Phytomedicine Plus 1 (2021) 100078

Contents lists available at ScienceDirect

Phytomedicine Plus

journal homepage: www.elsevier.com/locate/phyplu

20-hydroxyecdysone dilates muscle arterioles in a nitric oxide-dependent, estrogen ER-β receptor-independent manner

Nelson N Orie[a], Asmaa Raees[a], Maneera Y. Aljaber[a], Nada Mohamed-Ali[b], Halima Bensmail[c], Mostafa M. Hamza[c], Nasser Al-Ansari[a], Alka Beotra[a], Vidya Mohamed-Ali[a,b], Mohammed Almaadheed[a,b,*]

[a] Anti-Doping Laboratory Qatar, Sports City Road, P.O. Box 27775, Doha, Qatar
[b] Centre for Metabolism and Inflammation, Division of Medicine, Royal Free Campus, University College London, Rowland Hill Street, London, NW3 2PF, UK
[c] Qatar Computing Research Institute, Hamad bin Khalifa University, P.O. Box 5825, Doha, Qatar

A R T I C L E I N F O

Keywords:
20-hydroxyecdysone
Vasodilation
Muscle arterioles
Nitric oxide synthase 3
Estrogen receptor ER-β

A B S T R A C T

Background: 20-hydroxyecdysone is an ecdysteroid which is abundant in plants and insects and has anabolic potentials in mammals. It was recently shown to have affinity for estrogen ER-β receptor, which could potentially make it vasodilatory. Yet this possibility has not been previously investigated. Such an activity in muscle arterioles could have huge implications for muscle blood flow and performance.

Hypothesis/Purpose: We hypothesized that 20-hydroxyecdysone would dilate muscle arterioles by activating estrogen ER-β receptors. To test this, we investigated its vasodilatory properties in ovine muscle arterioles and further explored the mechanisms in human tissues and cells.

Study Design: The study was carried out experimentally, employing functional recording of arteriolar reactivity in intact ovine muscle arterioles and gene and protein expression analysis in human tissues and cells.

Methods: Direct effects of the compound on arteriolar tone were assessed by wire myography in abdominal muscle and mesenteric arterioles isolated from samples obtained from male sheep. The roles of endothelial nitric oxide synthase (NOS3), cyclooxygenase (COX) and estrogen ER-β receptor (ER-β), and their effects were determined with specific blockers. The NOS3 mRNA and protein expressions were analyzed in human coronary artery endothelial cells (HCAECs) and humanized liver of uPA+/+-SCID mice, before and after 20-hydroxyecdysone administration.

Results: Comparable dose-dependent relaxations were recorded for 20-hydroxyecdysone in both muscle and mesenteric arterioles with maximum relaxations of 46.94 ± 5.84% and 56.88 ± 7.04% respectively, which were not statistically different. Similar relaxation was recorded for β-estradiol in both arterioles. NOS inhibition with 100 μM L-NAME attenuated the relaxation to 20-hydroxyecdysone ($p < 0.001$) and β-estradiol ($p < 0.001$) in muscle arterioles. Neither COX inhibition with 10 μM indomethacin nor ER blockade with 1 μM PHTPP or 1 μM ICI182780 had any noticeable effect on 20-hydroxyecdysone relaxation in these arterioles. Transcriptome analysis revealed elevated NOS3 gene in the humanized liver of 20-hydroxyecdysone-treated mice, and, elevation of both NOS3 mRNA and protein in HCAECs treated with 20-hydroxyecdysone.

Conclusion: The data suggest that 20-hydroxyecdysone has a nitric oxide-dependent, but ERβ-independent, vasodilatory property in muscle arterioles. The benefits to muscle blood flow would however be dependent on the impact of its effects on other vascular beds.

Abbreviations. NOS3 (eNOS), endothelium nitric oxide synthase; ER, estrogen receptor; uPA, urokinase; SCID, severe combined immunodeficiency; PCR, polymerase chain reaction; qPCR, real time PCR; HCAEC, human coronary artery endothelial cell; mRNA, messenger ribonucleic acid; VEGFA, vascular endothelial growth factor A; VEGFR2, vascular endothelial growth factor receptor2; PBS, phosphate buffered saline; GTP, Guanosine-5′-triphosphate; cGMP, cyclic guanosine monophosphate; sGC, soluble guanylyl cyclase; PKG, protein kinase G or cGMP-dependent protein kinase

* Corresponding author.

https://doi.org/10.1016/j.phyplu.2021.100078
Received 21 March 2021; Received in revised form 29 April 2021; Accepted 30 May 2021
2667-0313/© 2021 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

1. Introduction

Ecdysteroids are polyhydroxylated ketosteroids that are abundant in plants as phytoecdysteroids (Dinan,1995, 2001; Schmelz et al.,2000) and serve as molting hormones in insects (Gorelick-Feldman et al., 2010; Slama and Lafont, 1995). Our knowledge of their role in the latter has in fact been enriched by their availability from plant sources (Dinan, 2001). Interests in these compounds have grown in recent years because of reported anabolic and favorable metabolic potentials in mammals, including humans (Dinan and Lafont, 2006; Gorelick-Feldman et al., 2008; Syrov and Kurmukov, 1976; Syrov, 2000; Tóth, 2008), which have made them attractive to fitness enthusiasts and athletes. One other selling point for these compounds is the absence of concomitant androgenic effects (Zwetsloot et al., 2014), consistent with their lack of binding affinity for the androgen receptor (Gorelick-Feldman et al., 2008). For these reasons, supplements containing these compounds are especially popular among recreational and elite athletes (Syrov, 2000) who crave to improve or maintain their fitness levels. The most studied of the ecdysteroids is 20-hydroxyecdysone, which has anabolic effects often at high doses (Todorov et al., 2000; Parr et al., 2015; Isenmann et al., 2019) and was added to the world anti-doping agency monitoring programme (World Anti-Doping Agency, 2020) in 2020.

The muscle's ability to maintain adequate blood flow during physical activity is essential to its performance. This ability depends on both cardiac output and local vascular resistance. The latter is decreased by vasodilation, which is enhanced by exercise training in humans (Green et al., 1994; Hickner et al., 1997) and animals (Delp, 1998; Lash and Bohlen, 1997) and can be modified by drugs to increase muscle blood flow.

Although the mechanisms of its anabolic effects in mammals are unclear, 20-hydroxyecdysone has been reported to bind to estrogen receptor beta (ER-β) at concentrations as little as 10 nM (Parr et al., 2014). Since estrogen receptors are vasodilatory and widely distributed in the vasculature, 20-hydroxyecdysone, acting on these receptors would potentially cause vasodilation and increase blood flow. Such an effect on skeletal muscle circulation could have huge implications for athletic performance, especially in endurance sports. To our knowledge, the direct effect of this widely consumed, supplement-derived compound on muscle circulation has not been reported. Here we describe both its direct effect on muscle vasculature and the mechanism of such effect.

