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Regulation of noradrenaline synthesis, uptake, and degradation in the left ventricle by fatty acid amide hydrolase (FAAH) inhibitor URB597 in the chronic unpredictable stress model of depression

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Abstract: Depression has been linked to the dysfunction of the autonomic nervous system, which may cause dysregulation of the cardiovascular system. One promising therapeutic strategy for treating different diseases is inhibiting the enzyme fatty acid amide hydrolase (FAAH), which increases the availability of endogenous cannabinoids. We examined the effect of chronic FAAH inhibition with URB597 treatment on the noradrenaline (NA) content, synthesis, transport, and degradation in the left ventricle of female and male rats exposed to chronic unpredictable stress (CUS). CUS decreased the levels of both NA and dopamine-β-hydroxylase (DBH) protein in male rats and decreased NA transporter (NET) protein levels in female rats while elevating monoamine oxidase A (MAO-A) in both sexes. Intraperitoneal URB597 application led to increased expression of DBH in stressed males, as well as elevated NET protein levels and decreased MAO-A protein levels in the left ventricle of stressed rats of both sexes. URB597 treatment may have a beneficial effect on the cardiovascular system in an animal model of depression with heightened sympathoneural activity.

Keywords: noradrenaline, left ventricle, chronic unpredictable stress, endocannabinoids, experimental rat

Abbreviations: ANS – autonomic nervous system; SNS – sympathetic nervous system; PFC – prefrontal cortex; TH – tyrosine hydroxylase; COMT – catechol-O-methyltransferase

INTRODUCTION

The rising incidence of anxiety and depression due to prolonged stress exposure is emerging as a major health issue [1]. Clinical depression stands out as the primary risk factor for cardiovascular disease. The autonomic nervous system (ANS) serves as the intermediary between the cardiovascular and central nervous systems, regulating internal body functions in response to physiological fluctuations [2]. The altered activity of the ANS may be responsible for the development of cardiovascular diseases. Data shows that hyperactivity of the sympathetic nervous system (SNS) in depression can be linked to an increased concentration of catecholamines in the plasma, vasoconstriction, and accelerated heart rate. Patients with depressive symptoms and cardiovascular disease have increased levels of noradrenaline (NA) [3]. Individuals with depression are two to four times more likely to experience cardiovascular disease [4]. Women are more susceptible to the co-occurrence of depression and cardiovascular disorders. Chronic unpredictable stress (CUS) is an animal model of depression that is characterized by depression-like behavior and elevated sympathetic cardiac tone [5]. Conventional antidepressants are widely used to treat mood disorders. The utilization of many antidepressant medications is limited due to their adverse effects on cardiovascular health [6]. A previous study demonstrated that prolonged administration of fluoxetine resulted in elevated plasma levels of noradrenaline and adrenaline [7]. Additionally, it was found that depressive patients taking fluoxetine for 40 days had higher catecholamine levels [8]. This raises the question of how these medications will affect the functioning of the heart, even though treating

cardiovascular patients with these medications reduces their depressive symptoms.

Modulation of the endocannabinoid system is increasingly recognized as a novel therapeutic approach for various neurodegenerative, cardiovascular, and inflammatory conditions [9]. The increased involvement of this system in both peripheral and central functions has raised interest in its potential therapeutic uses. Increasing the availability of endogenous cannabinoids by inhibiting their degradative enzyme fatty acid amide hydrolase (FAAH) represents a promising therapeutic approach in treating many diseases. One of the well-studied FAAH inhibitors with high potency and selectivity is URB597 [10]. Preclinical studies have indicated that the pharmacological suppression of FAAH activity may offer potential benefits across various mood disorders [11]. URB597 enhances noradrenergic transmission within the prefrontal cortex (PFC) and the basolateral amygdala neurons in stressed rats [12]. Furthermore, our findings illustrate that URB597 regulated catecholamine levels and the enzymes responsible for their synthesis and degradation in the medial prefrontal cortex (mPFC), hippocampus, and hypothalamus of stressed male and female rats [13]. However, its role and potential effects on cardiac catecholamine in stressed-induced depression remain unclear.

