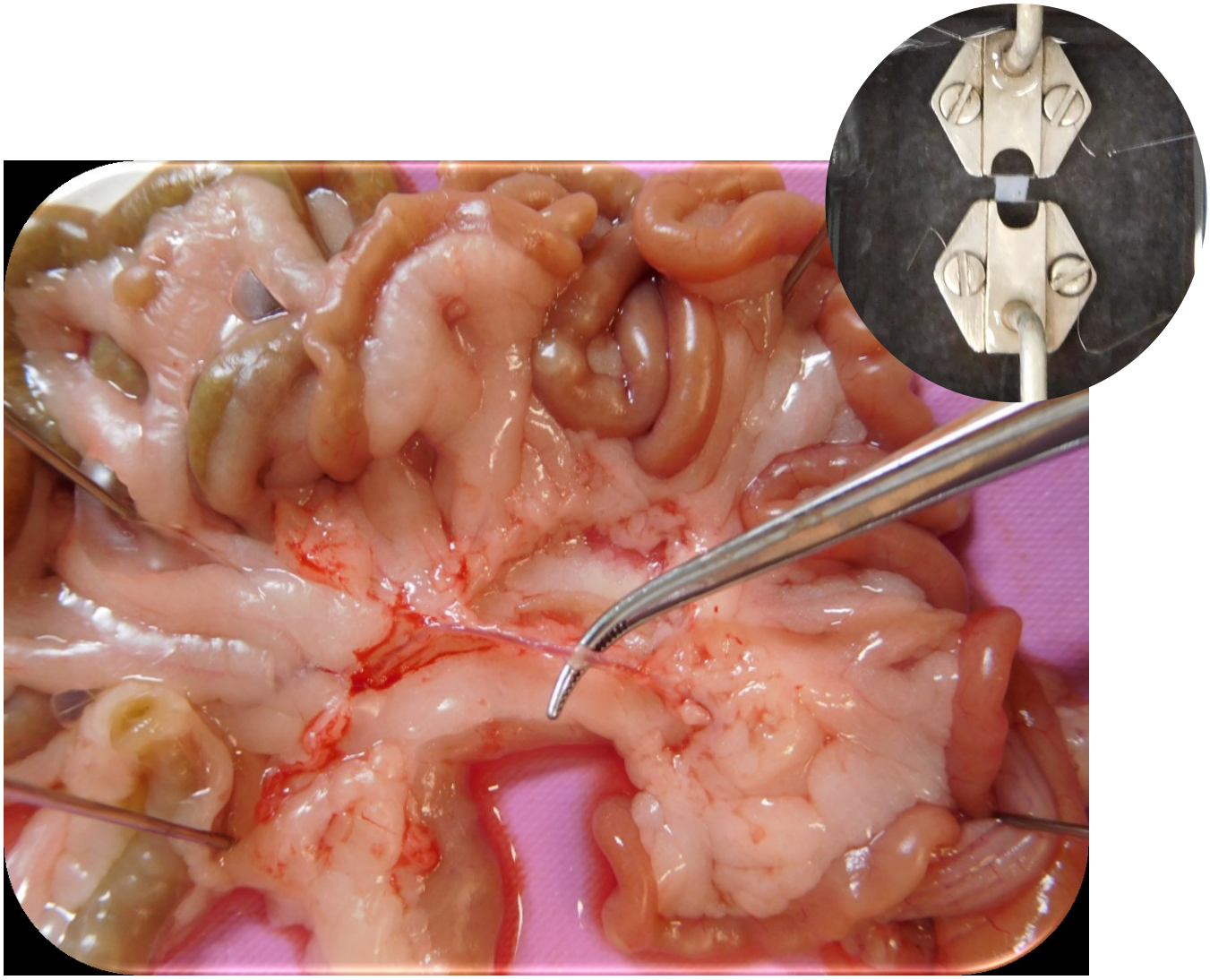


Figure 11: Rat mesenteric artery

3.5. Experiments on porcine coronary arteries

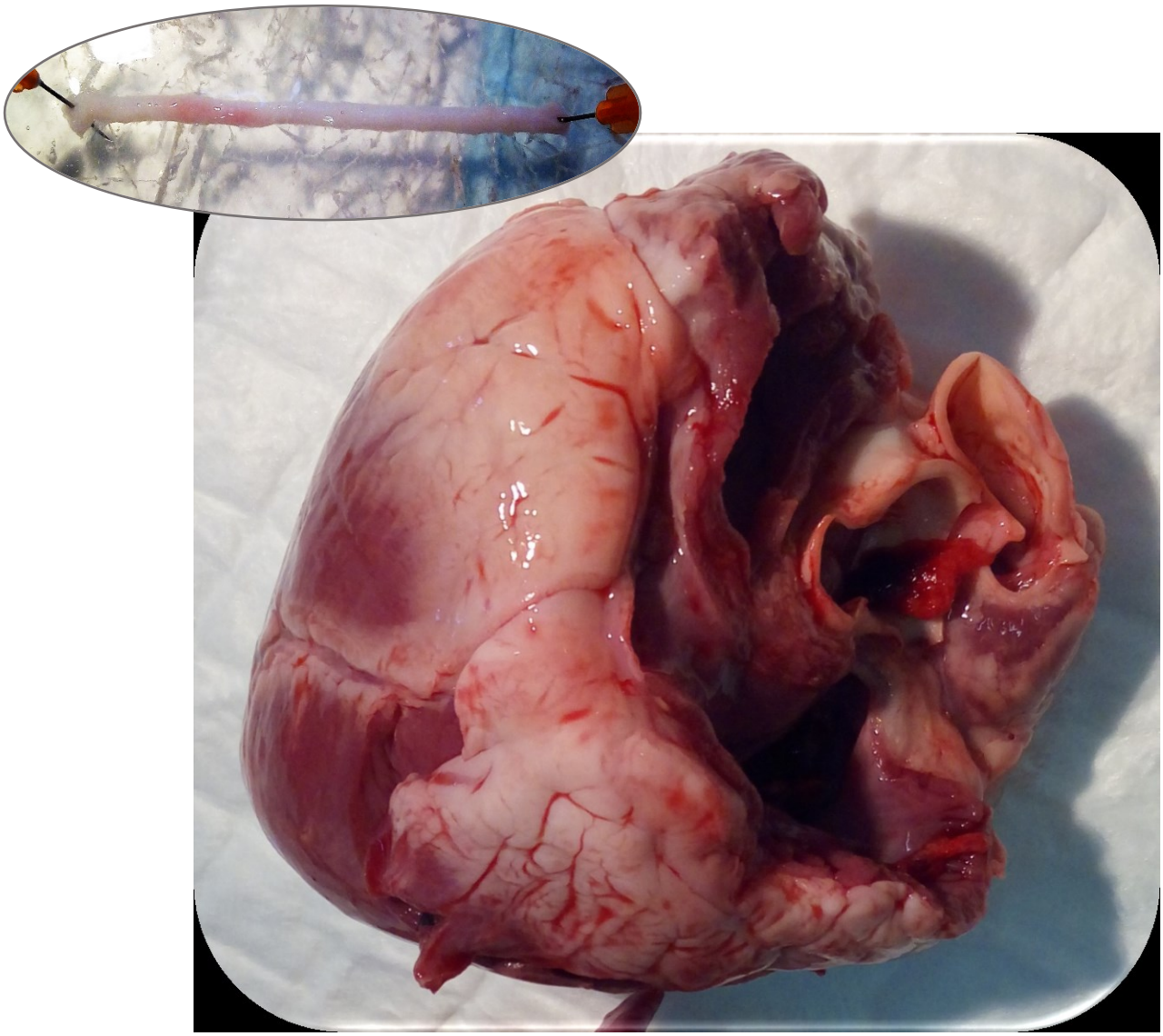


Figure 12: Porcine left circumflex coronary artery (above) isolated from a pig heart (below).

Pig hearts were collected from a local slaughterhouse (Copvial, Holtzheim, France), after pigs of both sexes (80-100 kg) were killed in the early morning. The left circumflex coronary arteries (Figure 12) were excised, cleaned of loose connective tissue, flushed with the Krebs solution to remove any remaining blood and cut into rings (4–5 mm in length). When needed, the endothelium was removed by rubbing the intimal surface of the rings with a pair of forceps.

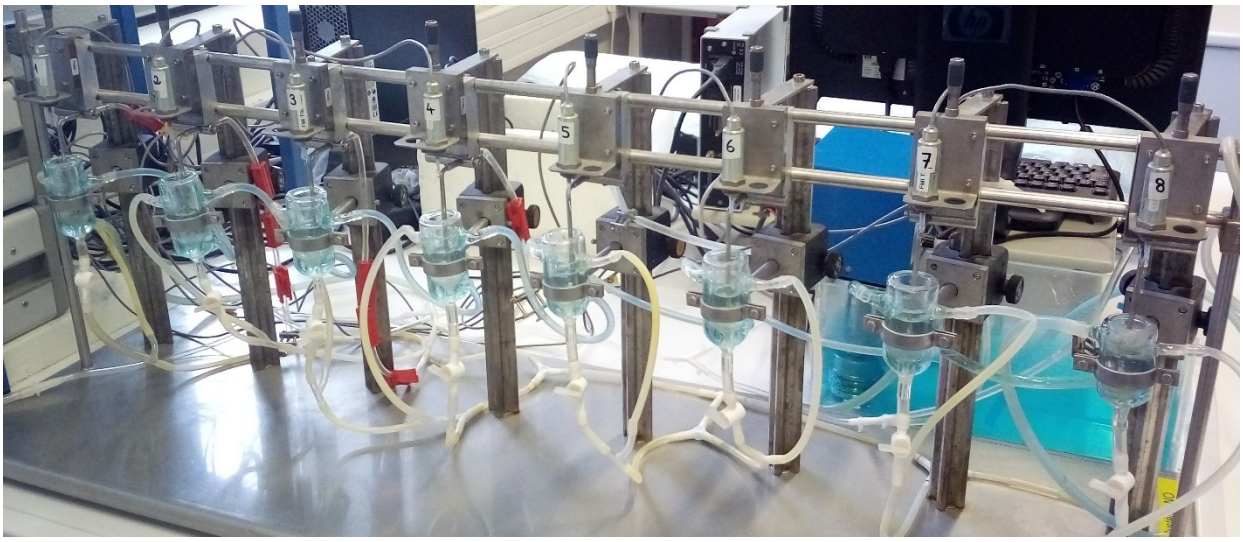


Figure 13: Apparatus used for isolated coronary artery rings. Apparatus was constructed by colleagues from the University of Strasbourg.

Similarly to the rat aorta, the rings of porcine coronary arteries were mounted in organ baths (Figure 13) containing an oxygenated (95% O_2 + 5% CO_2) Krebs solution at 37 °C for the determination of changes in isometric tension. Following equilibration for 90 min under a resting tension of 5 g, the rings were contracted with KCl (80 mM). After washing with the Krebs solution and resetting the basal tension, the rings were contracted by a thromboxane A_2 analog, U46619 (10-20 nM), to cause about 80% of the maximal constriction and then the relaxation induced by bradykinin (0.3 µmol/L) was used to confirm the presence or absence of endothelium. Thereafter, washout and a 30-min equilibration period were carried out.

3.5.1. Determination of the mechanism of the vasorelaxant effect

Subsequently the coronary arterial rings were again constricted with U46619 (6-16 nM) and cumulative doses of isoflavonoids (0.1-30 µM) or vehicle (DMSO, 0.001-0.3 %) were applied. The next dose was always added after stabilization of the relaxant response to the

previous dose. Enzyme inhibitors (ODQ - 10^{-6} M and indomethacin -10^{-5} M, dissolved in DMSO and 5% aqueous sodium carbonate solution, respectively) were added in the chamber 30 min prior to U46619 application when needed. The addition of sodium nitroprusside (10 μM) induced again the maximal (100%) relaxation of the arterial rings at the end of each experiment.

3.5.2. Inhibition of contractile responses

For the determination of the inhibition of contractile responses, equilibrated endothelium-denuded rings were exposed to three different concentrations (ranging from 3 - 30 μM) of the selected isoflavonoids or metabolites for 30 min before the cumulative administration of increasing concentrations of either KCl, 5-HT, U46619 or $CaCl_2$ in the presence of 40 mM KCl.

3.5.3. Inhibition of the effect of Bay K8644

Endothelium-denuded coronary rings were treated with four different concentrations (3, 10, 20 and 30 μM) of biochanin A, in the presence of 15 mM KCl. Then, Bay K8644, an L-type calcium channel activator, was applied cumulatively (10^{-10} - 10^{-6} M) to induce contraction of the smooth muscles. Experiments were carried out in darkness in order to avoid possible oxidation of the Bay K8644.

3.5.4. Effect on relaxation induced by activators of cyclic nucleotide synthesis.

To further investigate whether the mechanism of action of the tested compounds implicated the generation of cyclic GMP or cyclic AMP, endothelium-denuded coronary artery

rings were contracted with U46619 (6-16 nM) and then exposed to a low concentration of isoflavonoid or metabolite (2-4 μM), so that relaxation to about 15-20% of the maximum extent was induced. Subsequently, concentration-dependent relaxation was caused by the administration of cumulative doses (1 nM - 1 μM) of either sodium nitroprusside or forskolin, which activate guanylyl and adenylyl cyclase, respectively.

3.6. *In vivo* methodology

Four spontaneously hypertensive rats (SHR), obtained from the Czech Academy of Sciences (Czech Republic), were bred in the animal house of the Faculty of Pharmacy (Charles

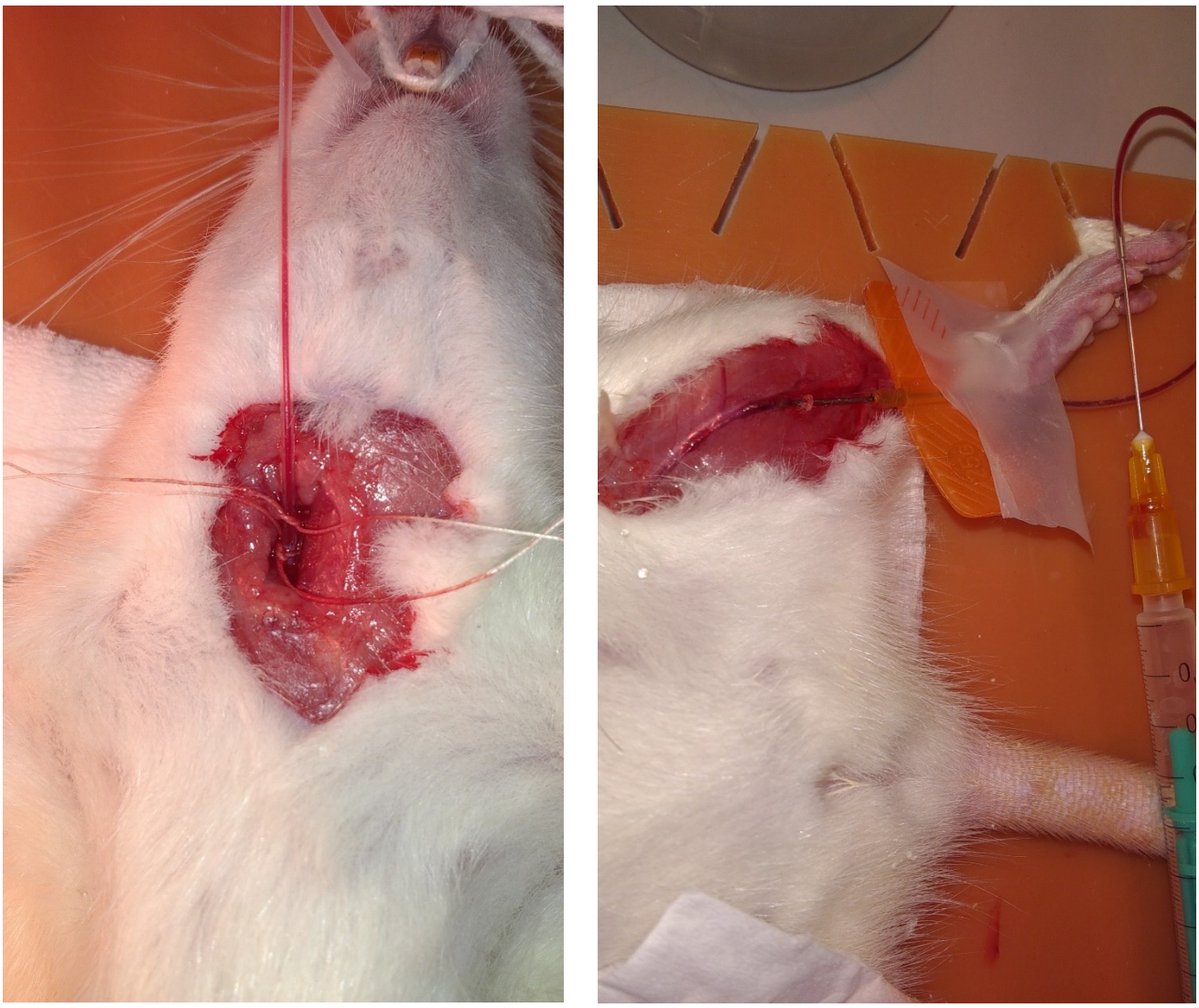


Figure 14: Cannulation of rat carotid artery (left) and saphenous vein (right).