2. Materials and methods

2.1. Reagent and chemicals

20-Hydroxyecdysone, Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), indomethacin, Noradrenaline hydrochloride, RIPA buffer, Isopropanol, and chloroform were obtained from Sigma Aldrich (Darmstadt, Germany). 17β-estradiol, ICI 182, 780 and PHTPP were from Tocris Bioscience (UK). Ethanol was from Ciba-Chem (Basel, Switzerland), Phosphate Buffered Saline (PBS) from Gibco (Waltham, Massachusetts, United States), and DEPC water and TRIzol reagent were from Ambion (Austin, Texas, United States). GeneChip WT PLUS Reagent Kit & High-Capacity cDNA Reverse Transcription kit were from Applied Biosystems (Foster City, California, United States). β-actin Primer Assays and ABI SYBR Green master mix were all obtained from Qiagen (Hilden, Germany). Human Coronary Artery Endothelial Cells (HCAECs) and Endothelial Cell Growth Media and supplement pack were obtained from Promocell (Heidelberg, Germany) while attachment factor was obtained from Gibco. SeeBlue plus2 protein standard marker was obtained from Invitrogen, mouse monoclonal anti-eNOS antibody (M221) was from Abcam and eNOS QuantiTect primer assay was obtained from Qiagen.

Stock solutions were prepared in water (Noradrenaline and L-NAME), ethanol (20-hydroxyecdysone and PHTPP) or DMSO (β-estradiol, ICI 182,780 and Indomethacin) and diluted in physiological salt solution, with final solvent concentration not exceeding 0.1 %. Under these conditions, the solvents had no discernible effects on responses.

2.2. Animal studies

uPA (+/+)-SCID mice, which were transplanted with primary human hepatocytes (chimeric mice) as previously described (Mercer et al., 2001) at KMT Hepatech lnc. (Edmonton, Canada) were used for in vivo experiments. All experiments with these animals were carried out at KMT Hepatech lnc. as per approval of the National Laboratory Ethics Committee (ECD06/09). KMT Hepatech is a production facility of PhoenixBio Group, which utilizes its proprietary platform technology of small animal model of chimeric mouse with highly humanized liver (>70% of the liver replaced with human hepatocytes), to provide services (https://kmthepatech.com/). Prior to administration of 20-hydroxyecdysone, human albumin concentration in each mouse plasma was determined and those with elevated human albumin, as a marker of successful human hepatocyte transplantation, were treated with daily doses of either 20-hydroxyecdysone (0.2 mg, n = 4) or vehicle (5% ethanol/PBS, n = 4) as previously described (Kraiem et al., 2021). 20-hydroxyecdysone was administered by oral gavage on days 0, 1 and 2 of the experiment. The animals were sacrificed on day 4 by Schedule 1 protocol and their livers harvested at the facilities of KMT Hepatech, flash frozen and shipped to the lab in Qatar for analysis.

2.3. Assessment of muscle and mesenteric arteriolar reactivity by wire myography

2.3.1. Vessel preparation

Arterioles were isolated from samples of abdominal muscle and mesentery obtained from male sheep, which were euthanized at a government-licensed abattoir in Doha, and therefore no further ethics approval was required for tissue collection (Bashraheel, et al., 2019). The samples were quickly collected after euthanasia and transported in normal physiological salt solution (PSS) to the laboratory. The PSS contained (in mM): NaCl 112, KCl 5, CaCl2 1.8, MgCl2 1, NaHCO3 25, KH2PO3 0.5, NaH2PO3 0.5, and glucose 10 and gassed with 95% O2/5% CO2 to pH 7.4. The arterioles were cut into segments (2 mm long) and mounted on wires in isometric myographs (510A JP Trading, Denmark) filled with PSS. The segments were pre-tensioned to an equivalent of 100 mmHg and continuously aerated at 37°C. The normalized luminal diameter of each segment was obtained automatically according to manufacturer's instructions and as described previously (Bashraheel, et al., 2019; Orie et al., 2006), and averaged 247.92 ± 10.21 μm (n = 42) in muscle arterioles and 325.71 ± 12.45 μm (n = 22) in mesenteric arterioles. An equilibration period of at least 1 h was allowed during which the segments were contracted with potassium chloride (KCl, 90 mM) to test for viability and noradrenaline (NA, 10-100 μM) to optimize tissue response.

2.3.2. Experimental protocol

Following equilibration period, the arterioles were precontracted with 10 μM noradrenaline, which produced 75-80 % of maximum contraction in both arterioles, to build a stable tone. Thereafter, graded concentrations of 20-hydroxyecdysone or β-estradiol were added cumulatively starting from the least concentration and changes in tone recorded. To determine whether nitric oxide synthase (NOS), cyclooxygenase (COX) or estrogen receptor-beta (ER-β) played any roles in the effects of these compounds, experiments were repeated in the presence of Nω-Nitro-L-arginine methyl ester (L-NAME, 100 μM, NOS inhibitor), indomethacin (10 μM, COX inhibitor), PHTPP (1 μM, ER-β blocker) or ICI 182, 780 (1 μM, ER blocker). The enzyme inhibitors and receptor blockers were added at least 30 mins before the addition of noradrenaline. Changes in tone were recorded with Power Lab 4/26 data ac-

quisition system and LabChart software (DMT-ADinstruments) and were expressed as the percentage of the initial tone due to noradrenaline.

2.4. Gene expression analysis

2.4.1. RNA extraction and cDNA synthesis

The liver tissues were ground to fine powder with the help of liquid nitrogen and TRIzol reagent (Invitrogen) added to disrupt the cells and ensure complete lysis. RNA was then precipitated with isopropanol and washed with 70% ethanol. RNA quality was checked using two methods; NanoDrop (Thermo Scientific) and Bioanalyzer (Agilent) and RNA Integrity Number (RIN) of greater than 6.0 was considered acceptable. cDNA was synthesized using first strand cDNA synthesis kit (Qiagen) and later used to confirm the chimeric phenotype by real time PCR expression of human ß-actin.

2.4.2. Transcriptome analysis

Whole Transcriptome expression analysis was carried out on the liver tissue samples using GeneChip WT PLUS Reagent Kit (Thermo Fisher). 100 ng RNA was used to synthesize the first and second strand cDNA respectively. This was followed by overnight in vitro transcription for the synthesis and amplification of cRNA (complimentary RNA). cRNA was then purified using magnetic beads and quantified using the NanoDrop. 15 μg of purified cRNA was used as input for the synthesis of 2nd cycle ss-cDNA (single stranded complimentary DNA) followed by RNA hydrolysis and removal by RNase H enzyme. The ss-cDNA was then purified and quantified using the NanoDrop and 5 μg was used as input for the fragmentation and the terminal labelling reactions. Finally, the labelled fragments of the ss-cDNA were hybridized to the human transcriptome array (Clariom^{TM} S Assay HT- Thermo Fisher) as per manufacturer instructions on the GeneTitan instrument (Thermo Fisher) for the automated staining and washing processes.