Given that the balance between NA synthesis, secretion, and reuptake is necessary for functional noradrenergic transmission, the effect of the FAAH inhibitor URB597 on the changes in key biosynthetic enzymes of catecholamine tyrosine hydroxylase (TH), DBH, degradative enzymes of MAO-A and catechol-O-methyltransferase (COMT), and NET in the left ventricle of male and female CUS rats was investigated.

MATERIALS AND METHODS

Ethics statement

All procedures involving animals in this study were executed with utmost care to minimize pain and discomfort, following the protocols set forth by the Ethical committee for Laboratory Animal Research at the "Vinča" Institute of Nuclear Sciences, University of Belgrade, and the Ministry of Agriculture and Environmental Protection. These protocols adhere strictly to the European Commission directive 2010/63/EU and were authorized under Veterinary Permissions Nos. 323-07-00704/2017-05 and 323-07-02772/2019-09.

Study Area

This study was carried out in 2020 at the Department of Molecular Biology and Endocrinology in the "Vinča" Institute of Nuclear Sciences, National Institute of the Republic of Serbia affiliated with the University of Belgrade.

Experimental animals

Albino Wistar rats of both sexes (three months old, weighing 250-300g) were used. The animals were bred at the Institute for Nuclear Sciences and housed in standard laboratory conditions at a temperature of 22±2°C, humidity ~45±5%, and a 12 h light/dark cycle in polycarbonate cages (37 cm × 21 cm × 13 cm), and were kept in groups of three or four animals per cage. In the experiment, food and water were available *ad libitum*. At the end of the experiment, the animals were sacrificed by decapitation using a guillotine (Harvard Apparatus, Holliston, USA). Heart tissue was isolated by dissection quickly on the ice; immediately after isolation, the heart was frozen in liquid nitrogen and stored at -80˚C until analyses were performed. In this study, vaginal swabs were collected and examined at multiple time points to monitor the estrous cycle phase. To mitigate the impact of female sex hormones on the outcomes, only samples from female animals in the diestrus phase at the experiment's conclusion were utilized.

Experimental procedure

After a week acclimatization period, 48 Albino Wistar male and female rats were divided into two groups: unstressed controls and animals exposed to CUS for six weeks (42 days). Each group was then subdivided into four subgroups based on the treatment they received: unstressed rats + vehicle (six rats of both sex); chronically stressed rats + vehicle (six rats of both sex); unstressed rats + URB597 (six rats of both sex); chronically stressed rats + URB597 (six rats of both sex).

Chronic unpredictable stress

Male and female rats subjected to chronic stress were exposed to several combinations of stressors twice daily for 6 weeks, randomly to prevent habituation. Stressors included: forced treadmill running stress at a constant speed (10 m/min,15 min); cage tilt (45°, 4-6 h); wet bedding (5 h); cold exposure (4°C, 5 h); food deprivation (18 h); water deprivation (18 h); grouping – (8 animals in one cage, 24 h); restraint stress (1 h); exposure to loud noise stress (1 h – screaming); social isolation (24 h); exposure to strobe light (8 h); immobilization stress (1 h), and reversed light and dark circle (24 h).

Fatty acid amide hydrolase (FAAH) inhibitor URB597 treatment

Male and female rats were treated with FAAH inhibitor URB597 (cyclohexyl carbamic acid 3'-carbamoyl-biphenyl-3-yl ester, cat. no. A4372, APExBIO, USA, in the literature also known as KDS-4103) or vehicle. URB597 was dissolved directly before application with a vehicle containing dimethyl sulfoxide (DMSO, Carlo Erba reagents, Spain), Tween 80 (Acros Organics, USA), and saline solution (0.9% NaCl) in a 1:1:18 ratio. Based on the previous studies pointing to the relatively short half-life of URB597, rats received intraperitoneal injections of URB597 (final volume, 0.3 mg/kg b.w./day) or the vehicle twice a day in the last two weeks of the stress protocol [14,15].

