Биолошки факултет
Број захтева: 33/10-1
Датум: 29.01.2016.

УНИВЕРЗИТЕТ У БЕОГРАДУ
ВЕЋУ НАУЧНИХ ОБЛАСТИ ПРИРОДНИХ НАУКА

З А Х Т Е В

за давање сагласности на реферат о урађеној докторској дисертацији за кандидата на докторским студијама

Молимо да, сходно члану 47. ст. 5. тач. 4. Статута Универзитета у Београду ("Гласник Универзитета", број 162/11-пречишћени текст, 167/12, 172/13 и 178/14), дате сагласност на реферат о урађеној докторској дисертацији:

КАНДИДАТ**:** **Маја М. Бундало**

студент докторских студија на студијском програму Молекуларна биологија, Молекуларна биологија еукариота.

пријавио је докторску дисертацију под називом:

„Утицај исхране богате фруктозом на експресију молекула ренин-ангиотензин система, нуклеарног транскрипционог фактора-кВ, матрикс металопротеиназе 9 і CXCL16 хемокина у ткиву срца пацова: полно специфичне разлике“.

из научне области: Биолошке науке.

Универзитет је дана 27.11.2014. године. својим актом под бр. 02 Број: 61206-5348/2-14 дао сагласност на предлог теме докторске дисертације која је гласила:

„Утицај исхране богате фруктозом на експресију компоненти ренин-ангиотензин система и инфламације у ткиву срца пацова: полно специфичне разлике“.

Комисија за оцену и одбрану докторске дисертације образована је на седници одржаној 13.11.2015. год, одлуком Факултета под бр. 33/258-13.11.2015. год. у саставу:

	Име и презиме члана комисије	звање	научна област	Установа у којој је запослен
1.	др Александра Станковић	научни саветник	молекуларна генетика	Универзитет у Београду-Институт за нуклеарне науке „Винча“
2.	др Гордана Матић	редовни професор	биохемија и молекуларна биологија	Универзитет у Београду-Биолошки факултет
3.	др Горан Корићанац	научни саветник	молекуларна ендокринологија	Универзитет у Београду-Институт за нуклеарне науке „Винча“

Напомена: уколико је члан Комисије у пензији навести датум пензионисања.

Наставно-научно веће факултета прихватило је реферат Комисије за оцену и одбрану докторске дисертације на седници одржаној 29. јануара 2016. године.

Декан Биолошког факултета

Проф. др Жељко Томановић

Прилог: 1. Реферат комисије са предлогом.
2. Акт Наставно-научног већа факултета о усвајању реферата
3. Примедбе дате у току стављања реферата на увид у јавности, уколико је таквих примедби било.
4. Електронска верзија.

УНИВЕРЗИТЕТ У БЕОГРАДУ
БИОЛОШКИ ФАКУЛТЕТ

Студентски трг 16
11000 БЕОГРАД
Република СРБИЈА
Тел: +381 11 2186 635
Факс: +381 11 2638 500
Е-пошта: dekanat@bio.bg.ac.rs

33/10-29.01.2016.

На основу члана 128. Закона о високом образовању и члана 59. став 1. тачка 1. Статута Универзитета у Београду-Биолошког факултета, Наставно-научно веће Факултета, на IV редовној седници одржаној 29.01.2016. године, донело је

О Д Л У К У

Прихвата се Извештај Комисије за преглед, оцену и одбрану докторске дисертације кандидата:

Маје Бундало, под називом:

„Утицај исхране богате фруктозом на експресију компоненти ренин-ангиотензин система и инфламације у ткиву срца пацова: полно специфичне разлике“.

Универзитет је дана 27.11.2014. године. својим актом под бр. 02 Број: 61206-5348/2-14 дао сагласност на предлог теме докторске дисертације кандидата.

Радови и конгресна саопштења из докторске дисертације:

Б1. **Радови у часописима међународног значаја:**

1. Bundalo M, Živković M, Tepavčević S, Ćulafić T, Korićanac G, Stanković A. Fructose-rich diet-induced changes in expression of the renin-angiotensin molecules in the heart of ovariectomised female rats could be reversed by estradiol. *Hormone and Metabolic Research* 2015;47:521-7. **M23**

2. Bundalo M, Živković M, Ćulafić T, Stojiljković M, Korićanac G, Stanković A. Oestradiol treatment counteracts the effect of fructose-rich diet on matrix metalloproteinase 9 expression and NFκB activation. *Folia Biologica (Praha)* 2015;61:233-240. **M23**

Декан Биолошког факултета

Проф. др Жељко Томановић

Доставити:
- Универзитету у Београду,
- докторанту,
- Стручној служби Факултета.