University) and maintained at a constant temperature of 23–25 °C with the 12-h dark/light cycle. Rats were provided a standard diet and tap water ad libitum. They had an average weight of 365 ± 24g (mean blood pressure under anesthesia: 202 ± 43 mmHg) and they were anaesthetized by intraperitoneal administration of pentobarbital at a dose of 50 $mg.kg^{-1}$, according our protocols published previously (Najmanova et al., 2016; Pourova et al., 2018).

A pressure transducer MLT0380/D was inserted to the left common carotid artery (*arteria carotis communis sinistra,* Figure 14) to record arterial blood pressure and heart rate with a PowerLab® System connected to the software LabChart 7 Software (AdInstruments, Australia). The ECG was recorded using an ECG unit that was also connected to the PowerLab.

Right saphenous vein (*vena saphena dextra,* Figure 14) was cannulated for intravenous administration of the tested compound or the solvent. The body temperature was maintained during the whole experiment at 36.5 ± 0.5°C. Administration started when each animal become haemodynamically stabilized (approx. after 15 min). First, each rat was given saline and the solvent (10% solution of Tween 80 in saline). Then, the O-DMA dissolved in the solvent was administered as the single bolus doses ranged from 0.001 $mg.kg^{-1}$ to 2.5 $mg.kg^{-1}$. The applied volume was the same in all cases (0.1 ml) as this volume by itself do not change the arterial blood pressure. The next dose was always administered after the return of cardiovascular parameters to stable values. The animals were euthanized by i.v. administration of 1ml of 1M KCl at the end of the experiment.

3.7. Experiments on human vascular smooth muscle cells

3.7.1. Cell Culture

To initiate primary cell cultures, human aortic (AoVSMC) and coronary artery (CASMC) smooth muscle cells (Lonza Australia Pty. Ltd.) were grown, according to the instructions of the provider, in smooth muscle cell basal medium (SmBM) (Lonza Australia Pty. Ltd.), containing 10% fetal bovine serum (FBS) and in SmBM, containing supplements and growth factors (namely: 0.1% hEGF, 0.1% insulin, 0.2% hFGF-B, 5% FBS and 0.1% gentamicin/amphotericin B) (Lonza Australia Pty. Ltd.), respectively. The cells were incubated at 37°C, in 5% CO_2 and 95% ambient air. Trypsin (0.05%)/EDTA (0.02%) for primary cells (Sigma-Aldrich) was used with HBSS and trypsin neutralizing solution (5% FBS in Dulbecco's phosphate buffered saline without calcium and magnesium /DPBS/, Sigma-Aldrich) to passage the cells. Cells were used at passage number 4 to 6 and were allowed to proliferate until they reached 80 to 90% confluency.

3.7.2. Measurement of intracellular calcium concentrations

Levels of intracellular calcium were measured with Fura-2 acetoxymethyl ester (Fura-2 AM) fluorescence dye (Abcam, Cambridge, UK). AoVSMC and CASMC grown to 70 to 90% confluency were washed twice with HBSS and gently harvested with 0.05% trypsin / 0.02% EDTA solution, neutralized with trypsin neutralizing solution. Detached cells were transferred into centrifuge tubes and centrifuged at 220 x g for 5 min. The collected cells were resuspended in growth medium (2-3 ml) and seeded on 15 mm coverslips, which eventually formed the base of the observation chamber. At least 24 h later, when the cells were adequately stuck, so as to withstand perfusion, they were incubated with Fura-2 AM at a concentration of

2 μM, in the presence of 0,02% pluronic F-127 (an emulsifying agent that assists the dispersion of the dye and thus cells loading), for 40 min – 1 h, at 37ºC. The medium with the remaining dye was then washed away with HBSS, followed by a 30 min incubation (time sufficient for the de-esterification of the dye to take place within the cells).

Subsequently each coverslip containing the muscle cells loaded with Fura-2 AM, was attached in an observation chamber and placed under live cell imaging Zeiss Axiovert 200 microscope (Carl Zeiss NTS Ltd. Oberkochen, Germany). Images were recorded using an ORCA-ERII (Hamamatsu Photonics K.K., Hamamatsu City, Japan) camera driven by the MetaFluor software package (v7.7.2, Molecular Devices LLC., San Jose, CA, USA). Fura-2 fluorescence was measured with a dual excitation at wavelengths of 340 nm and 380 nm. Fluorescence was detected in dark, using a filter for the emission wavelength of 510 nm, at 37 ºC and fluorescence intensity was recorded (Figure 15), while the cells were continuously perfused with HBSS containing the tested substances and reagents, as described below (Table 2). The change in cytosolic free calcium ($[Ca^{2+}]_i$) was calculated as a 340/380 nm fluorescence ratio of the emitted fluorescence signal.

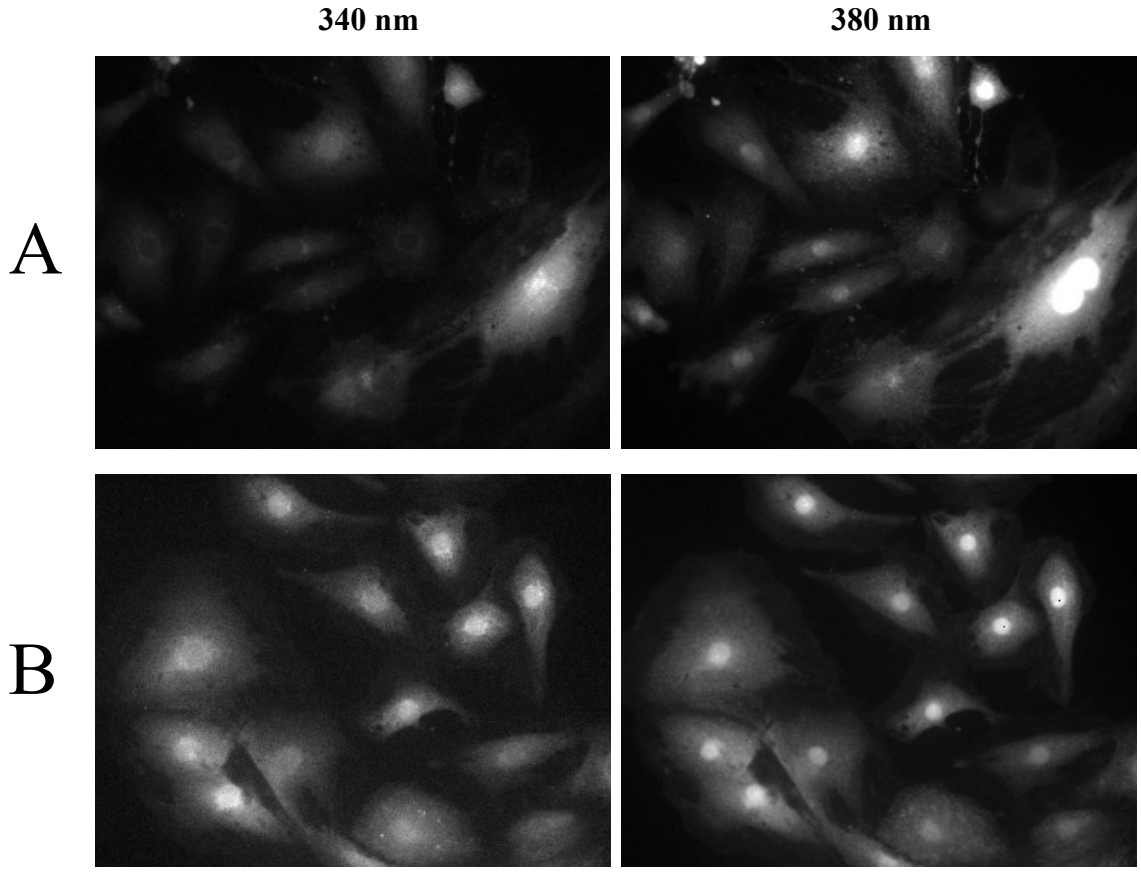


Figure 15: Human aortic (A) and coronary artery (B) smooth muscle cells emitting fluoresence, under 340 nm and 380 nm excitation wavelengths.

Table 2: Table describing the subsequent perfusions of human smooth muscle cells, with Hank's balanced salt solution containing different reagents.

Perfusion no.: / Tested compound:	**1**	**2**	**3**	**4**	**5**	**6**
Solvent control	KCl 15 mM	KCl 15 mM + DMSO 0.3%	DMSO 0.3% + KCl 1mM + Bay K8644 0.1 µM	DMSO 0.3% + KCl 1mM + Bay K8644 0.5 µM	DMSO 0.3% + KCl 1mM + Bay K8644 1 µM	DMSO 0.3% + KCl 1mM + Bay K8644 5 µM
Biochanin A	KCl 15 mM	KCl 15 mM + Biochanin A 30 µM	Biochanin A 30 µM + KCl 1mM + Bay K8644 0.1 µM	Biochanin A 30 µM + KCl 1mM + Bay K8644 0.5 µM	Biochanin A 30 µM + KCl 1mM + Bay K8644 1 µM	Biochanin A 30 µM + KCl 1mM + Bay K8644 5 µM
O-desmethylangolensin	KCl 15 mM	KCl 15 mM + O-DMA 30 µM	O-DMA 30 µM + KCl 1 mM + Bay K8644 0.1 µM	O-DMA 30 µM + KCl 1 mM + Bay K8644 0.5 µM	O-DMA 30 µM + KCl 1 mM + Bay K8644 1 µM	O-DMA 30 µM + KCl 1 mM + Bay K8644 5 µM

3.8. Statistical analysis

Data were presented as mean values ± standard errors of the means (S.E.M.). The number (n) of different experiments was always mentioned. The EC_{50} value corresponded to the concentration of the tested compound inducing 50% of the vasorelaxant response, determined by GraphPad Prism 8.0.2 (GraphPad Software, Inc., San Diego, CA, USA). The mean values were compared by analysing the variance followed by the Sidak post-hoc test employing GraphPad Prism 8.0.2. The difference was considered to be significant when p value of less than 0.05 was found. Concentration-relaxation curves were compared using 95% confidence intervals, plotted by the above-mentioned software.

Differences between *in vivo* effects of O-DMA and control were compared using a one-way ANOVA, followed by the Dunnett post hoc test employing GraphPad Prism 7.03.

For the comparison of live cell imaging results the two-way ANOVA was used, followed by either the Tukey's or the Sidak's multiple comparisons test (in the case of one or two or only one substance *vs.* the control, respectively), for AoVSMC and CASMC respectively.

4. RESULTS

4.1. Isoflavonoids

4.1.1. Several isoflavonoids relax rat aortic rings *ex vivo*

In the first series of experiments, a screening of the vasodilatory effects of sixteen isoflavonoids (Table 1) on NE-precontracted rat aortic rings was performed. All six glycosides, namely genistin, daidzin, glycitin, ononin, tectoridin and puerarin had no significant vasorelaxant effects, when compared to that of the control containing only the solvent (Figure 16-18). A direct comparison using EC_{50} on rat aortas reported the following order of increasing potency: glycosides < daidzein ≅ prunetin ≅ cladrin ≅ calycosin ≅ tectorigenin ≅ formononetin ≅ isoformononetin < genistein ≤ glycitein = biochanin A (Figure 19). In particular, biochanin A and glycitein appeared the most potent vasodilators, having EC_{50} below 30 μM, followed in ascending order by genistein, isoformononetin, formononetin, tectorigenin, calycosin, cladrin, prunetin and daidzein, with EC_{50} ranging from 30 to 110 μM (Figure 19).

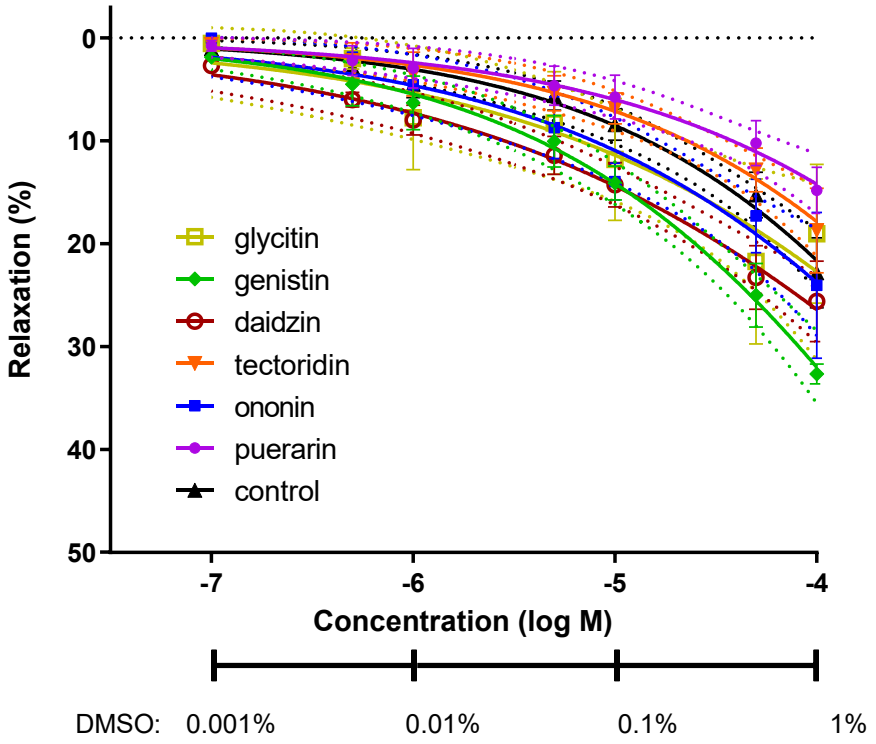


Figure 16: Concentration-response curves, with 95% confidence intervals, showing the vasorelaxant effect of isoflavonoid glycosides in endothelium-intact rat aortic rings. The individual points represent the mean ± SEM. Vessels were precontracted by norepinephrine (10^{-5} M), n=3-4. Relaxation is expressed as percentages of the sodium nitroprusside-induced maximal relaxation. A concentration-response curve to the solvent was used as the negative control and the final concentration of DMSO in the organ bath is indicated below the x axis.