2.4.3. Real time PCR (qPCR) analysis

qPCR analysis was performed on Human Coronary Artery Endothelial Cells (HCAECs) to validate the effect of 20-hydroxyecdysone on the expression of NOS3. Cells were starved for 24 hours and then treated with different concentrations of 20-hydroxyecdysone (0.1nm, 1nm, 10nm, 100nm and 1μm) for 24 hours. RNA was extracted, and reverse transcribed to cDNA and used for detection of NOS3 using RT2 First Strand Kit (Qiagen) in accordance with manufacturer recommendations.

2.4.4. NOS3 protein expression analysis

NOS3 protein expression analysis was performed on Human Coronary Artery Endothelial Cells (HCAECs) for the detection of NOS3 protein. Cells were starved for 24 hours and then treated with different concentrations of 20-hydroxyecdysone (0.1nm, 1nm, 10nm, 100nm and 1μm) for 24 hours. Thereafter, cells were lysed in RIPA buffer (Sigma) and total protein extracted and analyzed by western blot for NOS3 protein expression using anti-NOS3 antibody (Abcam [M221] ab76198). The amount of NOS3 protein was normalized to β-tubulin in the same sample.

2.5. Statistical Analysis

Data generated with myographs are expressed as mean ± SEM and were analyzed using GraphPad prism (version 6.02, GraphPad Inc. La Jolla, Ca. USA) software. Agonist concentration-response curves were constructed on log scale and data fitted using the sigmoidal fitting routine. Significant effects were determined using 1-way or 2-way ANOVA with Bonferroni test. Significance was defined as $p<0.05$. and "n" = number of samples or experiments.

Transcriptome data were analyzed using TAC 4.0 software comparing the 20-hydroxyecdysone treated group with vehicle treated group. Expression Analysis Settings used were Gene-Level Fold Change < -1 or > 1, $p < 0.05$, ANOVA Method. QPCR data were analyzed by comparative CT method and expressed as 2Λ-CT.

3. Results

3.1. 20-hydroxyecdysone-induced relaxation in muscle and mesenteric arterioles

20-hydroxyecdysone induced dose-dependent relaxation in both muscle and mesenteric arterioles with no significant difference between the curves (Fig. 1A). The maximum relaxation recorded in both arterioles were 46.94±5.84% and 56.88±7.04% respectively, but the difference was not statistically significant. A typical recording of 20-hydroxyecdysone-induced relaxation in the muscle arterioles is shown in Fig. 1B.

3.2. Role of NOS in 20-hydroxyecdysone-induced relaxation in muscle arterioles

To determine whether nitric oxide or prostanoids played any part in the relaxation induced by 20-hydroxyecdysone in these arterioles, experiments were repeated in the presence of the NOS inhibitor, L-NAME (100 μM) or COX inhibitor, indomethacin (10 μM). The relaxation was significantly reduced by L-NAME (Fig. 2A, $p < 0{,}001$ between the curves, 2-Way ANOVA, n = 12) but not by indomethacin (Fig. 2B, n = 5).

3.3. Role of estrogen receptor-beta (ER-β) in 20-hydroxyecdysone-induced relaxation in muscle arterioles

Since 20-hydroxyecdysone is reported to have an affinity for estrogen receptor-β (ER-β) (Parr et al., 2014) and it is the subtype most abundantly expressed in the vasculature (Gustafsson, 2000), its role in the vasodilation induced by 20-hydroxyecdysone was investigated. The presence of neither the selective ER-β blocker, PHTPP (1 μM, Fig. 3A) nor the more general ER-α/β blocker, ICI 182, 780 (1 μM, Fig. 3B) had any effect on 20-hydroxyecdysone -induced relaxation in these arterioles.

3.4. β-estradiol-induced relaxation in both muscle and mesenteric arterioles

β-estradiol like 20-hydroxyecdysone induced relaxation in both muscle and mesenteric arterioles (Figure 4A). Similarly, the presence of L-NAME (100 μM, NOS inhibitor) significantly attenuated the relaxation, with the curve significantly shifted to the right ($p<0.01$, 2-Way ANOVA, Figure 4B).

3.5. 20-hydroxyecdysone-induced changes in hepatic transcriptome

Transcriptome analysis of the humanized liver tissues revealed several differentially expressed genes. To interpret the data, pathway analysis (PA) or functional enrichment analysis, was done. PA allows for easier detection of related genes that may be differentially expressed in the treatment group, when compared to the control group (García-Campos et al., 2015). Initial PA found significant hits in the Vascular endothelial growth factor A - Vascular endothelial growth factor receptor2 (VEGFA-VEGFR2) signaling pathway. Of these, 34 (0.17%) passed filtering criteria, with 12 (35%) of these up-regulated and 22 (65%) down-regulated in the 20-hydroxyecdysone-treated group, compared with the control (Fig. 5A and B). Several genes involved in endothelial cell survival, migration and angiogenesis were flagged (Fig. 5C). Notably, NOS3 was significantly upregulated in this pathway in the 20-hydroxyecdysone -treated group (Fig. 5C $p = 0.04$).

3.6. Validation of 20-hydroxyecdysone-induced NOS expression in Human Coronary Artery Endothelial Cells (HCAECs)

To validate the effect of 20-hydroxyecdysone on NOS3 expression seen in the transcriptome analysis, qPCR of NOS3 mRNA was