НАСТАВНО-НАУЧНОМ ВЕЋУ БИОЛОШКОГ ФАКУЛТЕТА УНИВЕРЗИТЕТА У БЕОГРАДУ

На II редовној седници Наставно-научног већа Биолошког факултета Универзитета у Београду, одржаној 13.11.2015. године, прихваћен је извештај ментора проф. др Гордане Матић и др Александре Станковић, научног саветника о урађеној докторској дисертацији **Маје М. Бундало**, истраживача-сарадника у Институту за нуклеарне науке „Винча“, под насловом „**Утицај исхране богате фруктозом на експресију компоненти ренин-ангиотензин система и инфламације у ткиву срца пацова: полно специфичне разлике**“, и одређена је Комисија за преглед и оцену докторске дисертације у саставу др Александра Станковић, научни саветник, Универзитета у Београду-Институт за нуклеарне науке „Винча”, др Гордана Матић, редовни професор, Универзитета у Београду-Биолошки факултет и др Горан Корићанац, научни саветник, Универзитета у Београду-Институт за нуклеарне науке „Винча“.

Комисија је прегледала урађену докторску дисертацију кандидаткиње и Већу подноси следећи

И З В Е Ш Т А Ј

Општи подаци о докторској дисертацији:

Докторска дисертација **Маје М. Бундало** под насловом „**Утицај исхране богате фруктозом на експресију компоненти ренин-ангиотензин система и инфламације у ткиву срца пацова: полно специфичне разлике“**, написана је на 139 страна, и подељена у 7 поглавља: **Увод** (30 страна), **Хипотеза и циљеви** (1 страна), **Материјал и методе** (9 страна), **Резултати** (16 страна), **Дискусија** (20 страна), **Закључак** (2 стране) и **Литература** (61 страна). Рад садржи 577 литературних цитата и 2 *web site* цитата, 8 табела и 15 слика. Осим тога рад садржи и податке о менторима, резиме на српском и енглеском језику, податке о дисертацији, списак скраћеница, садржај, биографију кандидата и потписане изјаве.

Анализа докторске дисертације:

У поглављу **Увод** је свеобухватно дат преглед података релевантних за сагледавање теоретске основе дисертације и истраживања која се односе на разматрану проблематику. Кандидат даје преглед података о вези метаболичког синдрома и обољења срца, полно специфичним разликама у стопи обољевања од метаболичког синдрома, улози естрадиола у развоју метаболичког синдрома, повећаном уносу фруктозе, специфичном метаболизму фруктозе као и вези исхране богате фруктозом са настанком метаболичких и кардиоваскуларних поремећаја код људи и експерименталних животиња. Кандидат затим описује пет компоненти ренин-ангиотензин система [ангиотензин-конвертујући ензим (ACE), ангиотензин-конвертујући ензим 2 (ACE2), ангиотензински рецептор типа 1 (AT1R), ангиотензински рецептор типа 2 (AT2R) и колектрин). Наводе се маханизам деловања ензима и утицај молекула у чијој синтези учествују [ангиотензина 2 (ANG II) и ангиотензина-(1-7) (Ang-(1-7)] на витално важне процесе у срцу, као и сигнални путеви ангиотензинских рецептора. Такође се истиче улога компоненти ренин-ангиотензин система у настанку инфламације и поремећаја који леже у основи метаболичког синдрома. Даље кандидат даје детаљан преглед литературних података о

три компоненте инфламације и ремоделовања: металопротеинази матрикса-9 (MMP-9), нуклеарном фактору *kappa B* (NFκB) и лиганду 16 из фамилије хемокина CXC (CXCL16) и њиховој улози у настанку метаболичког синдрома.

У поглављу **Хипотеза и циљеви** дата је хипотеза и описани су циљеви. На основу литературних података се може очекивати да исхрана богата фруктозом доводи до полно специфичних промена у нивоу компоненти ренин-ангиотензин система и инфламације које би утицале на развој симптома метаболичког синдрома и са њим повезаних промена на нивоу срца.

У поглављу **Материјал и методе** наведене су хемикалије коришћене у експериментима и описане су експерименталне групе, дијететски и хормонски третмани животиња. Кандидат наводи све методе коришћене у раду, почев од биохемијских метода мерења концентрације глукозе, триглицерида, инсулина у циркулацији. Дат је детаљни опис поступка припреме протеина из лизата ћелија срца. Такође су изложене основе аналитичке методе електрофорезе на полиакриламидном гелу и *Western blot*-а, које су коришћене у анализи протеина. Кандидат даје и детаљан опис изолације iRNK молекула из ткива срца, поступка превођења iRNK молекула у cDNK молекул процесом реверзне транскрипције, као и *Real-time* методе која је коришћена за одређивање релативног нивоа iRNK у испитиваним узорцима ткива срца.

У поглављу **Резултати** су добијени резултати изложени у 7 табела и илустровани са 11 слика. Добијени резултати се могу укратко сумирати на следећи начин:

Исхрана обогаћена 10% раствором фруктозе је довела до развоја хипертриглицеридемије код пацова оба пола и код оваријектомисаних женки што је вероватно последица специфичног метаболизма фруктозе у јетри. Код оваријектомисаних женки је упоредо дошло и до хиперинсулинемије и развоја инсулинске резистенције. Третман естрадиолом није спречио развој хипертриглицеридемије, али је успео је превенира повећање концентрације инсулина у плазми и развој инсулинске резистенције. Код мужјака пацова који су пили 10% расвор фруктозе измерен је већи крвни притисак у односу на мужјаке који су пили воду. Код женки исхрана обогаћена фруктозом није утицала на крвни притисак.