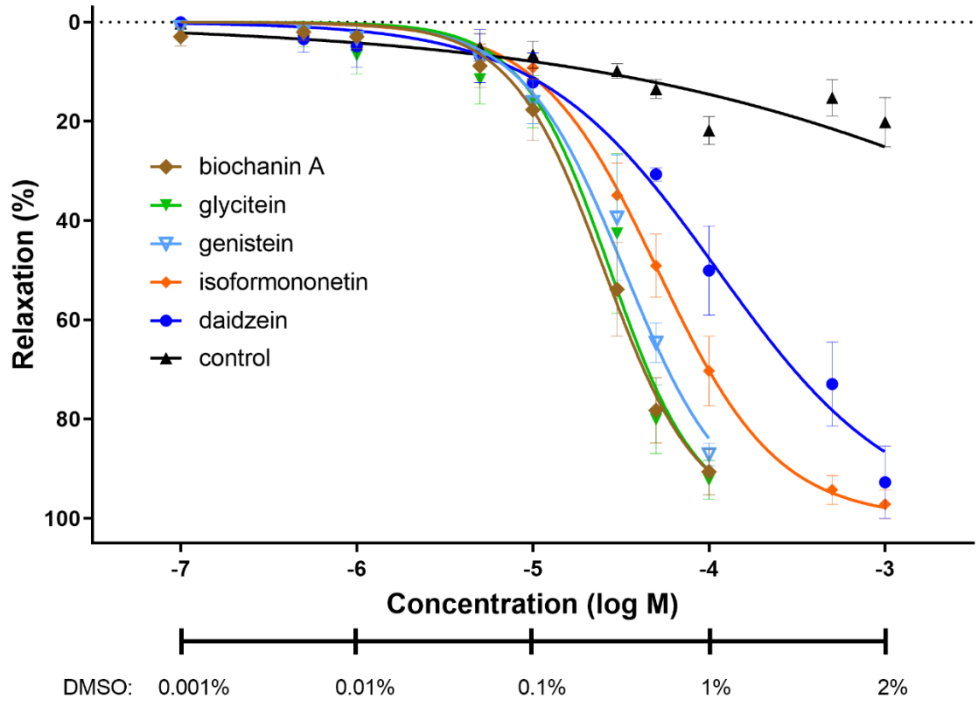


Figure 17: Concentration-response curves showing the vasorelaxant effect of the most potent isoflavonoid ***aglycones*** *in endothelium-intact rat aortic rings, precontracted by norepinephrine (10^{-5} M), n=5. Relaxation is expressed as percentages of the sodium nitroprusside-induced maximal relaxation of the vessels. The individual points represent the mean ± SEM. A concentration-response curve to the solvent was used as the negative control and the final concentration of DMSO in the organ bath is indicated below the x axis.*

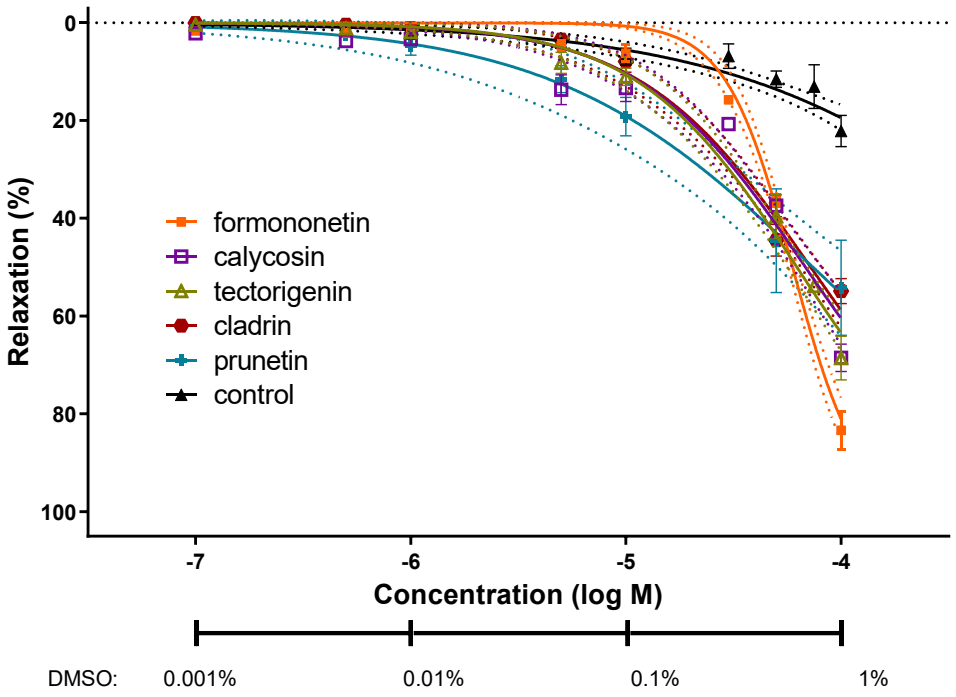


Figure 18: Concentration-response curves, with 95% confidence intervals, showing the vasorelaxant effect of other isoflavonoid ***aglycones.*** *Results are from endothelium-intact rat aortic rings precontracted by norepinephrine (10^{-5} M), n=4-6. The individual points represent the mean ± SEM. Relaxation is expressed as percentages of the sodium nitroprusside-induced maximal relaxation of the vessels. A concentration-response curve to the solvent was used as a negative control and final concentration of DMSO in the organ bath is indicated below the x axis.*

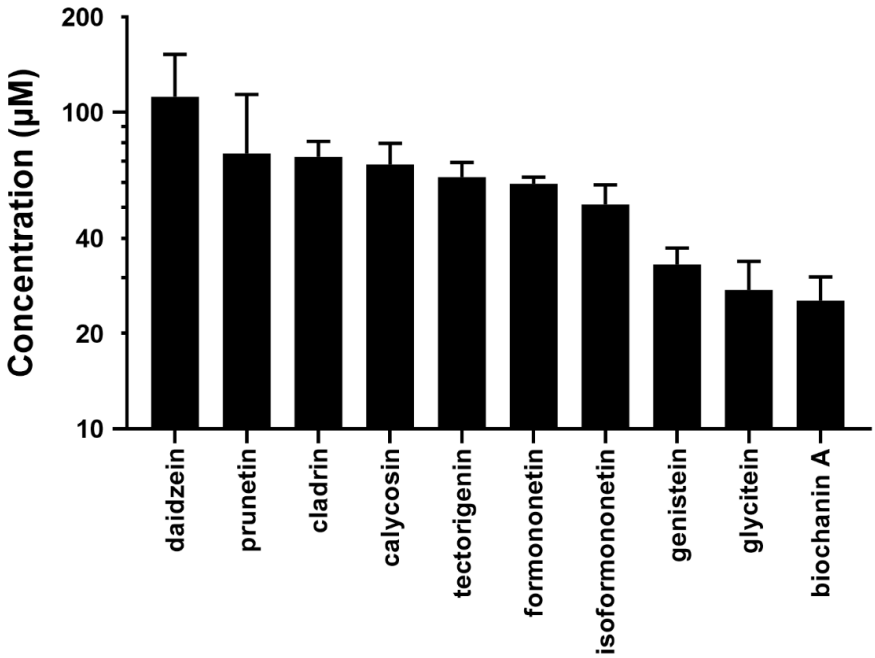


*Figure 19: **EC_{50}** of the vasodilatory response to isoflavonoid aglycones in isolated rat thoracic aortic rings ex vivo. Data are shown as the mean with 95% confidence intervals, n=5.*

4.1.2. Biochanin A and glycitein cause endothelium-independent relaxations of rat aorta and porcine coronary artery rings

Thereafter, the two most potent isoflavonoids, biochanin A and glycitein, were selected for further mechanistic experiments performed on both isolated rat aorta and porcine coronary arteries. Similarly to rat aorta, as is also the case of coronary arteries, both compounds induced dose-dependent vasorelaxation. The effect was not significantly modified by the absence of the vascular endothelium (Figures 20, 21) in any of the artery models. Interestingly, the EC_{50} of biochanin A on coronary arteries (5.5 ± 0.6 µM) was lower than that on rat aortas (23.4 ± 2.0 µM) and that of glycitein (16.2 ± 1.0 µM). For glycitein, the difference between EC_{50}s for the two types of vessels was smaller (EC_{50}s= 25.6 ± 2.5 µM and 16.2 ± 1.0 µM on rat

aortas and porcine coronary arteries, respectively). Since the effect on coronary arteries was achieved in lower concentrations, and given the similarities between porcine and human hearts (Lelovas, Kostomitsopoulos, & Xanthos, 2014), this model was used for further testing.

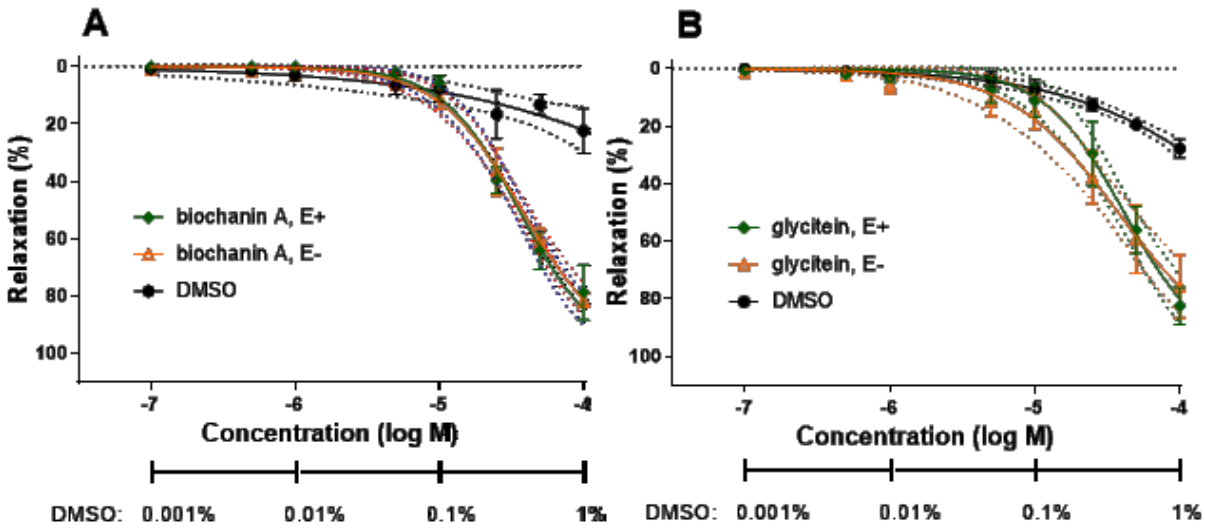


Figure 20: Concentration-response curves, with 95% confidence intervals, showing the vasorelaxant effect of ***biochanin A*** *(A) and* ***glycitein*** *(B) in endothelium-intact (E+, green line) or -denuded (E-, orange line) rat* ***aortic*** *rings, precontracted by norepinephrine (10^{-5} M), n=3. Relaxation is expressed as percentages of the sodium nitroprusside-induced relaxation of the vessels. A concentration-response curve to the solvent was used as a negative control and the final concentration of DMSO in the organ bath is indicated below the x axis.*

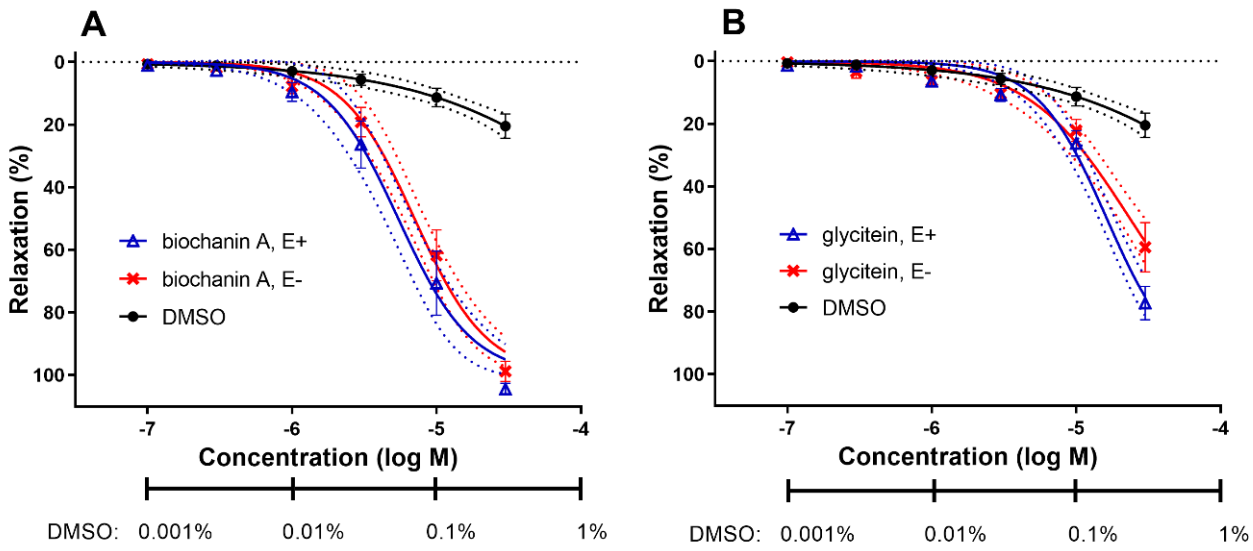


Figure 21: Concentration-response curves, with 95% confidence intervals, showing the vasorelaxant effect of ***biochanin A*** *(A) and* ***glycitein*** *(B) in endothelium-intact (E+, blue line) or -denuded (E-, red line)* ***coronary*** *artery rings, precontracted by U46619 (10^{-8} M). Relaxation is expressed as percentages of the initial basal tension of the rings, data are shown as the mean ± SEM, n=6. A concentration-response curve to the solvent was used as the negative control and the final concentration of DMSO in the organ bath is indicated below the x axis.*

4.1.3. Biochanin A, but not glycitein, inhibits contractile responses to KCl, $CaCl_2$, 5-HT and U46619

To further elucidate the mechanism(s) of the selected isoflavonoids, endothelium-denuded coronary arteries pretreated with biochanin A (3, 10 and 30 µM) or glycitein (10, 15 and 30 µM) were exposed to increasing concentrations of four different vasoconstricting stimuli. Contractions induced by both KCl and $CaCl_2$ were dose-dependently inhibited by biochanin A. In the highest dose, biochanin A equally blocked 73% of the maximal vasoconstriction caused by both inducers (Figure 22A). On the other hand, glycitein caused approximately only 25% reduction of the maximal effect of KCl and did not significantly alter

the contraction induced by the maximal concentration of $CaCl_2$. However, 30 μM of glycitein partly blocked the effect of lower concentrations of $CaCl_2$ (Figure 22B). Similarly, biochanin A demonstrated higher potency against receptor-mediated contractions induced by serotonin or thromboxane-analogue U46619. In the highest concentrations, it blocked 88% and 56% of their maximal effects, respectively (Figure 22A). On the contrary, only the highest used concentration of glycitein (30 μM) managed to block, at least partially (38%), the maximal effect of serotonin, as well as decreased the contractions induced by low concentrations of U46619 (Figure 22B).