Measurement of noradrenaline levels in the left ventricle

The levels of noradrenaline in the left ventricle of chronically stressed animals and unstressed animals were examined using a CAT Research ELISA kit (Catalog No: BAE 5600¸ Labor Diagnostic Nord GmbH & Co., Germany). The analyzed tissue was homogenized in a 0.01N HCL buffer containing 1 mM EDTA and 4 mM sodium metabisulfite at a ratio of 1:100 and centrifuged for 15 min at 12298 x g as recommended by the supplier instructions. Immunoassays were performed according to the manufacturer's instructions. Using a microplate reader, absorbance was measured at 450 nm and a reference wavelength of 650 nm (Vallac multilabel counter VICTOR2 1420, PerkinElmer, Turku, Finland). Levels of NA were expressed as mg of catecholamines per g of tissue (mg/g).

Western blot analysis

The left ventricle was homogenized in a RIPA Lysis Buffer System (Santa Cruz Biotechnology, Inc., Dallas, TX, USA, sc-24948) using a T8 IKA-WERKE homogenizer at 4˚C. After homogenization, the supernatants were lysed for 1 h at 4˚C and then centrifuged for 20 min at 12000 x g. The protein concentration in the samples was determined using the Western blot method proposed by Lowry et al. [16], following the methodology described previously [13]. The respective primary and secondary antibodies that were used for the specific labeling of the given proteins: for TH (dilution 1:1000, sc374048, Santa Cruz Biotechnology, USA), for DBH (dilution 1:250, Catalog No. sc-47707, Santa Cruz Biotechnology, USA); for NET (dilution 1:2000, Catalog No. ab41559, Abcam, United Kingdom); for COMT (dilution 1:5000, Catalog No. ab 126618, Abcam, United Kingdom), and for MAO-A (dilution 1:2000, Catalog No. ab126751, Abcam, United Kingdom). The following secondary antibodies were used: secondary anti-mouse (Goat Anti-Mouse IgG (HRP), Catalog No. sc2005, Santa Cruz Biotechnology, USA) or anti-rabbit antibody (Goat Anti-Rabbit IgG (HRP), Catalog No. ab6721, Abcam, United Kingdom). Proteins were visualized using Immobilon Western Chemiluminescent HPR Substrate (Catalog No. WBKLS 0100, Millipore Corporation, USA). To obtain relative quantification, blots were quantified by densitometry relative to the internal loading control, β-actin (dilution 1:5000, Catalog No. sc-47778, Santa Cruz Biotechnology, USA) using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

The statistical analysis of the data was performed using Origin software, ver. 9 (Jandel Corporation, USA), and Statistica, ver. 7 (StatSoft, Inc., Tulsa, USA). The results of this study are presented as the means±standard error of the mean (SEM). To assess the interaction between three independent variables (sex, stress, and URB597 treatment), a three-way ANOVA was used. Additionally, a two-way ANOVA, split by sex as an independent factor, was carried out to elucidate the

results further. Significant ANOVA results were examined using Tukey's post hoc test for between-group comparisons or interaction analysis. Statistical significance was set at P<0.05. Graphical representation was accomplished using GraphPad Prism software (version 8.3.0).

RESULTS

The results demonstrate the effect of the FAAH inhibitor URB597 treatment on sympathetic innervation in the rat's heart under chronic stress. These results show the modulatory action of endocannabinoids on the processes of synthesis, transport, degradation, and the quantity of catecholamines in the heart. Fig. 1 shows the levels of noradrenaline in the left ventricle. The presented results indicate that NA levels (P<0.001) were higher in the left ventricle of unstressed males compared to females, indicating sex difference in baseline noradrenaline levels. Two-way ANOVA revealed significant effects of stress (P<0.001). The three-way ANOVA analysis revealed the effect of sex on the NA levels in the left ventricle (Supplementary Fig.1, Table 1c). Chronic unpredictable stress exposure decreased NA (by 29%, P<0.001) in male rats, while it did not affect the female rats. Chronic URB597 treatment restored the NA content to control values in stressed males (P<0.001).