Исхрана богата фруктозом је у срцу мужјака пацова повећала ниво ACE, довела до смањења нивоа ACE2 и AT2R, није утицала на експресију гена за AT1R, док транскрипција гена за AT2R није била детектабилна. Исхрана богата фруктозом није довела до промене нивоа iRNK за колектрин, MMP-9 и CXCL16 код мужјака пацова. Исхрана фруктозом је утицала на већи ниво iRNK за AT1R и MMP-9 који је детектован је код мужјака у односу на женке. Код женки пацова храњених фруктозом није дошло до промена у нивоу компоненти ренин-ангиотензин система, као ни у транскрипцији гена за колектрин, MMP-9 и CXCL16 у ткиву срца.

Код оваријектомисаних женки је исхрана богата фруктозом повећала ниво ACE, експресију гена за AT1R и MMP-9 и ниво iRNK за CXCL16, док је дошло до смањења нивоа ACE2 и AT2R. Након третмана естрадиолом код ових оваријектомисаних женки је дошло до следећих промена: ниво ACE2, AT2R и ниво iRNK за CXCL16 су се вратили на ниво измерен код контрола, док је ниво ACE, AT1R и MMP-9 био чак мањи од нивоа измереног код контролних оваријектомисаних женки. Исхрана богата фруктозом је у ткиву срца оваријектомисаних женки довела до повећања фосфорилације p65 субјединице NFκB на серинском остатку који се налази на позицији 276, што указује на активацију овог транскрипционог регулатора. Третман естрадилом је утицао на смањење ове фосфорилације.

Може се сумирати да резултати показују да и пол и исхрана богата фруктозом утичу на смањену експресију компоненти ренин-ангиотензин система за које се сматра да имају заштитно дејство на ткиво срца − ACE2 и AT2R. Исхрана богата фруктозом није утицала

на експресију гена за AT1R и на ниво iRNK за колектрин, MMP-9 и CXCL16 ни код мужјака ни код женки пацова. Ипак, у срцу мужјака је утврђен већи ниво молекула са потенцијално штетним дејством на ткиво срца, AT1R и MMP-9 у односу на женке храњене фруктозом. Оваријектомија уз исхрану обогаћену фруктозом доводи до промена у експресији компоненти ренин-ангиотензин система активацијом ACE/AT1R/ANG II осе и деактивацијом ACE2/AT2R/ANG II, повећања ниво експресије компоненти укључених у инфламацију и ремоделовање срца (NFκB, CXCL16, MMP-9). Третман естрадиолом имао је позитиван ефекат на срце оваријектомисаних женки враћајући поремећени баланс свих испитаних молекула у равнотежу чиме показује заштитни ефекат на срце.

У поглављу **Дискусија** кандидат даје детаљну анализу добијених експерименталних резултата и интерпретира их у односу на одговарајућу литературу. Ово поглавље је подељено у неколико одељака што доприноси бољем разумевању и тумачењу резултата сопствених истраживања у односу на резултате објављене у постојећој научној литератури. У првом одељку кандидат дискутује о утицају исхране богате фруктозом на морфолошке и биохемијске карактеристике мужјака и женки пацова. Даље кандидат дискутује о утицају исхране богате фруктозом на крвни притисак мужјака и женки пацова. У наредном одељку кандидат анализира промене у нивоу компоненти ренин-ангиотензин система у срцу мужјака и женки пацова настале као последица исхране богате фруктозом. Ово је прва студија у којој је на свеобухватан начин испитивано полно специфично дејство исхране богате фруктозом на експресију компоненти ренин-ангиотензин система. Следећи део се бави анализом утицаја исхране богате фруктозом и естрадиола на експресију компоненти ренин-ангиотензин система код оваријектомисаних женки и дискутује се о заштитном деловању естрадиола на које упућују добијени резултати. Потом следе одељци у којима се дискутује о полно специфичним разликама у ефекту фруктозе на експресију MMP-9 и о утицају исхране богате фруктозом и естрадиола на активацију NFκB и транскрипцију гена за CXCL16. На основу увида у литературу, кандидат износи идеје о сигналном путу преко кога би активација ренин-ангиотензин система могла бити повезан са активацијом NFκB и MMP-9.

У поглављу **Закључци**, кандидаткиња Маја Бундало сумира најважније закључке који произилазе из експерименталних резултата добијених у докторској дисертацији и прегледане литературе. Главни закључци докторске дисертације су следећи:

1. Исхрана обогаћена 10% раствором фруктозе је довела до повећаног калоријског уноса код мужјака и женки пацова. Ово је резултовало повећањем телесне масе само код женки пацова вероватно услед повећања масе висцералног масног ткива. Исхрана богата фруктозом није довела до промене апсолутне и релативне масе срца ни код једног пола.
2. Исхрана богата фруктозом доводи до хипертриглицеридемије код пацова оба пола, док нема утицаја на инсулинску осетљивост.
3. Мужјаци пацова су подложнији за настанак кардиоваскуларних болести након исхране обогаћене 10% раствором фруктозе у односу на женке. Ово штетно дејство исхране богате фруктозом се огледа у:
    - повећању крвног притиска код мужјака
    - нарушавању равнотеже у нивоу протеинских компоненти ренин-ангиотензин система које би могло резултовати патофизиолошким променама у структури и функцији срца код мужјака
    - повећаној транскрипцији гена за MMP-9 и CXCL16 код мужјака у односу на женке, што указује на покретање инфламације и штетно ремоделовање ткива срца мужјака