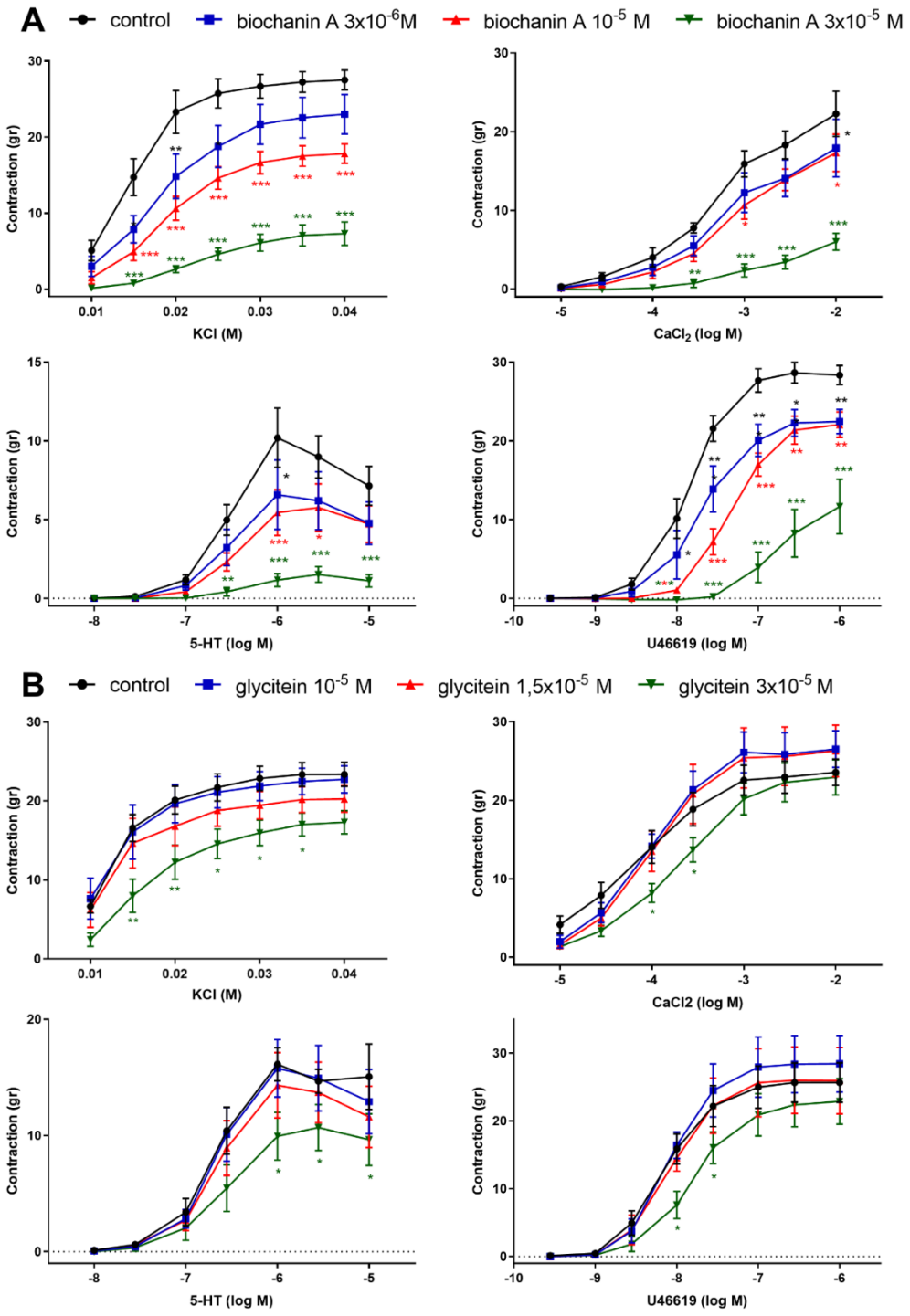


Figure 22: Concentration-dependent effects of ***biochanin*** *A (A) and* ***glycitein*** *(B) on the contraction of coronary arteries induced by KCl, $CaCl_2$, serotonin (5-HT) and U46619. Porcine coronary artery rings were pretreated with indicated concentrations of isoflavonoids before the addition of cumulative doses of the inducer. * $p < 0.05$, ** $p < 0.01$, *** < 0.001 vs. control, n=6.*

4.1.4. Biochanin A affects contractions induced by the activation of L-type calcium channels

Due to the higher potency of biochanin A, subsequent series of mechanistic experiments were performed with this isoflavonoid. Since biochanin A blocked constrictions induced by different inducers, intracellular calcium signalling could be the target of this compound. Hence, isolated porcine coronary arteries were firstly pretreated with different concentrations of biochanin A and then exposed to increasing concentrations of Bay K8644, an activator of L-type calcium channels. Under these conditions, biochanin A again dose dependently blocked the contractions (Figure 23).

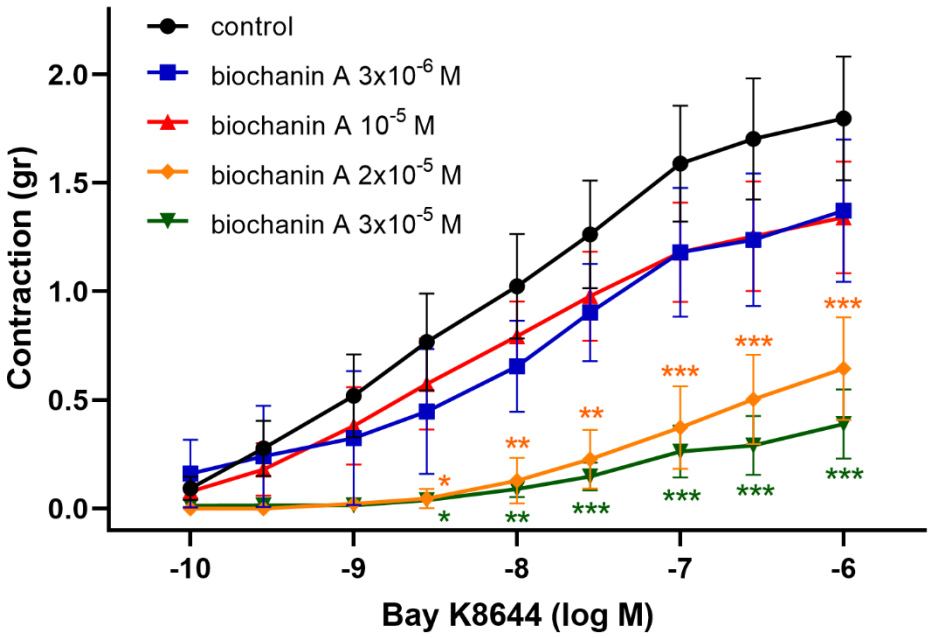


*Figure 23: Concentration-dependent effects of **biochanin A** on vessel contraction induced by the activation of L-type channels. Porcine coronary artery rings were treated with indicated concentrations of biochanin A before the addition of cumulative doses of calcium channel activator BAY K8644. * $p < 0.05$, ** $p < 0.01$, *** < 0.001 vs. control, n=5.*

4.1.5. Biochanin A potentiates the effect of sodium nitroprusside

In a separate series of experiments, sodium nitroprusside and forskolin were used to relax precontracted porcine coronary artery rings without endothelium, by raising the intracellular levels of cyclic GMP and cyclic AMP, respectively. Co-treatment of the vessels with low concentrations of biochanin A (2-4 µM) led to a significant potentiation of the sodium nitroprusside-induced relaxation (Figure 24A), decreasing its EC_{50} from 24.2 ± 4.5 nM to 9.4 ± 6.3 nM. However, no significant influence on the vasodilatory effect of forskolin was observed (Figure 24B), indicating that biochanin A might stimulate the cGMP, but not the cAMP, related vasodilatory pathway.

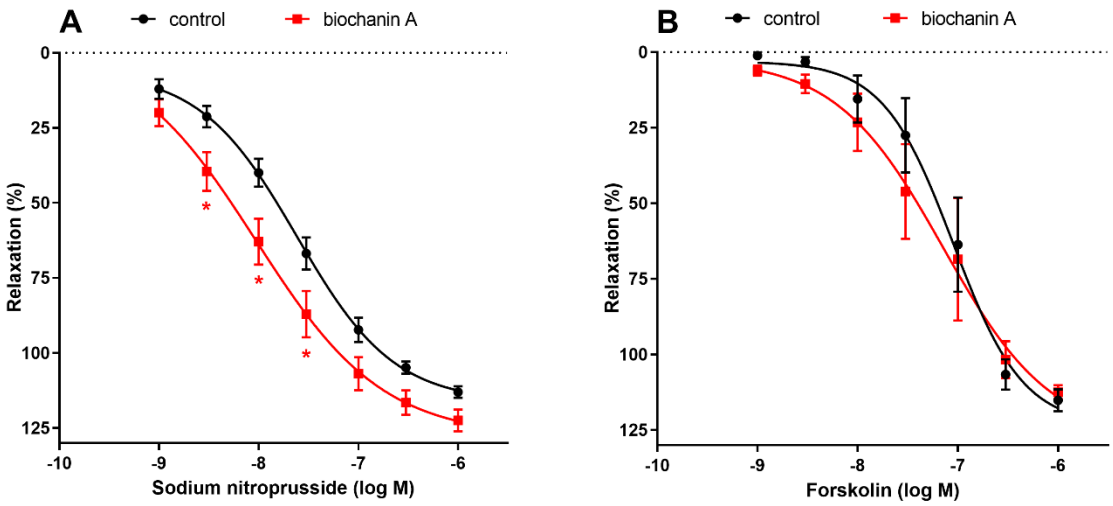


*Figure 24: The effect of **biochanin A** on relaxation induced by sodium nitroprusside (A) and forskolin (B). After exposure of endothelium-denuded coronary artery rings to biochanin A (concentrations slightly varied from 2 to 4 µM in order to trigger 20% of relaxation), further relaxation was induced by the addition of cumulative doses of either nitroprusside or forskolin. Relaxation is expressed as percentages of the initial basal tension of the rings. * indicates a difference at $p<0.05$ between corresponding values, n=5.*

4.1.6. Biochanin A-induced relaxation is partially influenced by the function of soluble guanylyl cyclase

A notable step within the cGMP pathway, activated by nitric oxide, is the conversion of GTP to cGMP, by soluble guanylyl cyclase. The presence of ODQ (10^{-6} M), a selective inhibitor of this enzyme, delayed the onset of the vasorelaxant response to biochanin A in endothelium-denuded coronary rings (Figure 25A). The EC_{50} shifted from 5.1 ± 0.8 to 6.8 ± 0.5 μM, while the achieved maximal relaxation was not affected. Additionally, in order to determine whether the production of prostanoids is involved in the mechanism of action of biochanin A, coronary artery rings without endothelium were incubated with indomethacin (10^{-5} M), a non-selective cyclooxygenase inhibitor, prior to the administration of the isoflavonoid. However, the inhibition of this pathway did not cause any alteration in the vasorelaxant effect (Figure 25B).

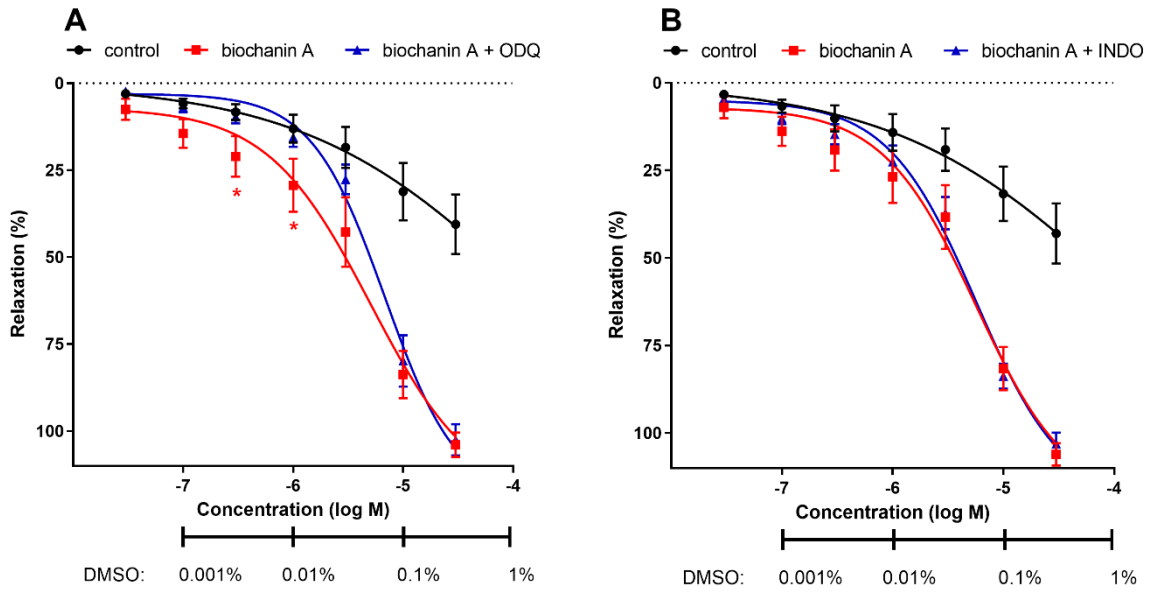


Figure 25: The influence of inhibition of guanylyl cyclase (A) and cyclooxygenase (B) on ***biochanin A*** *induced vasorelaxation. Endothelium-denuded coronary artery rings were incubated with* ***ODQ*** *(A, 10^{-6} M) or* ***indomethacin*** *(B, 10^{-5} M) before cumulative doses of biochanin A were administered. Relaxation is expressed as percentages of the initial basal tension of the rings. * indicates a difference ($p<0.05$) between the absence and presence of the inhibitor, n=6. A concentration-response curve to the solvent was used as the negative control and the final concentration of DMSO in the organ bath is indicated below the x axis.*

4.2. Isoflavonoid metabolites:

4.2.1. Several isoflavonoid gut metabolites relaxed rat aortic rings *ex vivo*

Initially the four known gut bacteria isoflavonoid metabolites as well as the racemate *R,S*-equol were screened for their vasodilating properties in isolated rat aortas. Out of the metabolites, the two smaller phenolic compounds, namely 4-ethylphenol and HPPA, failed to induce a vessel relaxation significantly different from that caused by the solvent itself, in the

range of the tested concentrations (Figure 26). On the contrary, O-DMA and *S*-equol, as well as the racemic mixture of the latter, demonstrated clear vasorelaxant effect of EC_{50} ranging from 43 to 66 µM (Table 3). Among them O-DMA appeared to be numerically slightly more active, but statistically there was no difference.

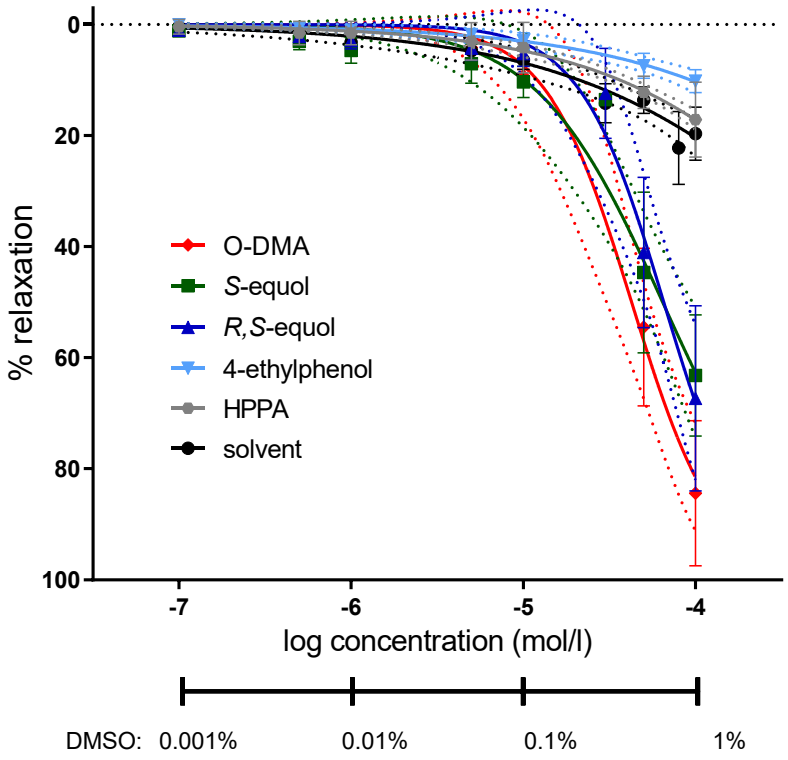


Figure 26: Concentration-response curves, with 95% confidence intervals, showing the vasorelaxant effect of the isoflavonoid gut ***metabolites*** *in endothelium-intact rat* ***aortic*** *rings, precontracted by norepinephrine (10^{-5} M). Relaxation is expressed as percentages of the sodium nitroprusside-induced relaxation of the vessels, n=3-4. A concentration-response curve to the solvent was used as the negative control and the final concentration of DMSO in the organ bath is indicated below the x axis.*

4.2.2. O-DMA, *S*-equol and *R,S*-equol caused endothelium-independent relaxations of porcine coronary artery rings

After they were screened in rat aorta, the three active metabolites were further investigated for their possible effects on porcine coronary arteries. O-DMA, *S*-equol and *R,S*-equol induced a dose-dependent dilation in this vessel model as well, with their potency being far higher when compared to rat aorta. Their EC_{50} ranged from 6 to 17 μM (Table 3). In this case *R,S*-equol appeared more active than the other two (Figure 27A). Furthermore, parallel experiments performed in endothelium-denuded arteries showed that the absence of endothelium did not affect the vasorelaxant capability of any of the compounds (Figure 27).