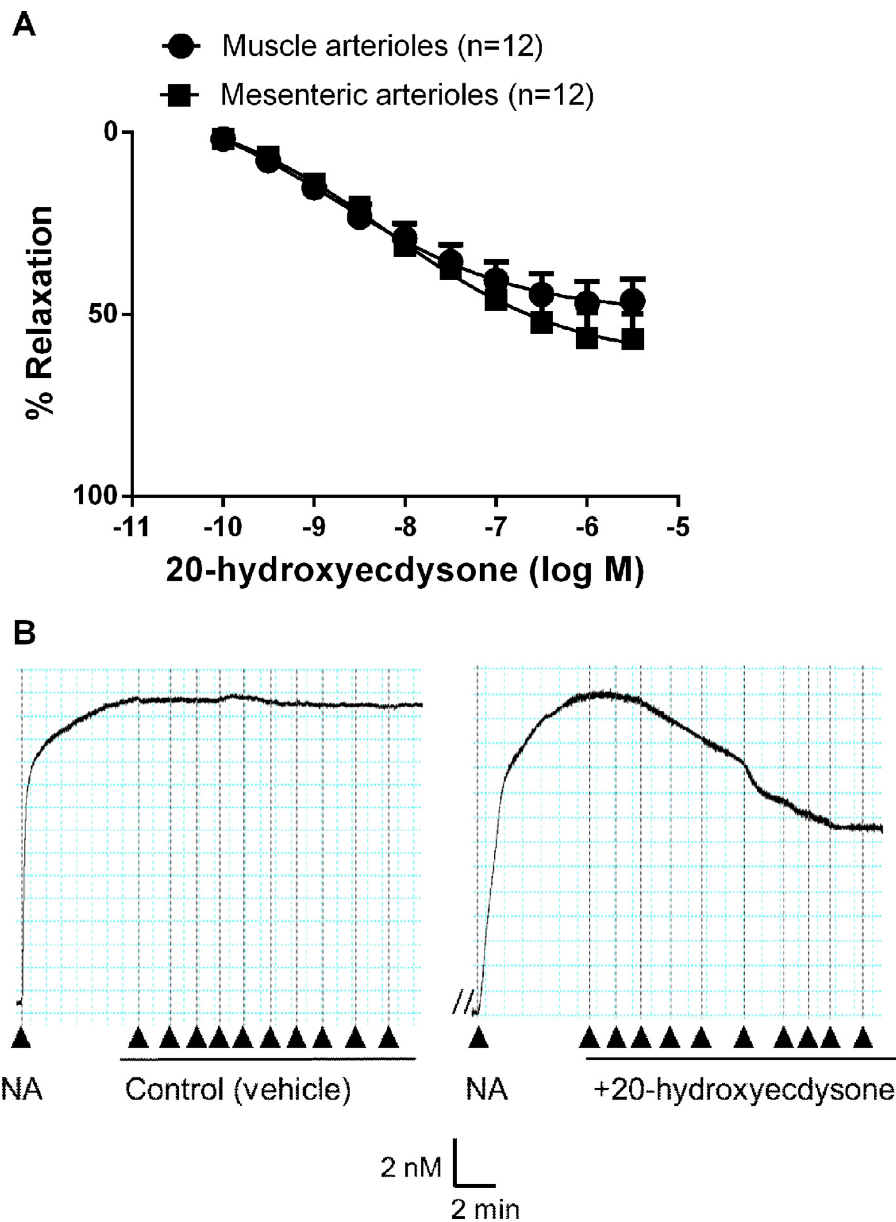


Fig. 1. 20-hydroxyecdysone-induced relaxation in muscle and mesenteric arterioles. (A) Concentration-relaxation curves for 20-hydroxyecdysone in muscle and mesenteric arterioles. Both curves were statistically comparable. (B) Typical recording of control (vehicle) tone vs. 20-hydroxyecdysone-induced relaxation in muscle arteriole. Vessels were pre-contracted with 10 μM noradrenaline and 20-hydroxyecdysone added in a cumulative fashion once the contraction had plateaued. Curves were compared by 2-Way ANOVA with Bonferroni test.

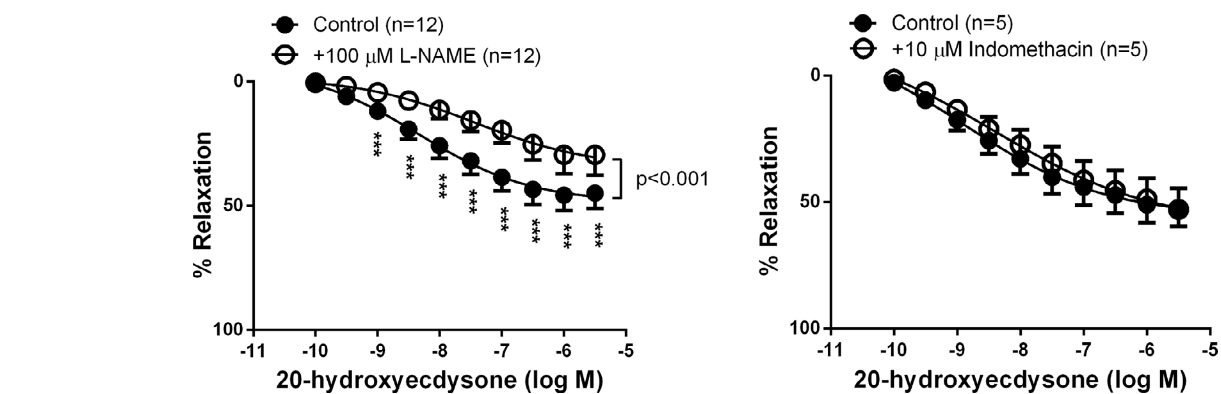


Fig. 2. Effect of NOS and COX inhibition on 20-hydroxyecdysone-induced relaxation in muscle arterioles. (A) Concentration-relaxation curves for 20-hydroxyecdysone in the absence and presence of 100 μM LNAME (NOS inhibitor). L-NAME caused a rightward shift in the curve ($p<0.001$). (B) Concentration-relaxation curves for 20-hydroxyecdysone in the absence and presence of 10 μM indomethacin (COX inhibitor). Indomethacin did not alter the relaxation curve. Curves were compared by 2-way ANOVA with Bonferroni test. ***$p<0.001$ compared with control responses

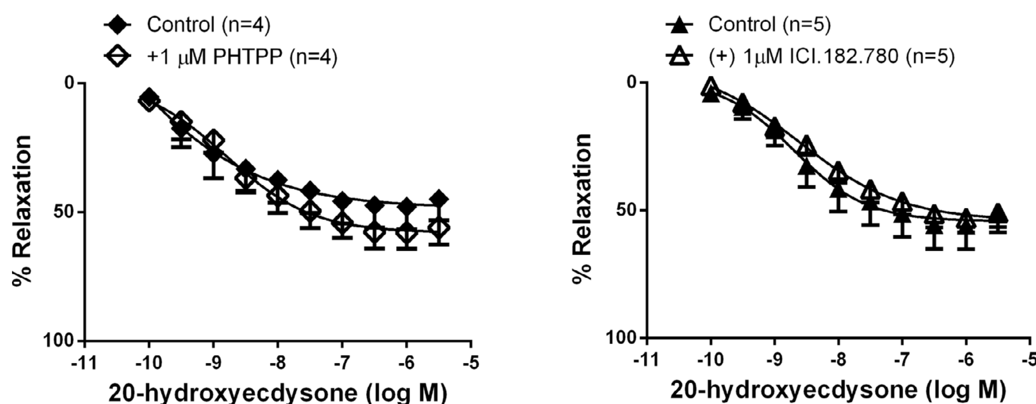


Fig. 3. Effect of blocking estrogen receptors (ERs) on 20-hydroxyecdysone-induced relaxation in muscle arterioles. (A) Concentration-relaxation curves for 20-hydroxyecdysone in the absence and presence of 1 μM PHTPP (selective ER-β blocker). (B) Concentration-relaxation curves for 20-hydroxyecdysone in in the absence and presence of 1 μM ICI 182, 780 (ER-α/β blocker). 20-hydroxyecdysone relaxation curves were unchanged under these conditions. Curves were compared by 2-way ANOVA with Bonferroni test.