4. Естрадиол поништава ефекте исхране богате фруктозом на повећање калоријског уноса код оваријектомисаних женки.
5. Естрадиол поништава штетно дејство исхране обогаћене 10% раствором фруктозе на развој хиперинсулинемије и инсулинске резистенције код оваријектомисаних женки пацова, али не спречава настанак хипертриглицеридемије.
6. Естрадиол поништава све штетне ефекте исхране богате фруктозом на промене у нивоу протеинских компоненти ренин-ангиотензин система код оваријектомисаних женки, што води смањењу инфламације и штетног ремоделовања у ткиву срца.
7. Исхрана обогаћена фруктозом, посредством сигналног пута ANGII/AT1R/p65 fosfoSer276/MMP-9, може довести до инфламације и штетног ремоделовања ткива срца код оваријектомисаних женки пацова, док естрадиол своје протективно дејство испољава смањујући ниво свих компоненти овог пута, као и активацију NFκB транскрипционог регулатора.

На крају ове докторске дисертације налази се поглавље **Литература**. Она садржи 577 литературних цитата који су адекватно и на одговарајућим местима цитирани у тексту докторске дисертације.

Радови и конгресна саопштења из докторске дисертације:

Б1. Радови у часописима међународног значаја

1. Bundalo M, Živković M, Tepavčević S, Ćulafić T, Korićanac G, Stanković A. Fructose-rich diet-induced changes in expression of the renin-angiotensin molecules in the heart of ovariectomised female rats could be reversed by estradiol. *Hormone and Metabolic Research* 2015;47:521-7. **M23**
2. Bundalo M, Živković M, Ćulafić T, Stojiljković M, Korićanac G, Stanković A. Oestradiol treatment counteracts the effect of fructose-rich diet on matrix metalloproteinase 9 expression and NFκB activation. *Folia Biologica (Praha)* 2015;61:233-240. **M23**

Б2. Радови у часописима домаћег значаја

1. **M**
2. **M**

Б3. Конгресна саопштења на скуповима међународног значаја

1. Bundalo Maja, Tepavčević S, Romić S, Korićanac G, Živkovič M, Stanković A. The expression of ACE, ACE2, AT1 and AT2 receptors in the heart of fructose fed ovariectomised rats-effects of estrogen. 10th International Congress on Coronary Arthery Disease, Florence-Italy, october 13-16, 2013. **M34**

Б4. Конгресна саопштења на скуповима домаћег значаја

1. **M**
2. **M**

Мишљење и предлог Комисије:

На основу анализе докторске дисертације кандидата **Маје М. Бундало**, под насловом „**Утицај исхране богате фруктозом на експресију компоненти ренин-ангиотензин система и инфламације у ткиву срца пацова: полно специфичне разлике**“, Комисија закључује да резултати представљају оригиналан и значајан допринос разумевању полно специфичног ефекта исхране обогаћене фруктозом на експресију компоненти ренин-ангиотензин система и инфламације у ткиву срца. Треба посебно нагласити да резултати добијени у овој докторској дисертацији могу имати апликативни потенцијал у медицини у циљу превенције и нутриционистичке препоруке прилагођене полно специфичним разликама. Такође, добијени резултати би могли бити од значаја у даљем испитивању фенотипова као што су инсулинска резистенција, метаболички синдром и пратеће кардиоваскуларне болести које могу имати полно специфичну основу. Током израде дисертације кандидаткиња је показала висок степен познавања научне основе проблематике, добро је дефинисала хипотезу и циљеве, применила адекватне методе у истраживању и обради добијених резултата које је критички дискутовала, уз исцрпне податке из литературе.

Неоспоран квалитет дисертације потврђује и чињеница да је део резултата до сада објављен у међународним часописима и саопштењу на међународном скупу што додатно потврђује мишљење Комисије да резултати тезе представљају значајан помак у области молекуларне генетике која се бави испитивањем интеракције генетске основе, хормона и исхране.

На основу свега наведеног, Комисија предлаже Наставно-научном већу Биолошког факултета Универзитета у Београду да прихвати овај извештај и одобри јавну одбрану докторске дисертације Маје М. Бундало.

КОМИСИЈА:

_____

др Александра Станковић, научни саветник,
Универзитет у Београду, Институт за
нуклеарне науке „Винча“

_____

др Гордана Матић, редовни професор,
Универзитет у Београду, Биолошки факултет

_____

др Горан Корићанац, научни саветник,
Универзитет у Београду, Институт за
нуклеарне науке „Винча“

У Београду, 10.12.2015. године.