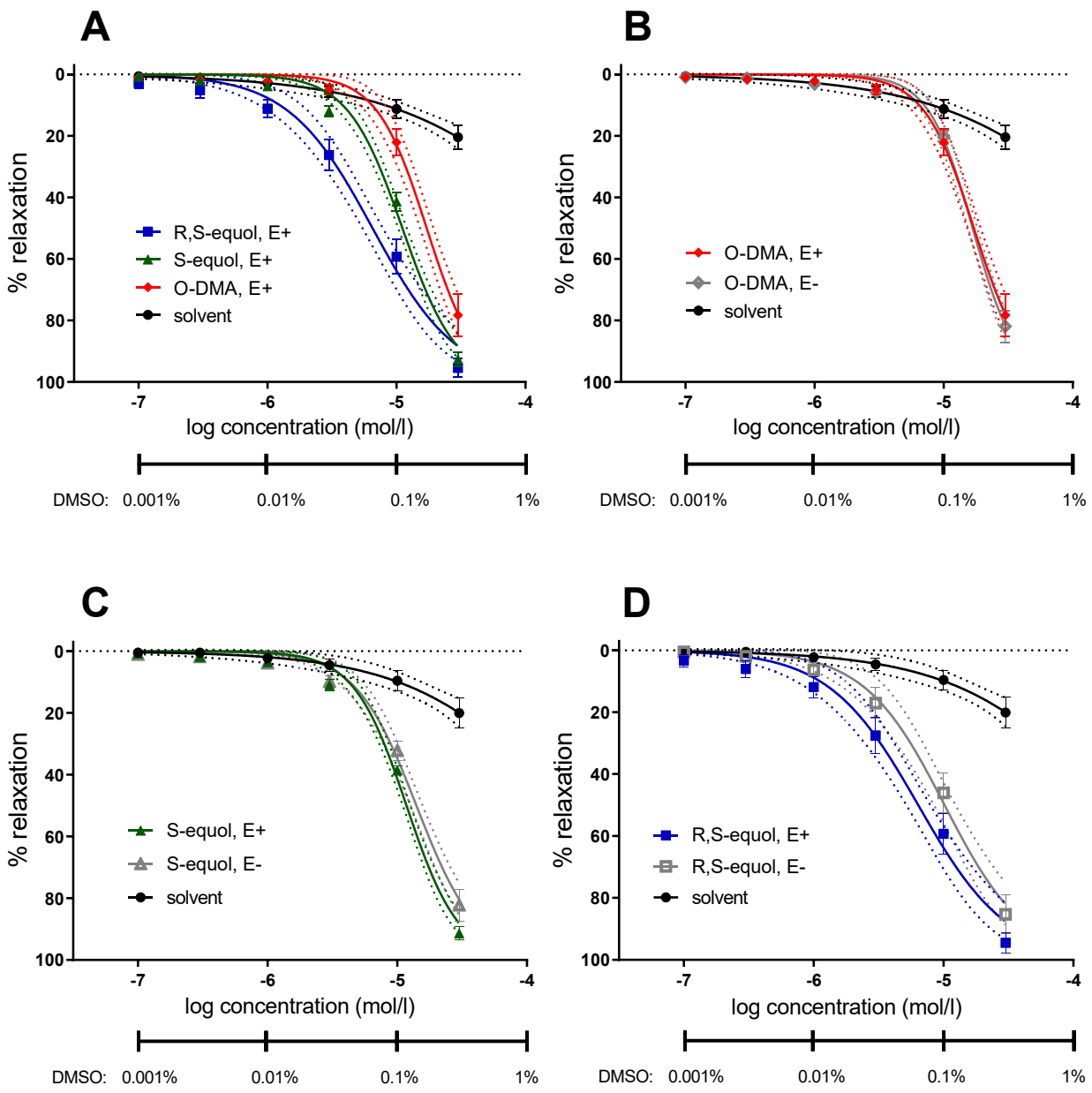
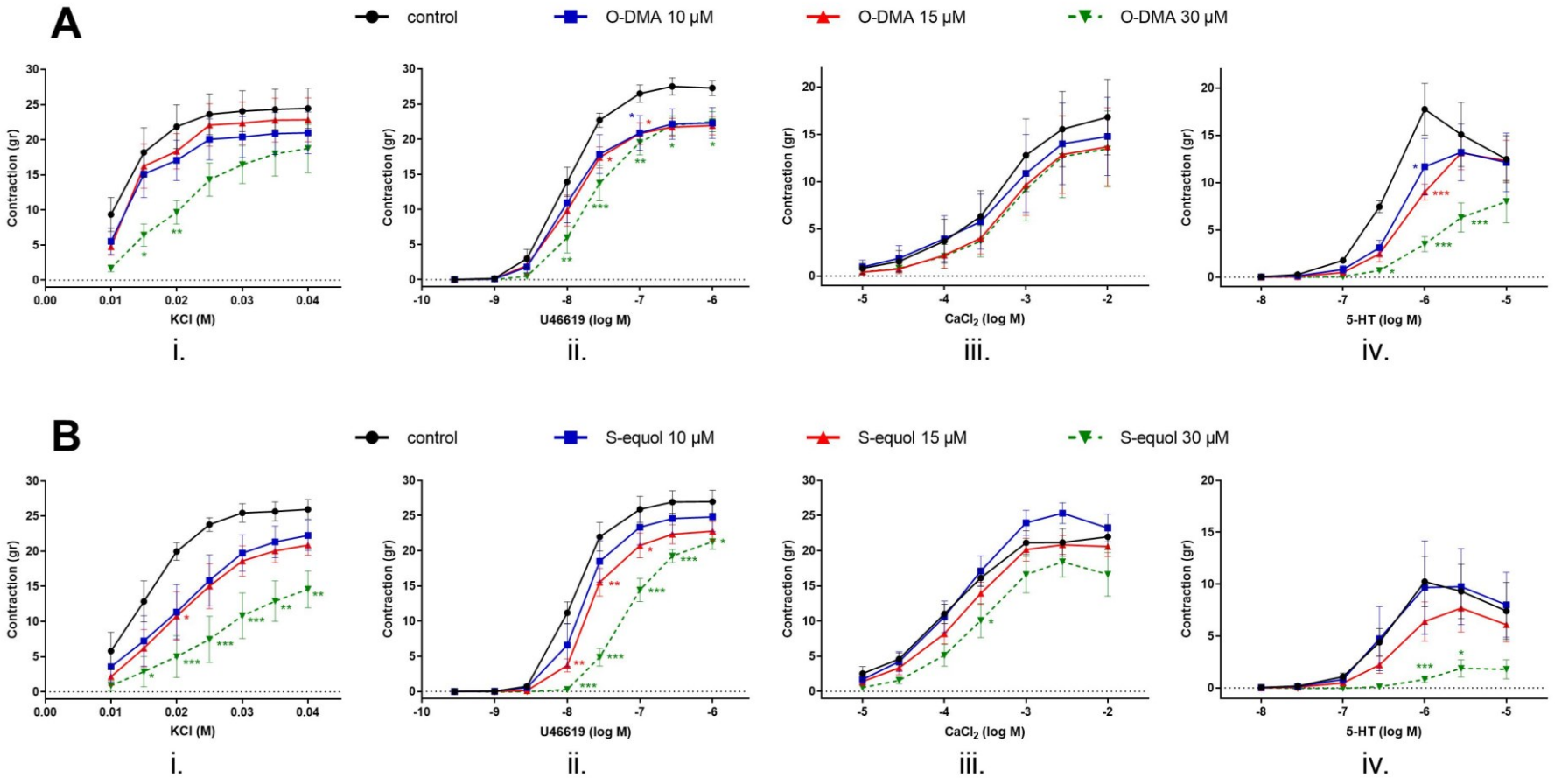


Figure 27: Concentration-response curves, with 95% confidence intervals, showing the vasorelaxant effect of all tested compounds (A) in endothelium-intact (E+) porcine ***coronary*** *artery rings and that of* ***O-desmethylangolensin*** *(B),* ***S-equol*** *(C) and* ***R,S-equol*** *(D) in endothelium-intact (E+) or -denuded (E-) porcine* ***coronary*** *artery rings, precontracted by U46619 (10^{-8} M). Relaxation is expressed as percentages of the initial basal tension of the rings, data are shown as the mean ± SEM, n=5-7. A concentration-response curve to the solvent was used as the negative control and the final concentration of DMSO in the organ bath is indicated below the x axis.*

4.2.3. *S*-equol and *R,S*-equol inhibited the contractile responses to KCl, $CaCl_2$, 5-HT and U46619

Due to the higher potency of all three active metabolites in coronary arteries and considering the similarities of porcine and human hearts (Lelovas, Kostomitsopoulos, & Xanthos, 2014), this model was also used also for the further elucidation of the mechanism of action in this case. Hence, endothelium-denuded coronary arteries pretreated with three different concentrations (ranging from 3 to 30 μM) of the vasodilators were exposed to increasing concentrations of four vasocontricting stimuli. O-DMA showed a partial inhibitory activity only concerning the receptor mediated contractions induced by U46619 and serotonin (Figure 28 A/ii, A/iv), while the effects of potassium and calcium (Figure 28 A/i, A/iii) were only minorly affected. *S*-equol and *R,S*-equol in the highest tested concentration (30 μM) managed to, at least partially, block the effect of all four vasoconstrictors (Figure 28 B, C). *R,S*-equol demonstrated such an inhibitory effect, against the constriction induced by $CaCl_2$ and serotonin, also in lower concentration (10 μM), in which *S*-equol was inactive (Figure 28 B/iii, B/iv, C/iii, C/iv). Nevertheless, the concentration-contraction curves (Figure 28) indicated a dose-dependent mode of action. These results provided once again evidence for *R,S*-equol having a higher vasodilatory potential. Therefore, a separate set of experiments was performed, trying to investigate if its activity in lower concentrations (2-4 μM) is related to cGMP or cAMP cascades. Such an assumption was found rather improbable, since *R,S*-equol did not manage to potentiate the effects of neither sodium nitroprusside nor forskolin (Figure 29).


A
control
O-DMA 10 µM
O-DMA 15 µM
O-DMA 30 µM
Contraction (gr)
KCl (M)
U46619 (log M)
CaCl2 (log M)
5-HT (log M)
i.
ii.
iii.
iv.
B
control
S-equol 10 µM
S-equol 15 µM
S-equol 30 µM
Contraction (gr)
KCl (M)
U46619 (log M)
CaCl2 (log M)
5-HT (log M)
i.
ii.
iii.
iv.

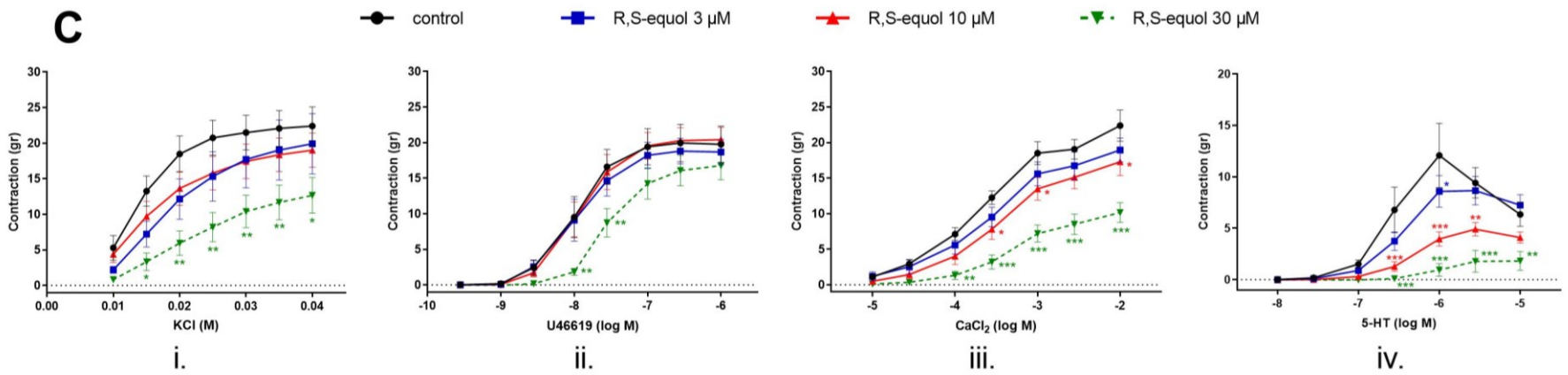


*Figure 28: Dose-dependent effects of **O-desmethylangolensin** (A), **S-equol** (B) and **R,S-equol** on the contraction of coronary arteries induced by KCl (i.), U46619 (ii.), $CaCl_2$ (iii.) and serotonin (iv.). Porcine coronary artery rings were pretreated with indicated concentrations of isoflavonoids before the addition of cumulative doses of the inducer. * $p < 0.05$, ** $p < 0.01$, *** < 0.001 vs. control, n=5-6.*

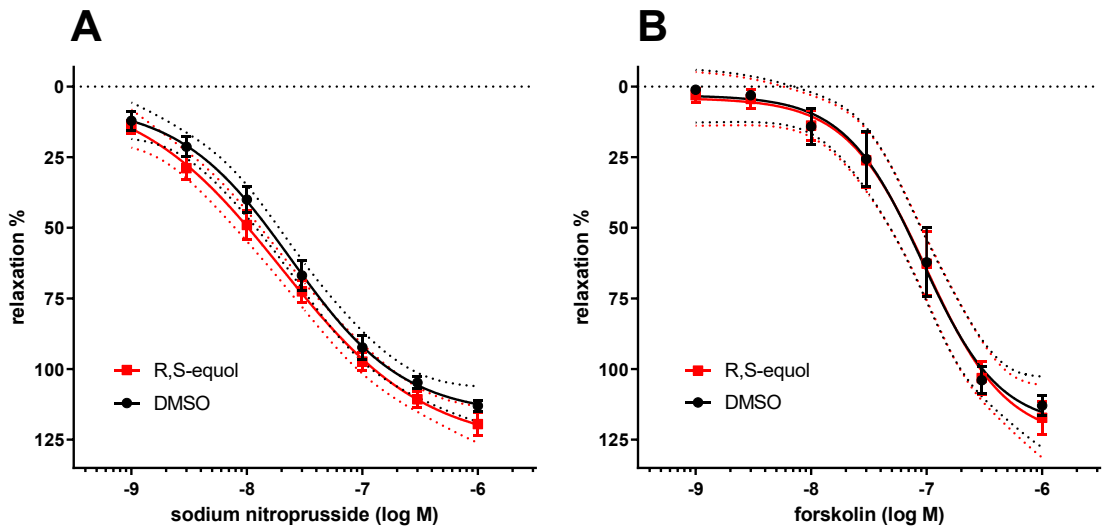


Figure 29: The effect ***R,S-equol*** *on relaxation induced by* ***sodium nitroprusside*** *(A) and* ***forskolin*** *(B). After exposure of endothelium-denuded coronary artery rings to the metabolite (2- 4 µM, able to cause 20% of relaxation), relaxation was induced by the addition of cumulative doses of either nitroprusside (A) or forskolin (B). Relaxation is expressed as percentages of the initial basal tension of the rings., n=5-7.*

4.2.4. Active metabolites relaxed mesenteric artery rings *ex vivo*

The *ex vivo* vascular effect of O-DMA, *R,S*-equol and *S*-equol was reproducible also when the compounds were tested in endothelium-denuded rat mesenteric arteries (Figure 30). However, only the activity of *S*-equol remained as high as in the case of porcine coronary arteries, having an EC_{50} of about 9 µM and a maximal effect approaching 100% relaxation. O-DMA and *R,S*-equol in this model exhibited lower potency than in coronary artery but higher than in rat aorta (EC_{50} and maximal effects shown in Table 3).