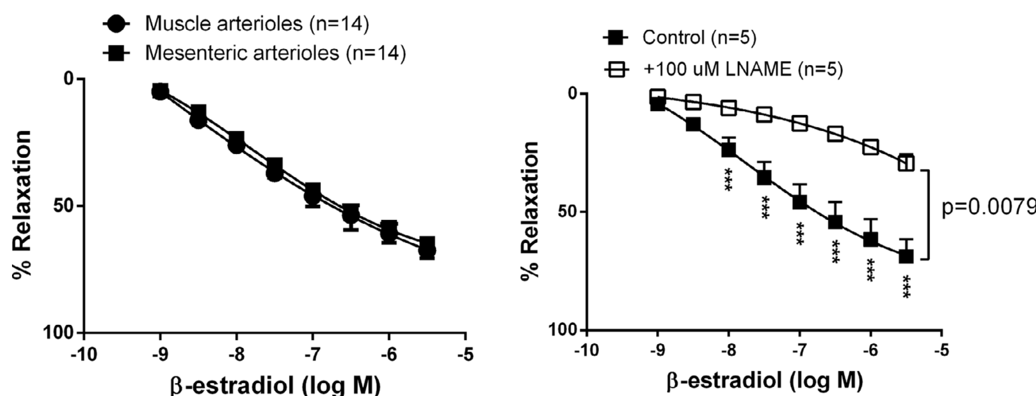


Fig. 4. β-estradiol-induced relaxation in muscle and mesenteric arterioles. (A) Concentration-relaxation curves for β-estradiol in muscle and mesenteric arterioles. Both curves were statistically comparable. (B) Concentration-relaxation curves for β-estradiol in muscle arterioles in the absence and presence of 100 μM LNAME (NOS inhibitor). L-NAME caused a rightward shift in the curve (p=0.0079). Curves were compared by 2-way ANOVA with Bonferroni test. ***p<0.001 compared with control responses

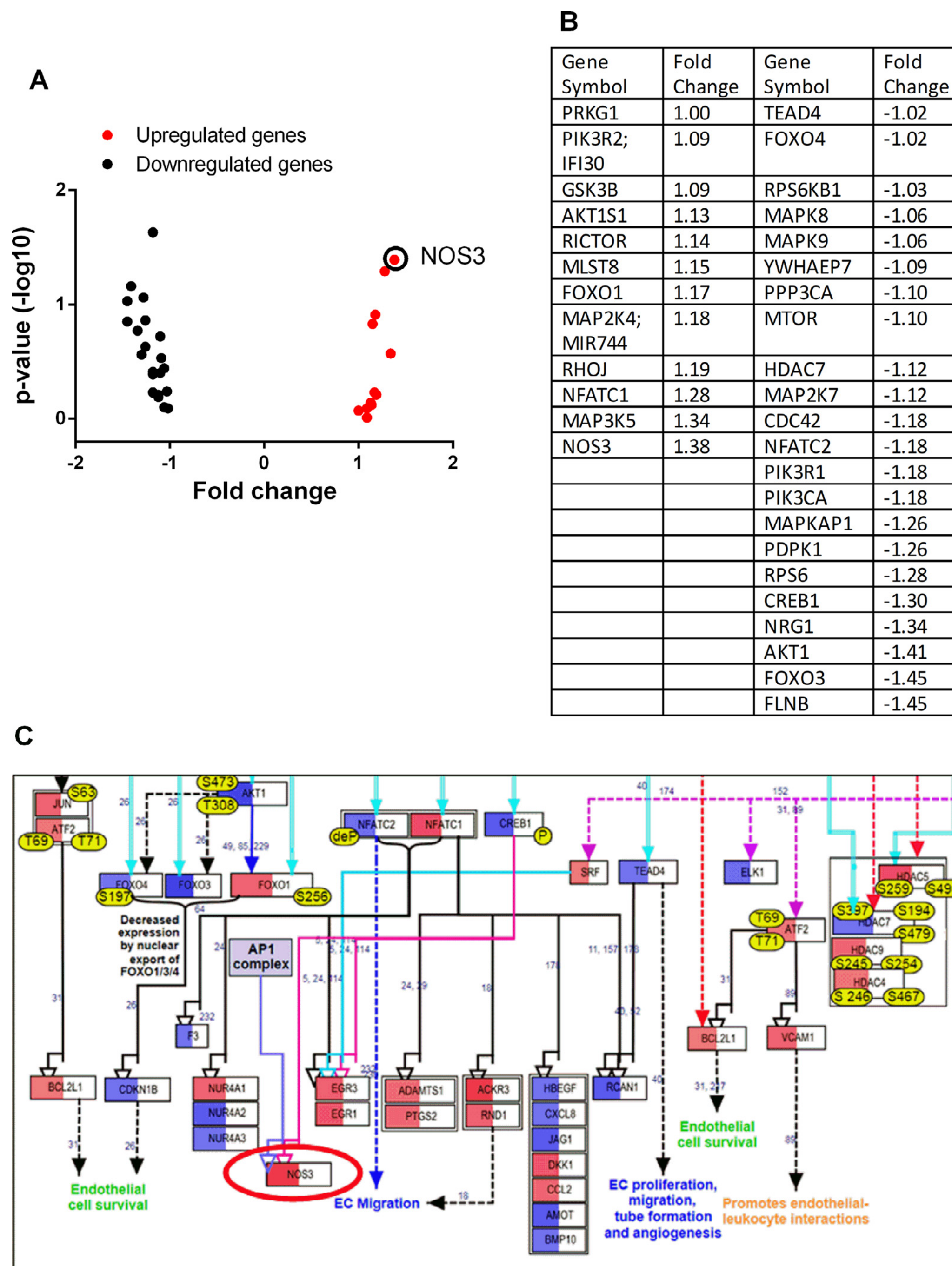
carried out in HCAECs treated with different concentrations of 20-hydroxyecdysone. NOS3 mRNA was increased at concentrations of 1–1000 nM of 20-hydroxyecdysone compared with the untreated control ($p < 0.001$, 1-way ANOVA, Fig. 6A). NOS3 protein was also increased in these cells, compared with the untreated control (p<0.001, 1-way ANOVA, Fig. 6B and C). At 1nM there was a 39% increase in protein expression, peaking at 68% in cells treated with 100 nM of the compound compared with untreated control ($p < 0.001$ Bonferroni). Images of the original western blots from which these figures were derived are shown in Supplementary Fig. 1. However, no further increase in protein expression was detected at 1000 nM, with an optimal dose for NOS3 induction at around 10 nM.