Fructose-Rich Diet-Induced Changes in the Expression of the Renin Angiotensin System Molecules in the Heart of Ovariectomized Female Rats Could be Reversed by Estradiol

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Fructose-Rich Diet-Induced Changes in the Expression of the Renin Angiotensin System Molecules in the Heart of Ovariectomized Female Rats Could be Reversed by Estradiol

Authors

M. Bundalo[1], M. Zivkovic[1], S. Tepavcevic[2], T. Culafic[2], G. Koricanac[2], A. Stankovic[1]

Affiliations

[1] Laboratory for Radiobiology and Molecular Genetics, Vinca Institute of Nuclear Sciences, University of Belgrade, Belgrade, Serbia
[2] Laboratory for Molecular Biology and Endocrinology, Vinca Institute of Nuclear Sciences, University of Belgrade, Belgrade, Serbia

Key words
- metabolic syndrome
- ovariectomy
- angiotensin converting enzyme
- angiotensin converting enzyme 2
- angiotensin II type 1 receptor
- angiotensin II type 2 receptor

Abstract

The renin-angiotensin system has been implicated in the development of metabolic syndrome and appears to be a key in the local tissue control of normal cardiac functions. Physiological concentrations of estrogens have been shown to be cardioprotective, especially against the damaging effects of fructose-rich diet. The aim of the study was to investigate the expression of the renin-angiotensin system molecules with potentially deleterious effect on the heart (angiotensin-converting enzyme and angiotensin II type 1 receptor) and those with potentially protective effects, (angiotensin-converting enzyme 2 and angiotensin II type 2 receptor), in ovariectomized fructose fed female rats with 17β-estradiol replacement. Real-time PCR and Western blot analysis were used for quantification of gene and protein expression in the heart. Fructose diet increased the expression of angiotensin-converting enzyme and angiotensin II type 1 receptor and decreased the expression of angiotensin-converting enzyme 2 and angiotensin II type 2 receptor. On the other hand, estradiol replacement seems to undo fructose diet effects on cardiac renin-angiotensin system. Downregulation of angiotensin-converting enzyme and angiotensin II type 1 receptor, and reversion of expression of both potentially protective molecules, angiotensin-converting enzyme 2 and angiotensin II type 2 receptor, to the control level in cardiac tissue took place. Obtained results suggest that estradiol may reverse the harmful effect of fructose-rich diet on the expression of renin-angiotensin system molecules. These findings may also be important in further research of phenotypes like insulin resistance, metabolic syndrome, and following cardiovascular pathology in females.

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Correspondence
A. Stankovic
Full Research Professor
Laboratory for Radiobiology and Molecular Genetics
Vinca Institute of Nuclear Sciences
University of Belgrade
P. O. Box 522
11000 Belgrade
Serbia
Tel.: +381/11/3408 566
Fax: +381/11/644 7485
alexas@vinca.rs

Introduction

Enhanced intake of fructose leads to the development of metabolic syndrome in humans and experimental animals [1, 2]. Metabolic syndrome is characterized by glucose intolerance, insulin resistance, central obesity, dyslipidemia, and hypertension. All these factors increase risk of cardiovascular disease [3]. Physiological concentrations of estrogens have been shown to be cardio protective [4] and they also have been assigned as protective against the damaging effects of fructose-rich diet (FRD) [5].

The changes in the expression of components of renin angiotensin system (RAS) have been implicated in the development of metabolic syndrome. It is known that all components of the RAS are generated in the heart [6]. The cardiac RAS maintains cell growth and proliferation in the heart, and mediates its adaptive response to myocardial stress in an appropriate cellular milieu [7]. In cardiomyocytes, similar to vascular smooth muscle cells and endothelium, pharmacological blockade of angiotensin II (AngII) action improves insulin sensitivity [8]. Previously, it was demonstrated that FRD affects the expression of ACE in aorta and angiotensin II type 1 (AT_1) receptor in heart and aorta of male rats [9]. Estrogens, including estradiol (E2), regulate several components of the RAS. Ovariectomy alone promotes changes in RAS that could be prevented by E2 replacement at physiological levels [10, 11] and even reversed by supraphysiological E2 treatment [12]. Still, this was not investigated in FRD. Based on these findings, we hypothesize that estradiol could have a beneficial effect on the expression of the heart tissue RAS after application of fructose-rich diet. The aim of the study was to investigate both protein and gene expression of molecules that are supposed to have

deleterious effect on cardiovascular tissue [(ACE and AT_1 receptor), and those with potentially protective effect (angiotensin-converting enzyme 2 (ACE2) and angiotensin II type 2 (AT_2) receptor] in ovariectomized females on FRD with E2 replacement.

Materials and Methods

Chemicals

The fructose was purchased from API-PEK (Becej, Serbia). The anti-ACE, anti-ACE2, anti-AT_1 receptors, and anti-actin antibodies were obtained from Santa Cruz Biotechnology, Inc. Additional anti-AT_1 receptor antibody was purchased from Pierce, Thermo Fisher Scientific. The anti-AT_2 receptor antibody was purchased from Abcam. Secondary anti-goat and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology. The 17β-estradiol was a product of Sigma-Aldrich Corporation.