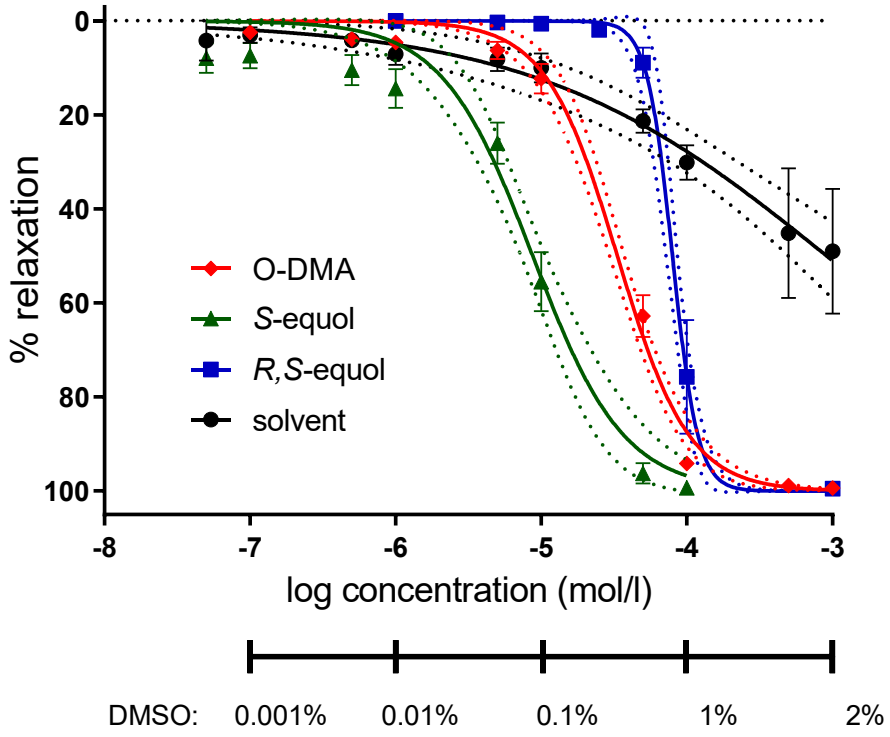


Figure 30: Concentration-response curves, with 95% confidence intervals, showing the vasorelaxant effect of ***O-desmethylangolensin****,* ***S-equol*** *and* ***R,S-equol*** *in endothelium-denuded rat* ***mesenteric*** *artery rings, precontracted by norepinephrine (10^{-5} M). Relaxation is expressed as percentages of the sodium nitroprusside-induced relaxation of the vessels, n=3-5. A concentration-response curve to the solvent was used as the negative control and the final concentration of DMSO in the organ bath is indicated below the x axis.*

Table 3: The EC_{50} and maximal effects of the active metabolites in three different artery models.

	rat aorta		rat mesenteric artery		porcine coronary artery	
	EC_{50} *(μM)*	E_{max} *(%)*[a]	EC_{50} *(μM)*	E_{max} *(%)*[a]	EC_{50} *(μM)*	E_{max} *(%)*[b]
O-desmethylangolensin	42.6 ± 5.2	81.5 ± 5.1	33.3 ± 1.9	87.4 ± 1.7	17.2 ± 1.0	98.1 ± 0.9
***S*-equol**	65.8 ± 9.6	61.9 ± 2.5	8.74 ± 0.8	96.7 ± 1.9	11.1 ± 0.4	98.9 ± 0.4
***R,S*-equol**	57.7 ± 5.8	77.6 ± 6.0	79.6 ± 3.4	75.7 ± 3.2	6.5 ± 0.6	97.4 ± 1.1

a: relaxation induced by 100 μM of the tested compound

b: hypothetical relaxation induced by 100 μM of the tested compound, as calculated by use of the GraphPad software

Data are shown as mean values ± standard errors of the means (S.E.M.), n=3-5.

4.2.5. O-desmethylangolensin reduced rat blood pressure *in vivo*

As O-DMA is the metabolite formed by majority in humans, and also it was the most potent compound in the resistance (mesenteric) artery model, it was selected for confirmation of its vasorelaxant effects under *in vivo* conditions. Indeed, such effects were verified. The intravenous administration of O-DMA dose-dependently decreased both diastolic and systolic arterial blood pressure in spontaneously hypertensive rats (Figure 31). The extent of the changes was comparable for the systolic and the diastolic blood pressures. Some effects were observable from the first dose given (0.001 $mg.kg^{-1}$) and they were significant at the dose of 1 $mg.kg^{-1}$, when compared with the solvent. The O-DMA administration did not significantly affect the heart rate (Figure 32).

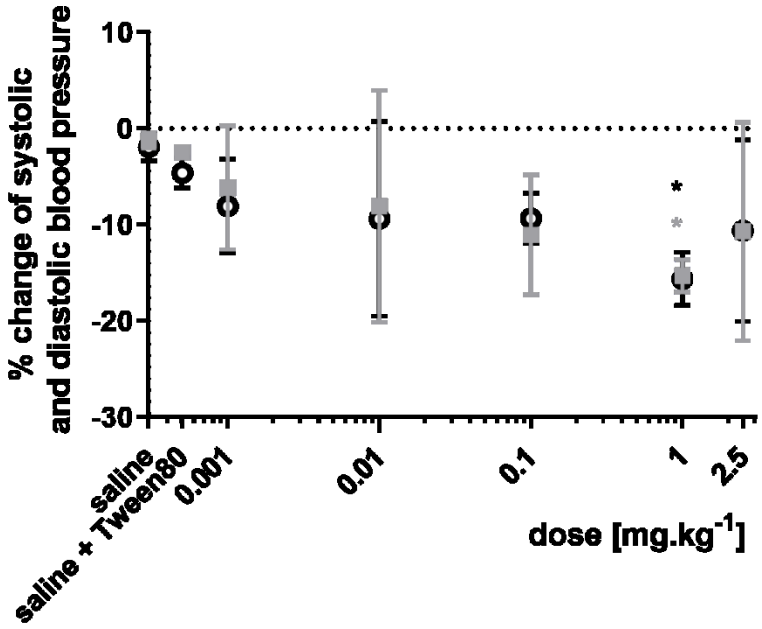


*Figure 31: Changes in the systolic (black rings) and diastolic (gray squares) arterial blood pressures in SHR rats, following successive i.v. bolus administration of O-desmethylangolensin (0.001 mg.kg^{-1} to 2.5 mg.kg^{-1}), dissolved in 10% Tween80 in saline. 0% stands for the baseline of a stable blood pressure. * indicates a difference at p<0.05 between the baseline and the corresponding values of decreased blood pressure, n=4.*

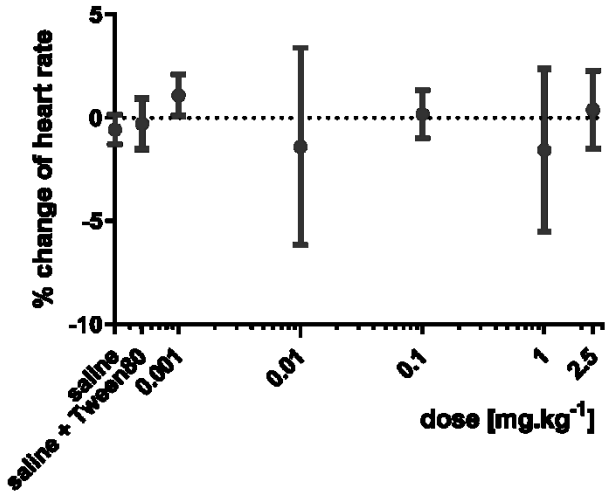


Figure 32: Heart rate in SHR rats following successive i.v. bolus administration of O-desmethylangolensin (0.001 mg.kg^{-1} to 2.5 mg.kg^{-1}), dissolved in 10% Tween80 in saline. 0% stands for the baseline of a stable heart rate, n=4.

4.3. Effects on human vascular smooth muscle cells - Biochanin A and O-desmethylangolensin blocked the activation of L-type calcium channels

As shown above, biochanin A possessed the highest vasodilatory potency, compared to other isoflavonoids, while the metabolite O-DMA affected animal vasculature both *ex vivo* and *in vivo*. Moreover, both compounds were able to relax the porcine coronary artery and the effect of biochanin A was likely mediated by inhibition of L-type channel currents. The next logical step was to test the intracellular calcium level changes. These further experiments were performed on human coronary and aortic smooth muscle primary cells, as more stable models than isolated vessels. While Bay K8644 (an L-type calcium channel agonist) increased the calcium concentration within smooth muscle cells, both biochanin A and O-DMA antagonized this effect. Specifically, biochanin A significantly blocked the calcium influx caused by all tested concentrations of Bay K8644, in both types of smooth muscle cells (moreover it introduced an initial calcium leakage, significant in AoVSMC) (Figure 33, 34). Instead, O-DMA exhibited a slightly lower but still very significant antagonizing effect on rise of intracellular calcium induced by Bay K8644, in AoVSMC (Figure 33). When comparing the effect of the two substances on AoVSMC, that of biochanin A appeared to be more potent (Figure 33).

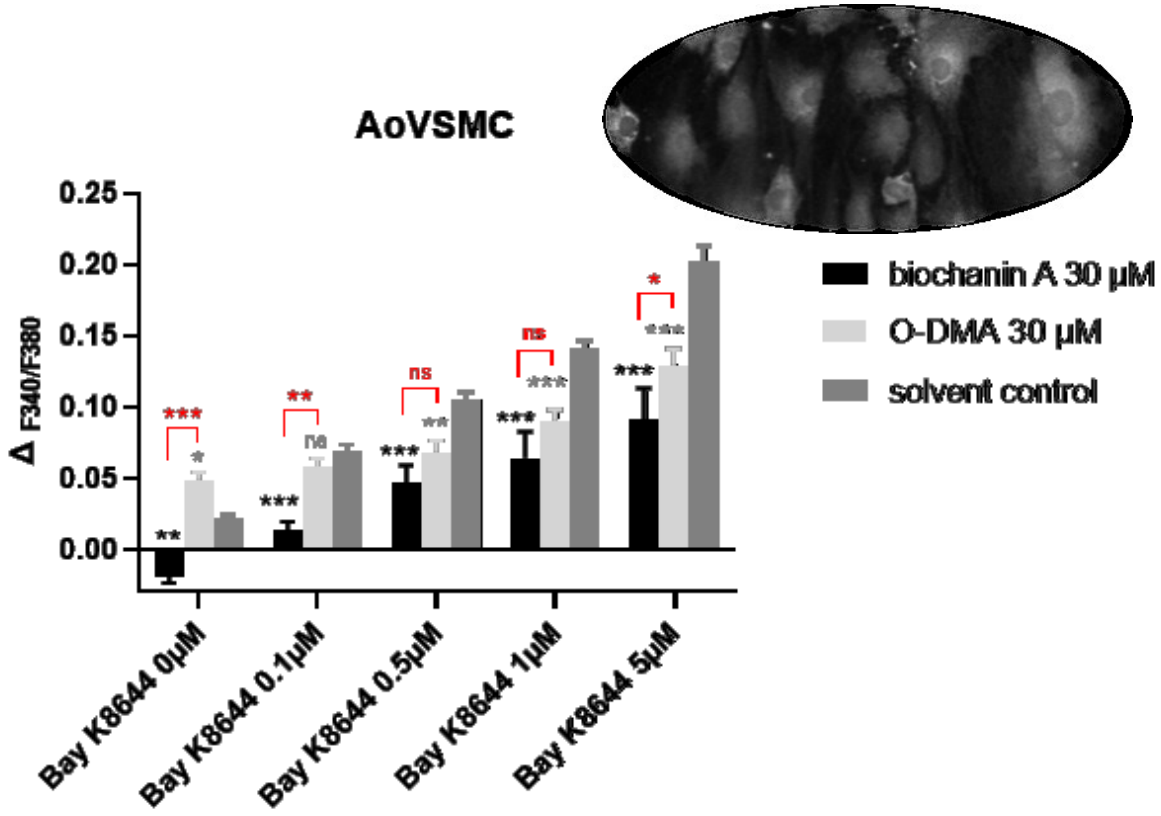


*Figure 33: Data obtained from live cell calcium imaging experiments on human aortic smooth muscle cell. Changes in the cytosolic Ca^{2+} concentration is expressed as a 340/380 nm fluorescence ratio of the emitted fluorescence signal, after the addition of Bay K8644 in different concentrations, in the presence of either the tested compound or the corresponding amount of solvent (DMSO 0.3%), as a control. P values were defined as significant by two-way ANOVA and are indicated as: n.s., not significant, * $p < 0.05$, ** $p < 0.01$, *** < 0.001. Black colour indicates the difference of the biochanin A treated cells vs. the control, gray colour O-DMA treated cells vs. the control and red colour biochananin A vs. O-DMA treated cells. Average values are given, error bars represent standard errors of the means (S. E.M.), n=3.*

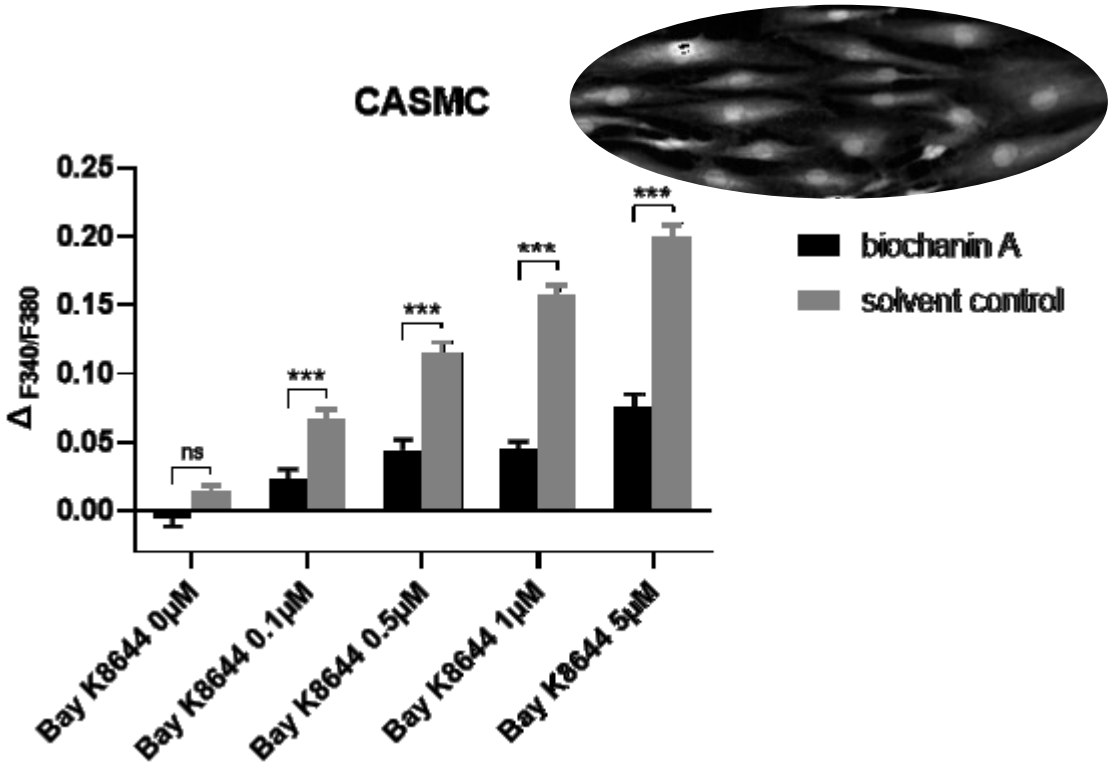


*Figure 34: Data obtained from live cell calcium imaging experiments on human coronary smooth muscle cell. Changes in the cytosolic Ca^{2+} concentration is expressed as a 340/380 nm fluorescence ratio of the emitted fluorescence signal, after the addition of Bay K8644 in different concentrations, in the presence of either the tested compound or the corresponding amount of solvent (DMSO 0.3%), as a control. P values were defined as significant by two-way ANOVA and are indicated as: n.s., not significant, * $p < 0.05$, ** $p < 0.01$, *** < 0.001, **** < 0.0001 vs. control. Average values are given, error bars represent standard errors of the means (S.E.M.), n=3.*