The effects of CUS and URB597 treatment on the protein levels of catecholamine-synthesizing enzymes TH and DBH in the left ventricle are shown in Fig. 2A and B. Neither the exposure to chronic stress nor treatment showed any significant effects on the TH protein levels in the left ventricle of stressed male and female rats. The two-way ANOVA results showed significant effects of stress (P<0.001) and the interaction between stress and treatment (P<0.05) on DBH protein levels in the left ventricle of male rats (Fig. 2B). Namely, CUS induced a statistically significant decrease in DBH (by 30%, P<0.001) protein levels in the left ventricle of male rats only (Fig. 2B). The URB597 treatment elevated the protein levels of DBH in stressed males (P<0.001, Tukey's test). The same treatment with URB597 did not affect the protein levels of DBH in the female rats (Fig. 2B).

The two-way ANOVA analysis showed a significant interaction of stress and URB597 treatment on the

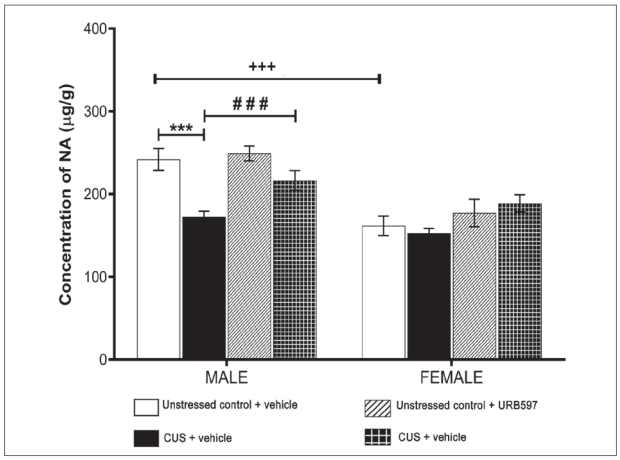

Fig. 1. Effect of URB597 treatment on noradrenaline (NA) concentration (μg/g) in the left ventricle of male and female rats exposed to chronic unpredictable stress for 6 weeks. Results are presented as the mean±SEM (n=6). Statistical significance: $^{+++}$P<0.001 male vs. female; ***P<0.001 unstressed controls vs. CUS; ### P<0.001 vehicle vs. URB597

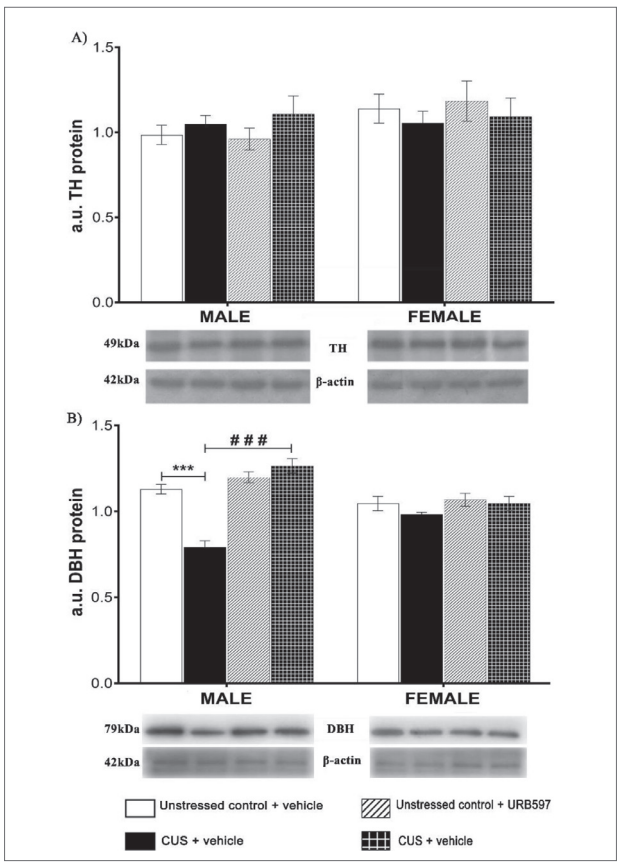

Fig. 2. Effect of URB597 treatment on tyrosine hydroxylase (TH) and dopamine beta-hydroxylase (DBH) in the left ventricle of male and female rats exposed to chronic unpredictable mild stress for 6 weeks. The result was expressed in arbitrary units and normalized relative to β-actin. The results are presented as the mean±SEM (n=6). Statistical significance: *** P<0.01 unstressed controls vs. CUS; $^{###}$ P<0.001 URB597 vs. vehicle.