4. Discussion

The data clearly demonstrates that 20-hydroxyecdysone dilates muscle but also mesenteric arterioles. Remarkably, vasodilation was recorded at concentration as low as 1 nM, much lower than had been previously reported for its anabolic effect (Todorov et al., 2000; Parr et al., 2015; Isenmann et al., 2019). This suggests that 20-hydroxyecdysone at doses too small to increase muscle size, can potentially increase muscle blood flow and by so doing enhance muscle performance. To our knowledge, this is the first report of the vasodilatory activity of 20-hydroxyecdysone in muscle arterioles.

The data also show that this effect was nitric oxide-dependent, consistent with the rightward shift in the relaxation curve when NOS was inhibited, which confirmed 20-hydroxyecdysone's ability to activate NOS3 and enhance NO production (Korkach et al., 2007; Omanakuttan et al., 2016). More so, 20-hydroxyecdysone increased NOS3 mRNA and protein in human coronary arterial endothelial cells (HCAECs) and NOS3 mRNA in humanized liver tissues. Although the increases in NOS3 mRNA and protein observed in HCAECs and humanized liver do not directly explain the acute vasodilatory effect, they can be linked to the transcriptional changes associated with the acute signaling pathways responsible for the activation of NOS3 activity (Menazza and Murphy, 2016). Both NOS3 activity as demonstrated with L-NAME in intact arterioles and its enhanced expression observed in HCAECS and humanized liver unequivocally show that 20-hydroxyecdysone specifically targets the NOS3 pathway in its vascular actions. NOS3 synthesizes NO from L-arginine in the endothelial cells. NO diffuses into adjacent vascular smooth muscle cells where it activates soluble guanylyl cyclase (sGC) to catalyze the dephosphorylation of GTP to cyclic GMP (cGMP). Cyclic GMP then activates cGMP-dependent kinase (PKG), which among other effects, activates the large-conductance Ca2+-activated potassium (K+) channels leading to K+ efflux, hyperpolarization and relaxation (Moncada et al., 1991).

Although the relaxation recorded for β-estradiol in these arterioles was comparable to that of 20-hydroxyecdysone, we could not confirm the activation of estrogen ER-β receptor in the actions of both compounds. Therefore, our hypothesis that 20-hydroxyecdysone would induce vasodilation by activating this receptor was not proven. The hypothesis had been based on the reported strong affinity of



Gene Symbol	Fold Change	Gene Symbol	Fold Change
PRKG1	1.00	TEAD4	-1.02
PIK3R2; IFI30	1.09	FOXO4	-1.02
GSK3B	1.09	RPS6KB1	-1.03
AKT1S1	1.13	MAPK8	-1.06
RICTOR	1.14	MAPK9	-1.06
MLST8	1.15	YWHAEP7	-1.09
FOXO1	1.17	PPP3CA	-1.10
MAP2K4; MIR744	1.18	MTOR	-1.10
RHOJ	1.19	HDAC7	-1.12
NFATC1	1.28	MAP2K7	-1.12
MAP3K5	1.34	CDC42	-1.18
NOS3	1.38	NFATC2	-1.18
		PIK3R1	-1.18
		PIK3CA	-1.18
		MAPKAP1	-1.26
		PDPK1	-1.26
		RPS6	-1.28
		CREB1	-1.30
		NRG1	-1.34
		AKT1	-1.41
		FOXO3	-1.45
		FLNB	-1.45

Fig. 5. Differentially expressed genes of the VEGFA-VEGFR2 Signaling pathway in the humanized liver of uPA(+/+)-SCID mice treated with 20-hydroxyecdysone . A. Volcano plot of genes altered by 20-hydroxyecdysone treatment. B. Table of the differentially expressed genes. C. Effect of 20-hydroxyecdysone on the expression of the VEGFA-VEGFR2 signaling pathway components. NOS3 (circled) was significantly upregulated.in the transcriptome. The red and blue colors represent over and under representation of the gene respectively in the treatment group compared with control. Pathway analysis was conducted using TAC 4.0 software.

20-hydroxyecdysone for ER-β (Parr et al., 2015; Isenmann et al., 2019), which is the predominant isoform expressed in blood vessels (Gustafsson, 2000) and abundantly expressed in these arterioles. The lack of effect of ICI 182,780, which is known to block both classical estrogen receptors (ERα and ERβ), suggests both classical receptors are unlikely mediators of 20-hydroxyecdysone relaxation in these arterioles. Paradoxically, ICI 182,780 is an agonist on the more recently described G-protein coupled estrogen receptor (GPER, Menazza and Murphy, 2016) which can mediate 17-β-estradiol -induced vasorelaxation via the release of nitric oxide (Lindsey et al., 2014). It is therefore not entirely surprising that ICI 182, 780 did not block β-estradiol -induced relaxation in the muscle arterioles in this study. Moreover, ICI 182, 780 did not block estrogen-induced vasorelaxation in either canine coronary or small rat arteries (Sudhir et al., 1995; Shaw et al., 2000; Scott et al., 2007; Meyer et al., 2010). Interestingly however, the relaxation curves for both 20-hydroxyecdysone and estrogen were comparable, although the similarity may be related to the ability of both compounds to activate NOS3 and release NO (Zhu et al., 2002). Additional studies are required to determine whether both share the same vascular receptor mechanisms.