Animals

All animal experiments conformed to the Directive 2010/63/EU of the European Parliament. The official Vinca institute's Ethical Committee for Experimental Animals approved the research. Female Wistar rats (21-days-old) were separated from their mothers and divided in the control group on a normal diet (ND) and fructose fed rats (FRD). The animals held on normal diet had free access to the tap water and normal rat chow. Animals held on FRD were fed standard food, but water was replaced with a 10% (w/v) fructose solution in tap water. Duration of diet regime was 9 weeks. All animals were kept under standard temperature and dark-light conditions. Ovariectomy (OVX) was performed 2 weeks prior to the sacrifice, under ketamine (40 mg/kg, intraperitoneally)/xylazine (5 mg/kg, intraperitoneally) anesthesia. Half of the FRD were subjected to E2 replacement treatment (40 µg/kg in volume of 100 µl/250 g) subcutaneously, every 2 days starting from the day after the ovariectomy, which continued until the day before sacrifice, in order to achieve the concentration near the physiological level [13]. The evidence collected in the past years indicates that the heart is regulated by a complex interplay of genomic and nongenomic signaling mechanisms of E2, and the integrated action of these machineries has important functional roles in a regulation of physiological processes. In view of these facts, we selected the experimental timeline that allows the E2 to realize both kinds of effects, particularly long-term genomic effects [14]. Non-estrogen treated animals were injected with vehicle (linseed oil, as estradiol was resolved in it) to avoid the effects of injection stress. Each experimental group contained 9 animals [OVX rats on ND (control group), OVX rats on FRD, OVX rats on FRD treated with E2; total of 27 animals] in accordance with the standards of statistics and ethics.

In order to assess the effect of FRD, we compared ovariectomized fructose fed rats with ovariectomized rats subjected to the normal diet. In order to estimate the effect of E2 in the context of FRD, we compared ovariectomized fructose fed rats with ovariectomized fructose fed animals treated with E2.

Biochemistry

After overnight fasting of animals, the blood samples were collected at decapitation in EDTA-pretreated tubes and centrifuged at 3000 rpm for 10 min in order to obtain the plasma samples. RIA method was used to determine plasma insulin levels. Homeostasis model assessment index (HOMA), an indicator of insulin resistance, was calculated from the fasted plasma insulin and the glucose concentration using previously described formula [15]. The triglycerides level was measured using a Multicare analyzer (Biochemical Systems International, Arezzo, Italy).

Preparation of cardiac cell lysate

After sacrifice, each heart was removed and washed with cold saline, quickly blotted with filter paper in order to remove surface liquid and weighed. The tissue was homogenized in cold with an Ultra-Turrax homogenizer in a buffer (pH 7.4) containing 10 mM Tris, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, protease inhibitors (2 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml aprotinin). The homogenates were centrifuged at 600×*g* for 20 min at 4 °C, and the obtained supernatants were centrifuged at 100 000×*g* for 60 min. BCA method was used to determine protein concentration. Supernatants were boiled in Laemmli sample buffer and used for Western blot analysis.

SDS-PAGE and western blotting

Cardiac lysate proteins (100 µg/lane) were loaded onto 10% SDS polyacrylamide gels and separated by electrophoresis. Then, proteins were transferred to PVDF membranes. PVDF membranes were blocked with 5% bovine serum albumin and incubated with primary antibodies against ACE (sc-12187, dilution 1:250), ACE2 (sc-17720, dilution 1:250), AT_1 receptor (sc-1173, dilution 1:200 and PA5-20812, dilution 1:500), AT_2 receptor (ab19134, dilution 1:2 500), and stayed overnight at 4 °C. Membranes were washed and incubated with secondary anti-rabbit or anti-goat horseradish peroxidase-conjugated antibody (dilution 1:10 000) for 2 h at room temperature and signals were detected with ECL reagents. To avoid ECL signal saturation we empirically optimized the protein quantity per well in SDS PAGE, as well as primary and secondary antibody concentration and the conditions of signal development. Since we did not observe cardiac hypertrophy [16,17], the actin antibody was used as internal standard to ensure that protein loading was equal in all lysate samples. Films were scanned and analyzed using the ImageJ software (NIH, USA).

RNA isolation and quantitative real-time reverse transcriptase-PCR (qRT-PCR)

Total RNA was extracted from the frozen (−70 °C) hearts using TRIzol Reagent (Ambion, Inc.). The quantity of RNA was measured spectrophotometrically (NanoDrop® ND-1000, Thermo Scientific). Contaminant genomic DNA in RNA samples were avoided by treatment with DNase I (1U/2 µg RNA) for 1 h at 37 °C. Reverse transcription was performed using 2 µg of total pure RNA and First Strand cDNA Synthesis kit, with oligodT18 primers, according to manufacturer's instructions (Fermentas, Lithuania). Mock reaction lacking reverse transcriptase was performed during the cDNA synthesis step.