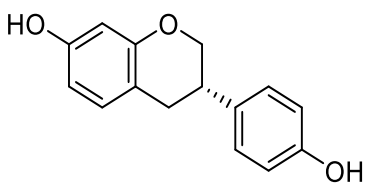
5. DISCUSSION

Isoflavonoids have always attracted scientific interest because of their potentially positive effects on human beings. Firstly, their phytoestrogenic property associated with ability to bind estrogen receptors has been for interest of both cardiovascular and non-cardiovascular diseases. Secondly, their vasorelaxant activity has been of similar interest, although studies reported that this is mostly an estrogen receptor-independent effect (Mishra et al., 2000; Wu et al., 2010). The issue is somehow controversial as there are exceptions in the literature: 1) in a case of smaller vascular beds, formononetin upregulated nitric oxide synthase in arterial endothelium through estrogen receptors and MAPK pathway (Sun et al., 2016) and 2) genistein caused relaxation of aorta from SHR rats *via* binding to membrane estrogen receptors-α with subsequent activation of a G protein-coupled, endothelial nitric oxide synthase-dependent pathway (Lin et al., 2011).

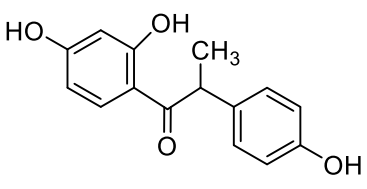
The clear limitation of the current literature was the absence of studies comparing isoflavonoid effects at the same conditions. The available studies, which tested vasoactive properties of isoflavonoids, have typically involved one, two or several isoflavonoids. Due to various experimental approaches, the comparison before we conducted this study, was at best difficult, if even possible at all. To the best of my knowledge, this data represents the first comparative investigation of the vascular effects of sixteen isoflavonoids. Some of them had never been tested before. Previous articles studied puerarin, prunetin, calycosin, formononetin, daidzein, genistein and biochanin A for their vasorelaxant properties. They showed different potencies on rat aortas, pulmonary and mesenteric arteries, rabbit basilar and coronary arteries, as well as porcine coronary arteries (Dong et al., 2004; Figtree et al., 2000; Kim et al., 2018; Lee et al., 2004; Lee & Man, 2003; T. Li et al., 2018; Mishra et al., 2000; Nevala, Korpela, & Vapaatalo, 1998; Sun, Liu, & Cao, 2011; Sun et al., 2007; Torregrosa et al., 2003; Tseng et al., 2016; Wang et al., 2006; Wang et al., 2005; Wu et al., 2010; Wu et al., 2006; Yeung et al.,

2006; Zhao et al., 2012). Small discrepancies between the aforementioned studies might derive from the use of different animal, breed or tissue models or maybe as well due to different vasoconstrictors used to pre-contract the tested arteries. In our experiments, porcine coronary arteries appeared to be more sensitive than rat aortas to both biochanin A and glycitein, but this difference was more pronounced in the case of biochanin A. Summing up all available studies, biochanin A has always been more potent than other isoflavonoids. There is only one partial exception (Figtree et al., 2000; Torregrosa et al., 2003); in the study conducted by Wu et al. (2010) formononetin reached the level of activity of biochanin A. Our findings cannot confirm such data, as the EC_{50} of formononetin appeared to be two times higher than that of biochanin A (Figure 19).

Concerning the structure-activity relationship, our results suggest that the methoxylation of 4' carbon in ring B of the isoflavonoid structure (Fig. 1) increased vasorelaxant potency, as biochanin and formononetin, which bear such modification were more efficient than genistein and daidzein, respectively, which are missing it. The role of 5-hydroxyl group in ring A was rather controversial, since genistein achieved higher potency than daidzein, but tectorigenin and prunetin were less active than the corresponding dehydroxylated structures of glycitein and isoformononetin, respectively. Nevertheless, this structure-activity relationship applied for the vasodilatory but not the other biological isoflavonoid effects, seeing that e.g. tectorigenin is a stronger antiplatelet compound than glycitein and others (Applova et al., 2017). The presence of a 3'-methoxy group in ring B did not improve the vasorelaxant effect, as can be deduced by making a comparison of calycosin with cladrin. The presence of a 6- or 7-



S-equol



O-desmethylangolensin

Figure 35: The chemical structures of the metabolites S-equol and O-DMA.

methoxy group in ring A can increase or decrease the potency, apparently depending on other substitutions. For example, in the case of tectorigenin, the methoxy group at C6 decreased the potency *vs.* biochanin A, while in the case of glycitein, the additional methoxy group at C6 increased the potency *vs.* daidzein. The probable reason for this paradoxical effect seems to be the different mechanism of action (see below). Additionally, in all cases, the presence of sugar moiety at any position of the isoflavonoid core abolished or significantly decreased the efficacy (Figure 16).

Concerning the isoflavonoid intestinal metabolites, the degradation of the polyphenolic structure to smaller phenolic compounds did not have a positive vasodilatory impact in all cases, as 4-ethylphenol and HPPA failed to induce vascular relaxation (Figure 26), on contrast to their precursor, genistein (Figure 17). However, cleavage of only the C ring, as in the case of O-DMA, was advantageous when comparing this metabolite to its precursor daidzein. Similarly, *S*-equol as well as its racemate *R,S*-equol, which are missing the C4 ketone group, showed a better vasorelaxant profile, than daidzein.

Based on the initial screening, we selected the two most active compounds, glycitein and biochanin A. Quite surprisingly, although glycitein is present in relatively high amounts in soy foods and some dairy products (Socas-Rodriguez et al., 2017), much less had been known about its vasoactive properties. In fact, it has been tested only as a part of mixed isoflavone preparations in the past (Yamaguchi et al., 2001). Data presented in this thesis showing that glycitein is a potent vasodilator, with EC_{50} which is comparable to that of biochanin A (Figure 19), were unexpected. Its effect was endothelium-independent (Figure 20, 21), but the inhibition of contraction by KCl, $CaCl_2$, serotonin and U46619 was achieved in high concentrations and was only partial (Figure 22). Due to this, we concentrated only on the more active isoflavone biochanin A in next experiments.

In the case of biochanin A, much more data showing its vasorelaxant properties are available. These include rabbit coronary and basilar arteries (Figtree et al., 2000; Torregrosa et al., 2003), as well as rat aortas (Choi et al., 2014; Wang et al., 2006; Wang et al., 2005). Biochanin A induces dose-dependent and endothelium-independent relaxation. This study confirmed these outcomes in rat aortas, and newly documented it in porcine coronary arteries as well. The endothelium-independent mode of action, is also in accordance with one study mentioning that biochanin A and formononetin are not altering neither the activity of the endothelial nitric oxide synthase nor the nitric oxide levels, in human umbilical vein endothelial cells (Simoncini et al., 2005).

Although these afore-mentioned studies confirmed the effects of biochanin A, the precise mechanism of action was unknown. Before this study was initiated, there was rather only negative evidence reporting that biochanin A did not affect the production of vasodilatory active nitric oxide or levels of prostaglandins (Choi et al., 2014; Wang et al., 2006). The only known mechanism of action was a partial effect of biochanin A on the activation of ATP-sensitive and large-conductance Ca^{2+}-activated K^+ channels (Choi et al., 2014; Wang et al., 2006; Wang et al., 2005). Since these effects cannot fully explain the ability of this compound to evoke complete or almost complete relaxation of the tested vessels, the mechanism of action was further sought. To investigate it, four different vasoconstrictive impulses were tested initially. In all these cases, biochanin A dose dependently blocked or markedly diminished their effects in endothelium-denuded coronary arteries (Figure 22). The inhibitory effects of biochanin A, as well as of daidzein and genistein, on KCl and $CaCl_2$ evoked contractions have been also previously reported in rat aortas and rabbit basilar arteries (Torregrosa et al., 2003; Wang et al., 2005).

Both $CaCl_2$ and depolarization induced by high K^+ levels lead to extracellular Ca^{2+} influx through the L-type voltage gated Ca^{2+} channels and thus result in smooth muscle

contractions (Nobe & Paul, 2001). Serotonin and thromboxane analogue U46619 cause receptor-mediated constriction through 5-HT_{2A} receptors and thromboxane A_2 receptors, respectively (Miyata et al., 2000; Nobe & Paul, 2001). Taking into consideration that the effects of both are inhibited by biochanin A, we can presume that this isoflavonoid acts rather on downstream contraction pathways than on the receptor level. Both 5-HT_{2A} and thromboxane receptors are coupled to Gq proteins and thus associated with Ca^{2+} release from the sarcoplasmic reticulum, extracellular Ca^{2+} influx and Ca^{2+} sensitization mediated by protein kinase C and Rho-kinase (Nobe & Paul, 2001). Biochanin A (1-10 μM) also managed to inhibit the contractions induced by phenylephrine, another agonist on Gq protein-coupled receptors, in rat aortas (Wang et al., 2005). Similarly, in this study, biochanin A caused the vasodilation of NE-precontracted vessels in the same experimental model (Figure 17). However, it remained unclear whether isoflavonoids have a direct effect on Ca^{2+} influx via L-type Ca channels from extracellular spaces or on Ca^{2+} release from the sarcoplasmic reticulum or both (Torregrosa et al., 2003; Wang et al., 2005). We suppose that the inhibition was rather mediated at the level of L-type channels, because these channels are involved in Gq protein-initiated vasoconstriction (Jackson, 2000, Nobe & Paul, 2001), and represent the main source for the cytoplasmatic calcium-increase. Thus their activation seems to be a common step between the ion and receptor-mediated pathways that we have investigated. For this reason, we decided to confirm this assumption experimentally for the first time. The effect of biochanin A on contractions induced by an L-type Ca^{2+} channel agonist, Bay K8644, was tested. The achieved results indicate that biochanin A potently blocks the effect of Bay K8644 and thus the influx of calcium through L-type channels into vascular smooth muscles in concentrations above 10 μM (Figure 23). Furthermore, the effects of biochanin A on calcium current via L-type channels was further investigated in primary human smooth muscle cells. Previously biochanin A effects were tested on various human cancer and umbilical vein endothelial cells (Hsu et al., 2018; Ming et al.,

2015; Spagnuolo et al., 2014) but, up to my knowledge, this was the first time that its effects were investigated on primary AoVSMC and CASMC. The aforementioned *ex vivo* ability of biochanin A to block the L-type calcium channels was confirmed in both types of human cells (Figure 33-34).

Although inhibition of calcium influx seems to be the major mechanism of action, looking at the vasorelaxant curves of biochanin A, it is apparent that this compound also has vasodilatory effects observable in lower concentrations in coronary arteries (Figure 21A).

For this reason, other mechanisms of action, which might be relevant in lower concentrations, were also considered. Indeed, low concentrations (2-4 μM) of biochanin A potentiated the relaxation induced by cumulative doses of sodium nitroprusside (Figure 24A). Moreover, biochanin A-induced relaxation was delayed in the presence of a soluble guanylyl cyclase inhibitor ODQ (Figure 25A). Such potentiating effect can be limited to coronary vasculature since it was not observed in rat aortic rings (Choi et al., 2014), and Torregrosa et al. (2003) reported that soluble guanylyl cyclase inhibitors (ODQ, NS2028) did not affect the biochanin A-induced dilation of rabbit basilar arteries. In addition to the afore-mentioned differences in vascular beds, or potentially interspecies differences, the discrepancy may also depend on the use of a high concentration of the inhibitors (10^{-5} M) by the above-mentioned researchers. Collectively, the presented data reveal that biochanin A activates the cGMP pathway in units of micromolar concentrations, while in slightly higher concentrations, it also acts as an L-type calcium channel blocker. From this point of view, it is interesting that biochanin A completely imitates the mechanism of action of estradiol (Han et al., 1995; Salom et al., 2001; White, Darkow, & Lang, 1995), confirming its phytoestrogenic effect in the vascular system. This action of biochanin A is summarized in Figure 36.

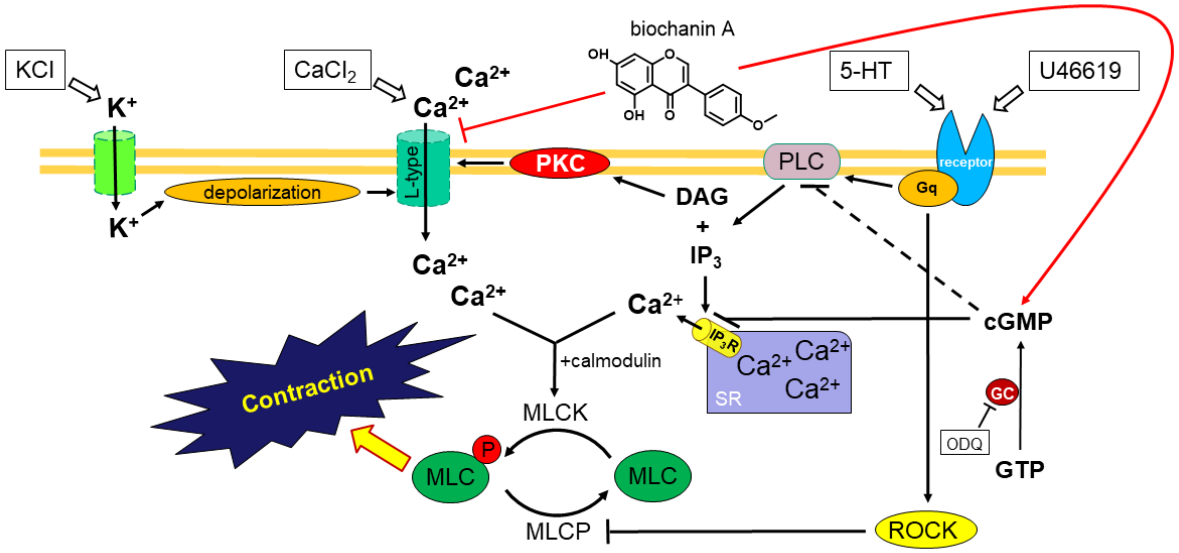


Figure 36: A scheme depicting the proposed mechanism of action of biochanin A on smooth muscle cells. cGMP= cyclic guanosine monophosphate, DAG= diacylglycerol, GC= guanylate cyclase, GTP= guanosine triphosphate, IP_3= inositol 1,4,5-triphosphate, IP_3R= inositol 1,4,5-triphosphate receptor, MLC= myosin light chain, MLCK= myosin light chain kinase, MLCP= myosin light chain phosphatase, ODQ= 1H-[1,2,4]oxadiazolo-[4, 3-a]quinoxalin-1-one (an inhibitor of soluble guanylate cyclase) P= phosphate group, PKC= protein kinase C, PLC= phospholipase C, ROCK= Rho-associated protein kinase, SR= sarcoplasmic reticulum, 5-HT= serotonin (5-hydroxytryptamine).