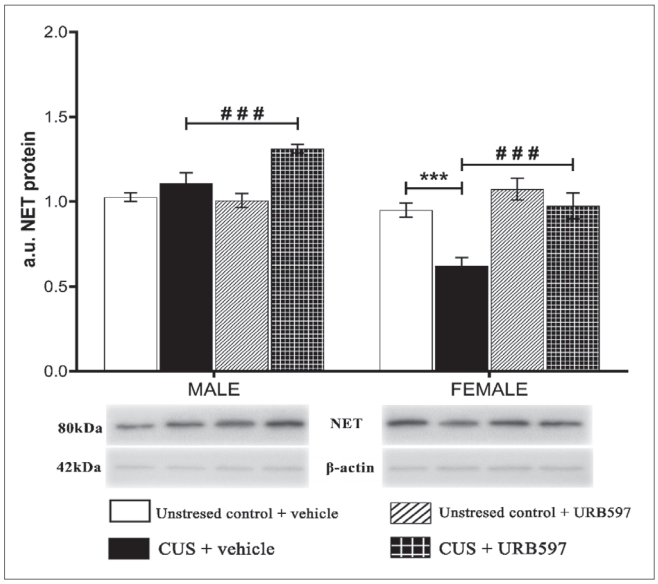

Fig. 3. Effect of URB597 treatment on monoamine noradrenaline transporter (NET) in the left ventricle of male and female rats exposed to chronic unpredictable stress for 6 weeks. The final result was expressed in arbitrary units and normalized relative to β-actin. The results are presented as the mean±SEM (n=6). Statistical significance: *** P<0.001 unstressed controls vs. CUS; ### P<0.001 URB597 vs. vehicle.

expression of NET in the left ventricle of female rats (P<0.05). NET protein levels significantly decreased in the left ventricle in female rats (by 34%, P<0.001) (Fig. 3). URB597 treatment had a positive effect on returning NET protein levels to baseline-like levels (P<0.001).

The effects of CUS (URB597 treatment) on catecholamine-degrading enzyme MAO-A and COMT levels in the left ventricle are presented in Figs. 4A and B. The protein levels of the COMT enzyme in the left ventricle of female and male rats were unchanged following chronic stress exposure and URB597 treatment. The two-way ANOVA, however, revealed a significant effect of stress (males: P<0.001; females: P<0.05) and URB597 (males: P<0.01; females: P<0.05) on MAO-A protein levels in the left ventricle of male and female rats (Fig. 4a). The post-hoc test showed that CUS caused increased MAO-A protein levels in both males (by 32%, P<0.001) and females (by 21%, P<0.001). FAAH inhibitor restored MAO-A protein levels to control values (P<0.001) (Fig. 4A).