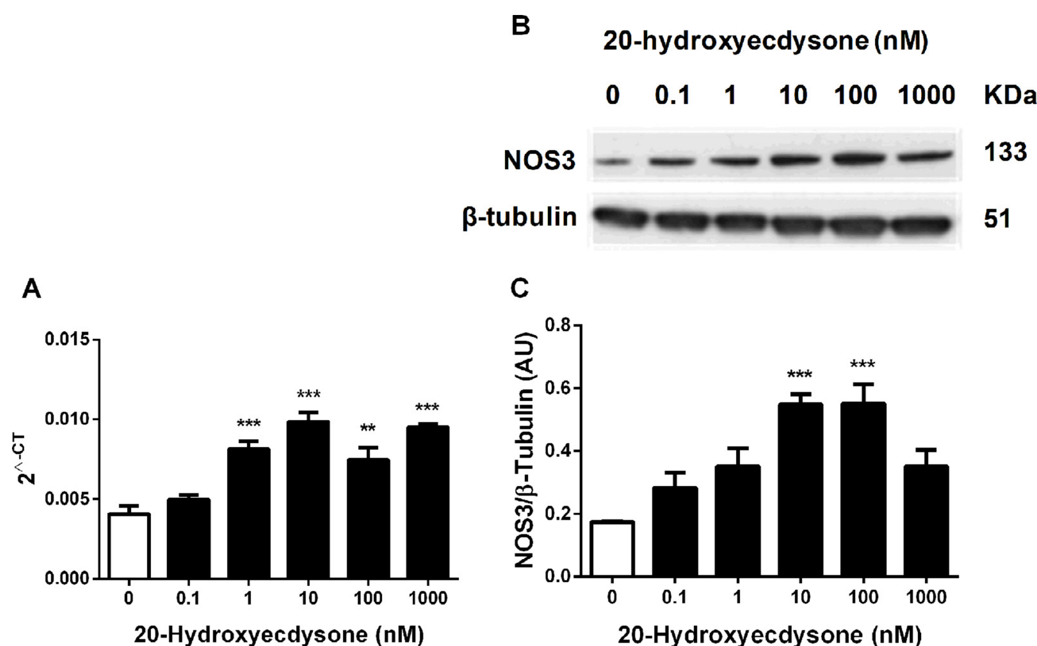


Fig. 6. Effect of 20-hydroxyecdysone on expression of NOS3 in HCAECs. Cultured HCAECs were exposed to 5 concentrations of 20-hydroxyecdysone (0.1, 1, 10, 100 and 1000 nM) for 24 hours and NO3 mRNA was analyzed by qPCR and protein expression by western blot. (A) mRNA expressions in response to graded concentrations of 20-hydroxyecdysone. NOS3 mRNA was significantly increased by 20-hydroxyecdysone ($p<0.001$). (B) Blot showing NOS3 and β-tubulin proteins in each sample. Whole blots for 3 repeats are shown in supplementary Fig. 1. (C) NOS3 protein expression normalized to β-tubulin expression in each sample. NOS3 protein was significantly increased by 20-hydroxyecdysone ($p<0.001$). Dose effects were compared by 1-way ANOVA with Bonferroni test. $^{**}p<0.01$, $^{***}p<0.001$ compared with vehicle alone control.

5. Conclusion

20-hydroxyecdysone dilates muscle arterioles at concentrations lower than required to demonstrate their anabolic effects. The mechanism is nitric oxide-dependent. Notably it causes elevation in NOS3 mRNA and protein in human coronary artery endothelial cells. The receptor mechanism could not be determined from the current data but did not appear to involve the activation of estrogen ER-β receptor contrary to our hypothesis and its reported affinity for this receptor. The effect was also not specific to muscle arterioles as it also dilated mesenteric arterioles comparably. Whether the nonspecific activity would impact negatively on the potential to increase muscle blood flow would require further investigation.

Author contributions

Nelson N Orie: Conceptualization, Investigation, Formal analysis, Visualization, Writing - Original Draft, Writing - Review & Editing, Asmaa Raees: Investigation, Maneera Yusuf AlJaber: Investigation, Validation, Nada Mohamed-Ali: Investigation, Validation, Halima Bensmail: Formal analysis, Mostafa M. Hamza: Formal analysis, Nasser Al-Ansari: Resources, supervision, Alka Beotra: Conceptualization, Supervision, Vidya Mohamed-Ali: Conceptualization, Project administration, Supervision, Resources, Writing - Original Draft, Writing - Review & Editing, Mohammed Almaadheed: Conceptualization, Supervision, Resources, Writing - Original Draft, Writing - Review & Editing

Declaration of Competing Interests

None.

Acknowledgments

This work was supported in-kind by the State of Qatar.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phyplu.2021.100078.

References

Bashraheel, S.S., AlQahtani, A.D., Rashidi, F.B., Al-Sul2aiti, H., Domling, A., Orie, N.N., Goda, S.K., 2019. Studies on vascular response to full superantigens and superantigen derived peptides: Possible production of novel superantigen variants with less vasodilation effect for tolerable cancer immunotherapy. Biomed. Pharmacother. 115, 108905. doi:10.1016/j.biopha.2019.108905.

Delp, M.D., 1998. Differential effects of training on the control of skeletal muscle perfusion. Med. Sci. Sports Exerc. 30 (3), 361–374. doi:10.1097/00005768-199803000-00005.

Dinan, L., 2001. Phytoecdysteroids: biological aspects. Phytochemistry 57 (3), 325–339. doi:10.1016/s0031-9422(01)00078-4.

Dinan, L., 1995. Distribution and levels of phytoecdysteroids within individual plants of species of the Chenopodiaceae. Eur. J. Entomol. 92, 295–300.

Dinan, L., Lafont, R., 2006. Effects and applications of arthropod steroid hormones (ecdysteroids) in mammals. J. Endocrinol. 191 (1), 1–8. doi:10.1677/joe.1.06900, PMID:17065383 .

García-Campos, M.A., Espinal-Enríquez, J., Hernández-Lemus, E., 2015. Pathway analysis: state of the art. Front. Physiol. 6, 383. doi:10.3389/fphys.2015.00383.

Gorelick-Feldman, J., Cohick, W., Raskin, I., 2010. Ecdysteroids elicit a rapid Ca2+ flux leading to Akt activation and increased protein synthesis in skeletal muscle cells. Steroids 75 (10), 632–637. doi:10.1016/j.steroids.2010.03.008.

Gorelick-Feldman, J., Maclean, D., Ilic, N., Poulev, A., Lila, M.A., Cheng, D., Raskin, I., 2008. Phytoecdysteroids increase protein synthesis in skeletal muscle cells. J. Agric. Food Chem. 56 (10), 3532–3537. doi:10.1021/jf073059z.

Green, D.J., Cable, N.T., Fox, C., Rankin, J.M., Taylor, R.R., 1994. Modification of forearm resistance vessels by exercise training in young men. J. Appl. Physiol. 77 (4), 1829–1833. doi:10.1152/jappl.1994.77.4.1829.

Gustafsson, J.A., 2000. Novel aspects of estrogen action. J. Soc. Gynecol. Investig. 7, S8–S9. doi:10.1016/s1071-5576(99)00060-x, 1 Suppl.

Hickner, R.C., Fisher, J.S., Ehsani, A.A., Kohrt, W.M., 1997. Role of nitric oxide in skeletal muscle blood flow at rest and during dynamic exercise in humans. Am. J. Physiol. 273, H405–H410. doi:10.1152/ajpheart.1997.273.1.H405, 1 Pt 2.

Isenmann, E., Ambrosio, G., Joseph, J.F., Mazzarino, M., de la Torre, X., Zimmer, P., Kazlauskas, R., Goebel, C., Botrè, F., Diel, P., Parr, M.K., 2019. Ecdysteroids as non-conventional anabolic agent: performance enhancement by ecdysterone supplementation in humans. Arch. Toxikol. 93 (7), 1807–1816. doi:10.1007/s00204-019-02490-x.

Korkach, Iu.P., Kotsiuruba, A.V., Prysiazhna, O.D., Mohyl'nyts'ka, L.D., Sahach, V.F., 2007. NO-dependent mechanisms of ecdysterone protective action on the heart and vessels in streptozotocin-induced diabetes mellitus in rats. Fiziol. Zh. 53 (3), 3–8.