Interestingly, in our further experiments, two of the human microbiota metabolites of isoflavonoids, O-DMA and *S*-equol, were able to relax precontracted rat aorta in a much more potent way /O-DMA (EC_{50}= 42.6 ± 5.2 µM) and *S*-equol (EC_{50}= 65.8 ± 9.6 µM)/, than their parent isoflavone, daidzein (EC_{50}= 112.1 ± 16.8 µM). However other study reported that when the racemic mixture of equol (*R,S*-equol) was applied in carotid artery of both normotensive and hypertensive rats (EC_{50} ≈ 10 µM), its vasodilatory potency was higher than our findings in rat aorta (EC_{50}= 57.7 ± 5.8 µM), and very similar to that of daidzein (Jackman et al., 2007).

Such a large discrepancy, might derive either from the different experimental protocol followed (e.g. the different vasoconstrictors used for the initial precontraction) or from the fact that smaller arteries are more sensitive to vasotonic and vasodilatory stimuli. We also observed such an increased sensitivity, when mesenteric arteries were used for testing the isoflavonoid metabolites. This finding was, however, limited only to the case of *S*-equol (EC_{50}= 8.74 ± 0.8 μM). Considering the interspecies differences, all three tested compounds (O-DMA, *S*-equol and *R,S*-equol) appeared more active vasodilators of the porcine coronary artery as well (Table 3). As in the case of rat carotid and cerebral basilar arteries (Jackman et al., 2007; Yu et al., 2016), the mechanism of action did not depend on the presence of functional endothelium (Figure 27). This is in contrast to the findings about other intermediate metabolites, namely dihydrodaidzein, *cis/trans* 4-hydroxy-equol and dehydroequol, which are all able to relax NE-precontracted rat aorta, but in an endothelium-depended manner (Chin-Dusting et al., 2001). Such a vasodilatory action of dehydroequlol, was confirmed even in human forearm resistance arteries, *in vivo*. It produced a dose-dependent increase in forearm blood flow via a nitric oxide-dependent mechanism (Chin-Dusting et al., 2004).

There is also one study claiming that „equol causes endothelium- and nitric oxide-dependent relaxation of isolated aortic rings from rats“, but it seems that no experiments with endothelium-denuded aortas were conducted to fully confirm that hypothesis (Joy et al., 2006). Instead, the negative impact of a nitric oxide synthase inhibitor on the equol-induced relaxation was monitored, but a complete dose-response curve was not plotted and it is unclear whether *R,S*- or *S*-equol was used (Joy et al., 2006). Regardless, this is a common general source of possible confusion, as all the available vascular studies for equol concern the racemic mixture, whose biological activity might differ from that of the *S*-equol enantiomer, which is exclusively produced by the gut microbiota. Indeed, our results on mesenteric artery suggest a much higher

vasodilatory potency of *S*-equol in this model (Figure 30), while *R,S*-equol showed a better profile in counteracting vasoconstriction in coronary arteries (Figure 28 B, C).

Previous investigation of the mechanism of action showed that *R,S*-equol is related to the activation of large-conductance, voltage- and Ca^{2+}-activated K^+ (BK, also called BK_{Ca} or Maxi K) channels in rat cerebral arteries. *In vitro*, it also stimulates BK channel currents by acting on its β_1-subunit, as was documented in human embryonic cells (Yu et al., 2016). On the other hand, when human tissue was used, in particular endothelium-denuded uterine artery (sliced in rectangular fragments), neither non-specific nor voltage-gated potassium channel blockers were able to antagonize the equol-induced relaxation (Kim, Lee, & Park, 2015). As observed in human uterine artery, we showed that both *R,S*-equol and *S*-equol are able to block not only the voltage- but also the receptor-mediated contraction in coronary artery. Hence, similarly to the case of biochanin A, the mechanism of action of the colonic metabolite and its racemic mixture might implicate blockage of the calcium channels too (Kim, Lee, & Park, 2015).

Surprisingly, studies for the vascular activity of the more abundant metabolite of daidzein and of its structurally related isoflavonoid precursors, O-DMA, were missing before we have conducted this study. The vasorelaxant activity of O-DMA was confirmed in all three *ex vivo* models (Table 3) and moreover, this study brought for the first time an evidence that its effects are also relevant *in vivo* conditions, as it decreased the blood pressure in rats (Figure 31). Furthermore, the pharmacodynamic action of O-DMA was investigated in primary human smooth muscle cells. Previously O-DMA was tested solely on human cancer cells (Choi & Kim, 2013, 2014) but, up to my knowledge, this was the first time that the effect of this metabolite was investigated on primary AoVSMC. Likewise biochanin A, the O-DMA antagonized the calcium influx in AoVSMC (Figure 33).

The last crucial question, if the effects observed by us are biologically relevant, is the most complicated and not fully resolved in this study. There are only few studies reported plasma levels of biochanin A, equol and O-DMA. According to the available data, the peak plasma concentrations, after oral administration, is approximately ranging from 0.03 to 0.17 μM for biochanin A (Howes et al., 2002; Setchell et al., 2001) and from 0.5 to 1 μM for S-equol (Setchell et al., 2001; Setchell et al., 2009) and from 0.01 to 0.19 for O-DMA (Gardana, Canzi, & Simonetti, 2014). Equol is rapidly absorbed, having a much higher apparent bioavailability and slower clearance rate than the isoflavones (Setchell & Clerici, 2010b). Moreover, the biological activity of equol should be enhanced by its reduced binding to serum proteins and hence larger availability for receptor binding. Interestingly, the apparent systemic bioavailability of *R*-equol was significantly greater than that of *S*-equol (Setchell & Clerici, 2010b). This observation could have a useful clinical outcome in the future, concerning also our results indicating a higher coronary potency of the racemic mixture, probably because of the presence of R-equol.

It would be of real interest to test also directly the effect of S-equol, and possibly of the more bioavailable R-equol, in a rat model of hypertension. Here, the major obstacle represents the low solubility of equol in some biologically friendly solvent. DMSO can well dissolve S-equol but it is well known that it affects arterial blood pressure by itself (Kaneda et al., 2016). We previously resolved the problem by preparing solid dispersions of quercetin (Porcu et al., 2018). Although this solution is feasible, preparation of such dosage form is highly time and resource-consuming, and hence it was not possible to include it in my thesis experimentation. Another interesting fact is that the final biological effect of flavonoid administration might be based on a synergistic effect of more metabolites with different mechanisms of action. Also this hypothesis was confirmed for two flavonoid gastrointestinal tract-microbiota metabolites, 4-methylcatechol and 3-(3-hydroxyphenyl) propionic acid

(Najmanova, Pourova, & Mladenka, 2020), but it remains to be answered in the case of isoflavonoids and their metabolites.

6. CONCLUSIONS

It can be concluded that this study brings novel data suggesting that the effects of isoflavonoids and their metabolites on arterial blood pressure might be of clinical relevance. In general, isoflavonoids started to exhibit direct vasorelaxant activity in tens of μM, while even lower concentrations of biochanin A significantly potentiated the effect of sodium nitroprusside. Such results are of potential clinical interest, since such concentrations might be feasible in human blood circulation, after eating a special diet or being administered food supplements rich in isoflavones. This study provided for the first time evidence about the vasorelaxant action of glycitein and deeper insight into the mode of action of biochanin A, which was also for the first time investigated on human vascular smooth muscle cells.

In addition, previous studies showing that *S*-equol and O-DMA are biologically more active that their precursors, were confirmed. However, the assumptions that equol represents the main active metabolite of daidzein and that it largely contribute to the health benefits of isoflavones (Jackman, Woodman, & Sobey, 2007) was challenged by the results of this study. O-DMA was not only shown to be potent vasodilator *ex vivo*, but it was as well able to decrease blood pressure, in a biologically relevant *in vivo* rat model. Future studies are however needed to see if these effects are relevant in human and if there can be some synergistic effects between low concentrations of isoflavonoids presented in plasma and their metabolites formed by human microbiota.

7. LIST OF SCIENTIFIC CONTRIBUTION OF THE CANDIDATE

7.1. Original articles related to the topic of the dissertation

- **Migkos T.**, Pourová J., Vopršalová M., Auger C., Schini-Kerth V., Mladěnka P. Biochanin A, the Most Potent of 16 Isoflavones, Induces Relaxation of the Coronary Artery Through the Calcium Channel and cGMP-dependent Pathway. Planta Medica, 2020, vol. 86, s. 708-716. ISSN 0032-0943. **(IF= 3.356 , Q2)**

7.2. Original articles unrelated to the topic of the dissertation

- Catapano M., Tvrdý V., Karlíčková J., **Migkos T.**, Valentová K., Křen V., Mladěnka P. The stoichiometry of isoquercitrin complex with iron or copper is highly dependent on experimental conditions. Nutrients, 2017, vol. 9. ISSN 2072-6643. **(IF= 4.196 , Q1)**
- Porcu E., Cossu M., Rassu G., Giunchedi P., Cerri G., Pourová J., Najmanová I., **Migkos T.**, Pilařová V., Nováková L., Mladěnka P., Gavini E. Aqueous injection of quercetin: An approach for confirmation of its direct in vivo cardiovascular effects. International Journal of Pharmaceutics, 2018, vol. 541, s. 224-233. ISSN 0378-5173. **(IF= 4.213 , Q1)**
- Pourová J., Najmanová I., Vopršalová M., **Migkos T.**, Pilařová V., Applová L., Nováková L., Mladěnka P. Two flavonoid metabolites, 3,4-dihydroxyphenylacetic acid and 4-methylcatechol, relax arteries ex vivo and decrease blood pressure in vivo. Vascular Pharmacology, 2018, vol. 111, s. 36-43. ISSN 1537-1891. **(IF= 3.330 , Q2)**
- **Migkos T.**, Applová L., Horký P., Tvrdý V., Karlíčková J., Macáková K., Hrubša M., Catapano M., Tomanek M., Pour M., Mladěnka P. The influence of microbial

isoflavonoid specific metabolites on platelets and transition metals iron and copper. Phytomedicine, 2019, vol. 62. ISSN 0944-7113. **(IF= 4.268 , Q1)**

- Pourová J., Applová L., Macáková K., Vopršalová M., **Migkos T.**, Bentanachs R., Biedermann D., Petrásková L., Tvrdý V., Hrubša M., Karlíčková J., Křen V., Valentová K., Mladěnka P. The effect of silymarin flavonolignans and their sulfated conjugates on platelet aggregation and blood vessels ex vivo. Nutrients, 2019, vol. 11. ISSN 2072-6643. **(IF= 4.546 , Q1)**

7.3. Oral presentations related to the topic of the dissertation

- **Migkos T.**, Pourová J., Vopršalová M., Horký P., Najmanová I., Pour M., Mladěnka, P. Comparison of *in vitro* vascular effects of isoflavonoids and their metabolites formed by human microflora. 7th Postgradual and 5th Postdoctoral Scientific Conference, 07-08 February 2017, Hradec Králové, Czech Republic.
- **Migkos T.**, Pourová J., Vopršalová M., Horký P., Najmanová I., Pour M., Mladěnka, P. Intergender differences in the vasoactive effect of selected isoflavonoids and the their colonic metabolites. 8th Postgradual and 6th Postdoctoral Scientific Conference, 24-25 January 2018, Hradec Králové, Czech Republic.
- **Migkos, T.**, Pourová, J., Vopršalová, M., Auger, C., Schini-Kerth, V., Mladěnka, P. Biochanin A and glycitein induce an endothelium-independent dilation of rat aorta and porcine coronary artery. 9th Postgradual and 7th Postdoctoral Scientific Conference, 23-24 January 2019, Hradec Králové, Czech Republic.

7.4. Poster presentations related to the topic of the dissertation

- **Migkos T.**, Najmanová I., Vopršalová M., Pourová J., Horký P., Pour M., Mladěnka, P. Comparison of *in vitro* vascular effects of isoflavonoids and their metabolites formed

by human microflora. 11th World Congress on Polyphenols Applications, 20-21 June 2017, Vienna, Austria.

- **Migkos T.**, Najmanová I., Vopršalová M., Pourová J., Horký P., Pour M., Mladěnka, P. Can the vasodilatory effect of isoflavonoids be related to their gut microfloral metabolites? 67th Czech-Slovak Pharmacological Days, 2-4 October 2017, Stará Lesná, Slovakia.
- **Migkos, T.**, Pourová, J., Vopršalová, M., Auger, C., Schini-Kerth, V., Mladěnka, P. Isoflavonoids have a direct effect on coronary smooth muscles. 68th Czech-Slovak Pharmacological Days, 5-7 September 2018, Hradec Králové, Czech Republic.
- **Migkos, T.**, Pourová, J., Vopršalová, M., Auger, C., Schini-Kerth, V., Mladěnka, P. Several isoflavonoids induce endothelium-independent relaxations and prevent contractile responses of porcine coronary artery rings. 12th World Congress on Polyphenols Applications, 25-28 September 2018, Bonn, Germany.
- **Migkos, T.**, Pourová, J., Vopršalová, M., Auger, C., Schini-Kerth, V., Mladěnka, P. Biochanin A relaxes porcine coronary arteries by blocking L-type calcium channels. 2019 XXIII ISHR (International Society for Heart Research) World Congress, 3-6 June 2019, Beijing, People's Republic of China.

7.5. Grant projects

Principal investigator

- Grant Agency of Charles University; 2017-2019; Grant number: 1080217/C/2017; Title of project: Flavonoid metabolites, their interaction with transition metals and pharmacokinetics. – evaluated as exceptionally good by the grant committee after termination of the project

- A travel grant from European Union ERASMUS+ programme; 2018; Application number: 2698000
- Rector's Mobility Fund; 2019; Grant number: FM/c/2019-1-002

Co-investigator

- Grant Agency of Charles University; 2017-2019; Grant number: 1134217/C/2017; Title of project: Phytochemical analysis and biological activity of the algae *Haematococcus pluvialis*.

7.6. Scientific experience abroad

- 5-month internship in the INSERM UMR 1260 laboratory, Faculté de Pharmacie, Université de Strasbourg, Illkirch, France, 2018.
- 4-month internship in the Bosch Institute, Medical Foundation, Faculty of Medicine and Health, University of Sydney, Sydney, Australia, 2019/20.

7.7. Theses in which the candidate was a consultant

- Author: Diana Katiová

  Title: Štúdium mechanizmu vazorelaxačného účinku biochanínu A *ex vivo* na izolovanej aorte potkana, (Mechanism of the vasorelaxant effect of biochanin A studied *ex vivo* on isolated rat aorta). Successfully defended in September 2020.

7.8. Courses attended during Ph.D. studies

- Training course for acquiring certificate of professional competence to design experiments and experimental projects. 1st Faculty of Medicine, Charles University, 4-8 March 2019, Prague, Czech Republic. Certificate obtained.

- TOX-OER project, Intense Learners Course Cardiopulmonary toxicity, 9-14 May 2017, Hradec Králové, Czech Republic
- TOX-OER project, Intense Learners Meeting Pharmaco/Toxicokinetics, 23-29 October 2017, Porto, Portugal.

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9. ANNEX

Biochanin A, the Most Potent of 16 Isoflavones, Induces Relaxation of the Coronary Artery Through the Calcium Channel and cGMP-dependent Pathway.

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Candidate's contribution:

Design of the work; data collection, analysis and interpretation; statistical analysis; drafting the manuscript; critical revision of the manuscript.