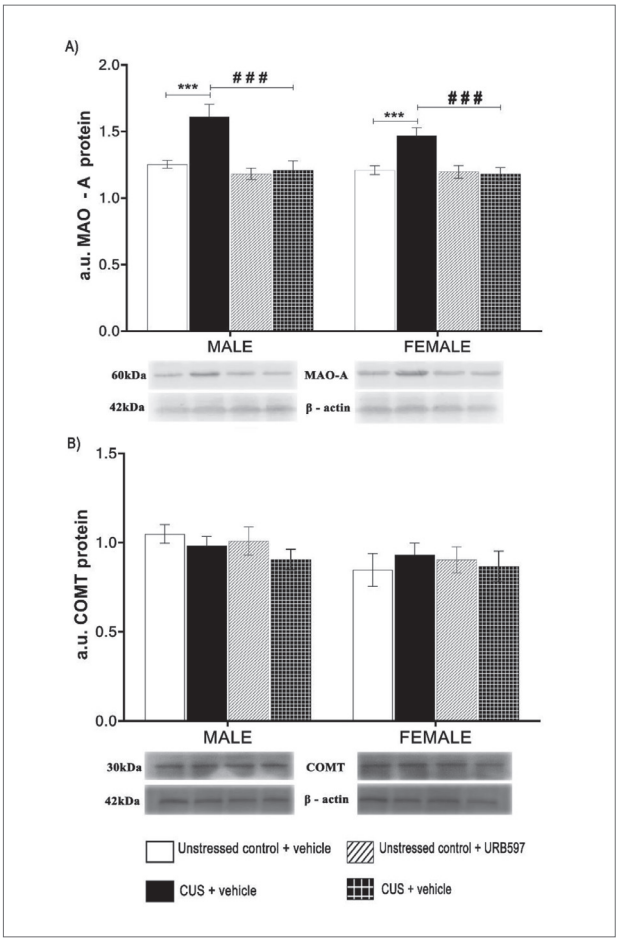

Fig. 4. Effect of URB597 treatment on **A** – monoamine oxidase A (MAO-A) and **B** – catechol-O-methyltransferase (COMT) protein levels in the left ventricle of male and female rats exposed to chronic unpredictable stress for 6 weeks. The final result was expressed in arbitrary units and normalized relative to β-actin. The results are presented as the mean±SEM of 6 rats. Statistical significance: *** P<0.001 unstressed controls vs. CUS; ### P<0.001 URB597 vs. vehicle.

DISCUSSION

In patients with depression, alterations in the heart's autonomic regulation are frequently observed. Grippo et al. found that chronic unpredictable mild stress increases the sympathetic tone [17]. Prolonged increased cardiac sympathetic outflow can lead to a reduction in cardiac NA levels, which is associated with a significantly higher risk of cardiovascular diseases. In a study by Shyu et al. [18], it was discovered that elevated NA concentrations induced apoptosis *in vitro* in neonatal rat cardiac myocytes. Recent findings have indicated that both male and female rats subjected

to CUS developed left ventricular hypertrophy and exhibited depression-like behaviors [19].

In the present study, we showed that CUS reduced the level of NA in the left ventricle of male rats, but did not change its level in females, which is partially consistent with the study of Carlsson and Carlsson [20], who reported that exposure to chronic mild stress causes sex-specific catecholamine changes in the heart, with significantly decreased NA levels in male rats. On the other hand, in contrast to our results, these authors demonstrated that female rats have 20% higher basal concentrations of NA than males, while our results showed that male rats have higher NA levels than females. Different factors can influence changes in catecholamine turnover and the levels of enzymes involved in the synthesis and degradation of catecholamines. Previous studies have reported the role of sex hormones in regulating catecholamine levels. In addition to the role of sex hormones, the genes located on sex chromosomes such as SRY (sex-determining region on the Y chromosome), which is expressed in various tissues including the adrenal glands and heart, were investigated [21]. Preliminary studies suggest that SRY regulates the transcription of genes for TH and MAO-A, potentially participating in sex-dependent regulation of catecholamine synthesis and degradation. This experiment has shown that CUS did not change the expression of TH in the left ventricle of males. However, the levels of DBH decreased. TH is considered a rate-limiting enzyme; in contrast, DBH is required for the synthesis of NA from dopamine, suggesting that reduced concentrations of NA may be due to decreased DBH expression [22]. We previously reported that CUS induced left ventricular hypertrophy [19]. Consistent with this result, Takechi et al. [23] reported a depletion of cardiac NA in cardiac hypertrophy. The concentration of NA remained unchanged, as well as the expression of TH and DBH in chronically stressed female rats. Maintaining homeostasis of NA in the heart depends on its release and reuptake, which is mainly carried out by NET transporters (more than 90%), with EMT non-neuronal transporters playing a small role [24]. Current data showed a decreased level of NET in the left ventricle of stressed females. Reduced NA uptake in sympathetic fiber terminals is associated with the onset of cardiovascular disease [25]. Additionally, this is associated with a poorer prognosis in patients experiencing cardiac arrest and an increased risk of sudden death [24]. Current findings indicated that CUS did not alter the expression of COMT, but it led to increased expression of MAO-A in the left ventricle of rats of both sexes. Elevated MAO activity is associated with detrimental effects on cardiac function [26].