Real-time PCR was performed in duplicate in an ABI Real-time 7500 system (ABI, Foster City, CA). Detection of AT_1 receptor and AT_2 receptor gene expression was done by amplification in the total volume of 25 µl using the pre-developed TaqMan® Gene Expression Assays Rn02758772_s1 (AT_1 receptor) and Rn00560677_s1 (AT_2 receptor) (ABI, Foster City, CA). Detection of internal reference 18s rRNA was done by the predeveloped TaqMan® Gene Expression Assays ID Hs99999901_s1 (ABI, Foster City, CA, USA). Cycling parameters were as follows: an initial denaturation at 95 °C for 10 min, followed by 40 cycles of a dena-

turation step at 95 °C for 15 s, and annealing step at 60 °C for 60 s. The 18s rRNA served as an internal reference to standardize the amount of sample RNA added to a reaction.

Statistical analysis

Data are presented as mean ± standard deviations for 9 animals per each experimental group (total of 27 animals). Each Western blot experiment was performed at least 3 times independently. Data were analyzed by Mann-Whitney U-test. The GraphPad Prism 5 statistical package was used. Values of $p < 0.05$ were considered statistically significant. Differences in mRNA expression between groups were analyzed using $2^{-\Delta\Delta Ct}$ method [18].

Results

Biochemical characteristics of animals

Fructose-rich diet elevated plasma insulin and triglycerides levels, as well as HOMA index ($p < 0.05$, ND-OVX vs. FRD-OVX). Estradiol treatment decreased insulin level and HOMA index in fructose fed animals ($p < 0.05$, FRD-OVX vs. FRD-OVX + E2) and reverted these parameters on the level detected in ND-OVX animals ($p > 0.05$, ND-OVX vs. FRD-OVX + E2). Triglycerides level in fructose fed animals remained on the higher level compared to ND-OVX animals even after E2 treatment ($p < 0.05$, ND-OVX vs. FRD-OVX + E2) (**Fig. 1**).

Effects of the fructose-rich diet and estradiol status on the expression of ACE and ACE2 in the rat heart

The present study showed that in ovariectomized females FRD had increased ACE protein levels in the heart ($p < 0.01$, ND-OVX vs. FRD-OVX), while E2 treatment had significantly decreased it ($p < 0.01$, FRD-OVX vs. FRD-OVX + E2) even lower than in rats that were fed a normal diet ($p < 0.01$ ND-OVX vs. FRD-OVX + E2) (**Fig. 2a**).

As presented in **Fig. 2b**, the FRD significantly decreased ACE2 protein expression in the heart ($p < 0.05$, ND-OVX vs. FRD-OVX), while E2 replacement significantly increased ACE2 protein expression, compared to FRD-OVX ($p < 0.05$, FRD-OVX vs. FRD-OVX + E2), reversing it to the level detected in rats that were fed a normal diet ($p > 0.05$, ND-OVX vs. FRD-OVX + E2).

Effects of the fructose-rich diet and estradiol status on the expression of angiotensin II receptors in the rat heart

The FRD increased AT_1 receptor protein expression in the heart of ovariectomized females ($p < 0.001$, ND-OVX vs. FRD-OVX, **Fig. 3a**), but there was no change in the AT_1 receptor mRNA expression (**Fig. 3b**). E2 treatment in fructose fed ovariectomized females significantly decreased AT_1 receptor protein and mRNA expression in the heart compared to the FRD-OVX group ($p < 0.001$ for protein, and $p < 0.05$ for mRNA expression, FRD-OVX vs. FRD-OVX + E2) as well as AT_1 receptor protein compared to ND-OVX females ($p < 0.001$, ND-OVX vs. FRD-OVX + E2). As the specificity of commercial antibodies against the AT1 receptor has been recently called into question [19], we performed Western blotting in parallel, with additional primary antibody against the AT1 receptor (PA5-20812, Pierce). The quantification with both antibodies gave the same results.

The FRD decreased AT_2 receptor protein expression ($p < 0.05$, ND-OVX vs. FRD-OVX, **Fig. 4**), while E2 treatment increased it in the heart of FRD-OVX ($p < 0.05$, FRD-OVX vs. FRD-OVX + E2) and reversed it to the level detected in animals on normal diet ($p > 0.05$ ND-OVX vs. FRD-OVX + E2). We did not detect AT_2 receptor mRNA expression in any of the comparison groups. To ensure that PCR reaction was successful, the positive control sample with known AT_2 receptor mRNA expression was used (cDNA from adipose tissue and heart of intact female rats, unpublished data).