Kraiem, S., Al-Jaber, M.Y., Al-Mohammed, H., Al-Menhali, A.S., AlThani, N.J., Helaleh, M., Samsam, W., Touil, S., Beotra, A., Georgakopoulas, C., Bouabdallah, S., Mohamed-Ali, V., Almaadheed, M., 2021. Analytical strategy for the detection of ecdysterone and its metabolites in vivo in uPA(+/+)-SCID mice with humanised liver, human urine samples and estimation of prevalence of its use in anti-doping samples. Drug Test Anal. 10.1002/dta.3032. Advance online publication doi:10.1002/dta.3032.

Lash, J.M., Bohlen, H.G., 1997. Time- and order-dependent changes in functional and NO-mediated dilation during exercise training. J. Appl. Physiol. 82 (2), 460–468. doi:10.1152/jappl.1997.82.2.460.

Menazza, S., Murphy, E., 2016. The expanding complexity of estrogen receptor signaling in the cardiovascular system. Circ. Res. 118 (6), 994–1007. doi:10.1161/CIRCRESAHA.115.305376.

Mercer, D.F., Schiller, D.E., Elliott, J.F., Douglas, D.N., Hao, C., Rinfret, A., Addison, W.R., Fischer, K.P., Churchill, T.A., Lakey, J.R., Tyrrell, D.L., Kneteman, N.M., 2001. Hepatitis C virus replication in mice with chimeric human livers. Nat. Med. 7 (8), 927–933. doi:10.1038/90968.

Meyer, M.R., Baretella, O., Prossnitz, E.R., Barton, M., 2010. Dilation of epicardial coronary arteries by the G protein-coupled estrogen receptor agonists G-1 and ICI 182,780. Pharmacology 86, 58–64. doi:10.1159/000315497.

Moncada, S., Palmer, R.M., Higgs, E.A., 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. 43 (2), 109–142.

Omanakuttan, A., Bose, C., Pandurangan, N., Kumar, G.B., Banerji, A., Nair, B.G., 2016. Nitric Oxide and ERK mediates regulation of cellular processes by Ecdysterone. Exp. Cell Res. 346 (2), 167–175. doi:10.1016/j.yexcr.2016.07.019.

Orie, N.N., Fry, C.H., Clapp, L.H., 2006. Evidence that inward rectifier K+ channels mediate relaxation by the PGI2 receptor agonist cicaprost via a cyclic AMP-independent mechanism. Cardiovasc. Res. 69 (1), 107–115. doi:10.1016/j.cardiores.2005.08.004.

Parr, M.K., Botrè, F., Naß, A., Hengevoss, J., Diel, P., Wolber, G., 2015. Ecdysteroids: a novel class of anabolic agents? Biol. Sport 32 (2), 169–173. doi:10.5604/20831862.1144420.

Parr, M.K., Zhao, P., Haupt, O., Ngueu, S.T., Hengevoss, J., Fritzemeier, K.H., Piechotta, M., Schlörer, N., Muhn, P., Zheng, W.Y., Xie, M.Y., Diel, P., 2014. Estrogen receptor beta is involved in skeletal muscle hypertrophy induced by the phytoecdysteroid ecdysterone. Mol. Nutr. Food Res. 58 (9), 1861–1872. doi:10.1002/mnfr.201300806.

Schmelz, E.A., Grebenok, R.J., Ohnmeiss, T.E., Bowers, W.S., 2000. Phytoecdysteroid turnover in spinach: Long-term stability supports a plant defense hypothesis. J. Chem. Ecol. 2, 2883–2896.

Scott, P.A., Tremblay, A., Brochu, M., St-Louis, J., 2007. Vasorelaxant action of 17 -estradiol in rat uterine arteries: role of nitric oxide synthases and estrogen receptors. Am. J. Physiol. 293 (6), H3713–H3719. doi:10.1152/ajpheart.00736.2007.

Shaw, L., Taggart, M.J., Austin, C., 2000. Mechanisms of 17 beta-oestradiol induced vasodilatation in isolated pressurized rat small arteries. Br. J. Pharmacol. 129, 555–565. doi:10.1038/sj.bjp.0703084.

Slama, K., Lafont, R., 1995. Insect hormones - ecdysteroids: their presence and actions in vertebrates. Eur. J. Entomol. 92 (1), 355–377 ISSN 1210-5759.

Sudhir, K., Chou, T.M., Mullen, W.L., Hausmann, D., Collins, P., Yock, P.G., Chatterjee, K., 1995. Mechanisms of estrogen-induced vasodilation: in vivo studies in canine coronary conductance and resistance arteries. J. Am. Coll. Cardiol. 26, 807–814. doi:10.1016/0735-1097(95)00248-3.

Syrov, V.N., 2000. Comparative experimental investigation of the anabolic activity of phytoecdysteroids and steranabols. Pharm. Chem. J. 34 (4), 193–197. doi:10.1007/BF02524596.

Syrov, V.N., Kurmukov, A.G., 1976. Ob anabolicheskoĭ aktivnosti fitoékdizona-ékdisterona, vydelennogo iz Rhaponticum carthamoides (Willd.) Iljin [Anabolic activity of phytoecdysone-ecdysterone isolated from Rhaponticum carthamoides (Willd.) Iljin]. Farmakol. Toksikol. 39 (6), 690–693.

Todorov, I.N., Mitrokhin, Y.i, Efremova, O.I., Sidorenko, L.I., 2000. The effect of ecdysterone on the biosynthesis of proteins and nucleic acids in mice. Pharm. Chem. J. 34 (9), 455–458. doi:10.1023/A:1005272922071.

Tóth, N., Szabó, A., Kacsala, P., Héger, J., Zádor, E., 2008. 20-Hydroxyecdysone increases fiber size in a muscle-specific fashion in rat. Phytomedicine 15 (9), 691–698. doi:10.1016/j.phymed.2008.04.015.

Zhu, Y., Bian, Z., Lu, P., Karas, R.H., Bao, L., Cox, D., Hodgin, J., Shaul, P.W., Thoren, P., Smithies, O., Gustafsson, J.A., Mendelsohn, M.E., 2002. Abnormal vascular function and hypertension in mice deficient in estrogen receptor beta. Science 295 (5554), 505–508. doi:10.1126/science.1065250.

Zwetsloot, K.A., Shanely, A.R., Merritt, E.K., McBride, J.M., 2014. Phytoecdysteroids: a novel, non-androgenic alternative for muscle health and performance. J Steroids Horm. Sci. doi:10.4172/2157-7536.S12-e001, S12: e001.

World Anti-Doping Agency, 2020. The 2020 monitoring program. https://www.wada-ama.org/en/resources/science-medicine/monitoring-program, (accessed March 2021).