Enhanced MAO-A expression may contribute to neuronal damage induced by oxidative stress. Previous research has confirmed that monoamine oxidases are the main source of reactive oxygen species (ROS) and are directly associated with the development of heart failure [27,28]. A study by Manni et al. [29] has shown that cardiac ischemia in humans is associated with increased activity of both isoforms of the MAO enzyme in the left and right ventricles. Studies have shown that endocannabinoids have a cardioprotective effect. Some studies have shown that FAAH inhibitors are antihypertensive agents [30]. Cannabinoid receptors CB1 and CB2 play direct roles in physiological regulation of signal transduction pathways in the heart. Additionally, TRPV1 contributes to the cardiovascular effects of cannabinoids [31]. Activation of presynaptic CB1 receptors during the early phase of myocardial infarction inhibits the release of NA from sympathetic nerve endings, thereby contributing to the suppression of tachycardia [32]. The effects of endocannabinoids on noradrenaline storage, biosynthesis, uptake, and degradation in the hearts of depression model rats remain poorly understood. In this experimental model, URB597 treatment increased the reduced NA stores and protein levels of DBH in the left ventricle of stressed males. It also elevated the expression of NET in both sexes. Heart disease has been linked to a decreased sympathoneural uptake of NA, and increased synthesis and uptake of NA after treatment in this study, which could suggest that endocannabinoids have a cardioprotective effect.

Additionally, the elevated levels of MAO-A in the left ventricle of both sexes following exposure to CUS are ameliorated by URB597 treatment. Mazor et al. [33] demonstrated that THC exhibits a dose-dependent inhibitory effect on MAO-A enzyme activity. Current findings strongly support the hypothesis that URB597 also plays a cardioprotective role, attributed to its capability to decrease MAO-A levels in the left ventricle of male and female rats subjected to CUS. An excessively elevated cytoplasmic NA level has been

associated with neuronal toxicity and oxidative stress. Increased sequestration and decreased deamination of NA by MAO-A during the URB597 treatment lowered the production of reactive oxygen species (ROS). These findings strongly indicate the role of URB597 as an FAAH inhibitor due to the cardioprotection it provides against CUS in both sexes. Together with our findings, the literature suggests that endocannabinoids may have beneficial effects by scavenging ROS. Notably, Ribeiro et al. [34] showed that the selective CB2 receptor agonist AM1241 completely blocked ROS generation in response to lipopolysaccharide (LPS) treatment in BV-2 cells.

URB597 reduces the impact of chronic stress on cardiac NA, suggesting that endocannabinoids play a key cardioprotective role in male and female animal models of depression. Based on the presented results, it can be concluded that, in addition to inhibiting NA release from sympathetic nerves, URB597 treatment has additional positive effects, including enhanced NA synthesis, which helps restore reduced NA levels in the left ventricle of males, reduced degradation in both sexes, and protection of cardiomyocytes from the harmful effects of excessive NA stimulation during chronic unpredictable stress (CUS). In this study, we can conclude that both male and female stressed rats provided an adequate response to CUS after treatment with the URB597 inhibitor; however, this process occurs through different molecular mechanisms. Evidence that sex, as a biological variable, influences stress-related functions through both hormones and sex chromosomes underscores the importance of including both sexes in experiments. Previous studies have demonstrated sex differences in the response to exogenous cannabinoids, with sex hormones, particularly estradiol, influencing the effects of exogenous cannabinoids in adults. Possible mechanisms that are likely related to the sex-dependent actions of URB597 in the stress response could include differences in the distribution of cannabinoid receptors, baseline activity of endocannabinoids (ECs), receptor desensitization rates, and differences in cannabinoid signaling [36,37].

In conclusion, given that URB597 positively affects anxiety and mood in depression and also regulates cardiac noradrenergic turnover, this FAAH inhibitor shows promise as a potential treatment for the comorbidity of depression and heart disease. Further preclinical and clinical trials are needed to explore and expand its therapeutic applications.

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Conflict of interest disclosure: The authors have no competing interests to declare.

Data availability: Data underlying the reported findings have been provided as a raw dataset available here: https://www.serbiosoc.org.rs/NewUploads/Uploads/Ferizovic%20et%20al_Raw%20Dataset.pdf

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