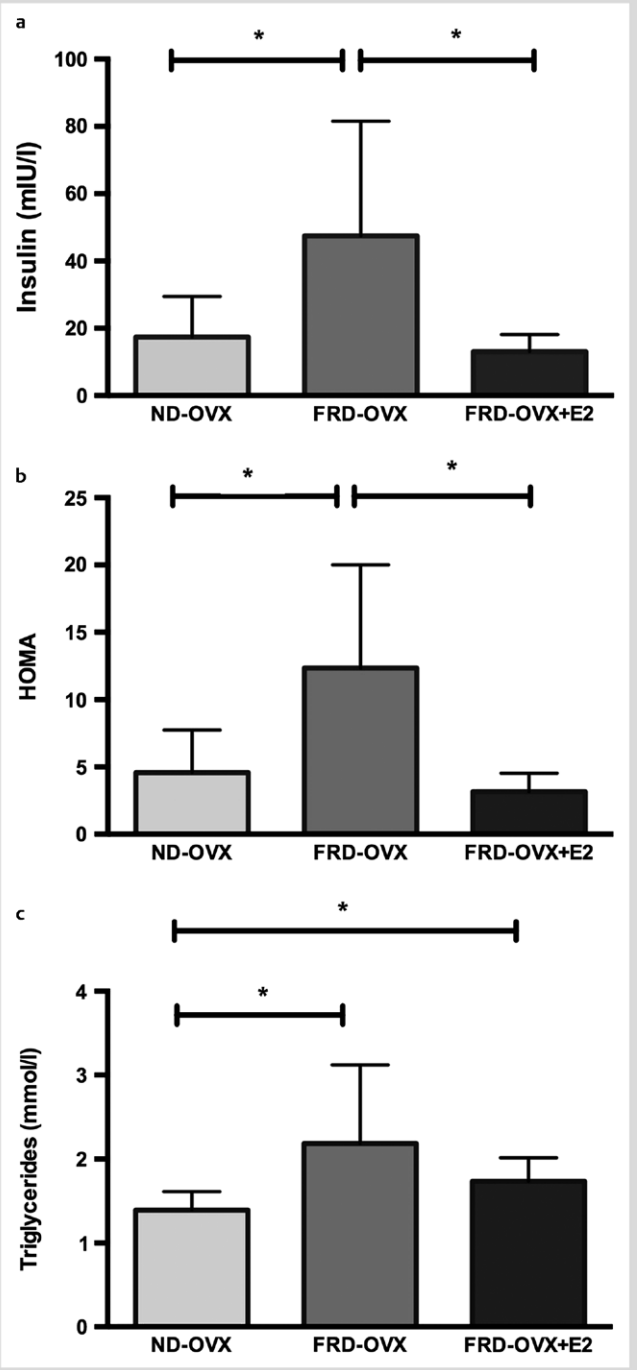

Fig. 1 Effects of fructose-rich diet and estradiol on the biochemical characteristics of ovariectomized female rats. Values are means, with their standard deviations represented by vertical bars. OVX: ovariectomy; ND: Rats on normal diet; FRD: Rats on fructose-rich diet; E2: Estradiol treatment. * $p < 0.05$.

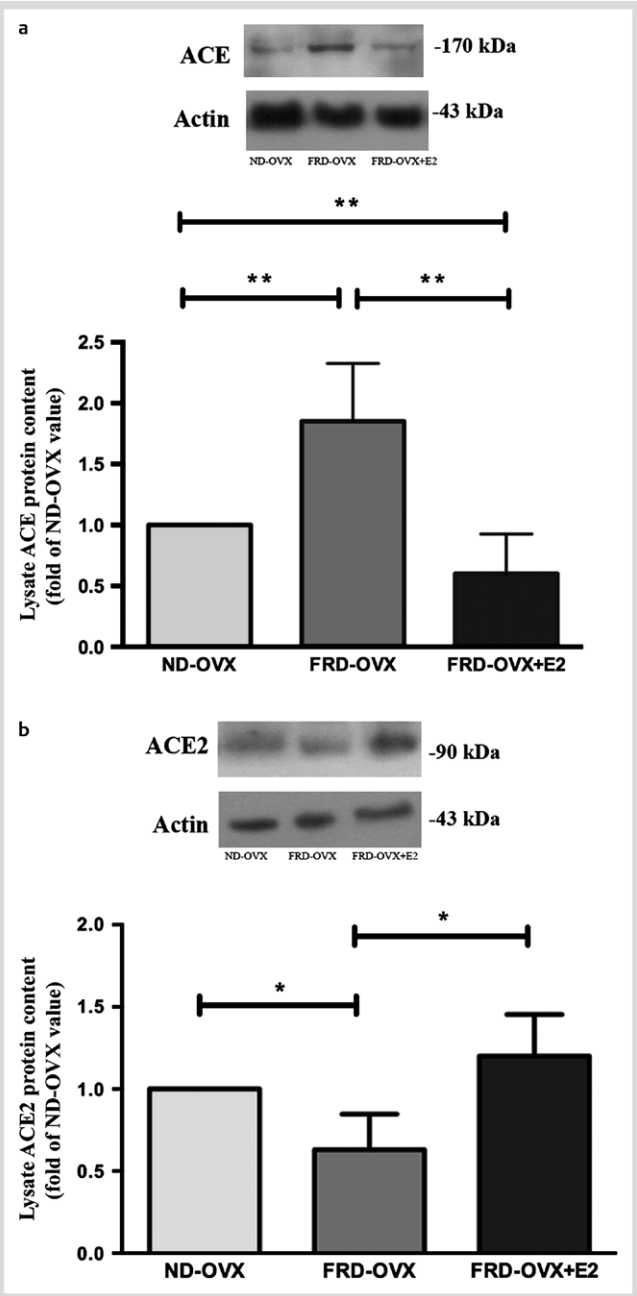

Fig. 2 ACE and ACE2 protein expression in the ovariectomized fructose fed rats in the presence or absence of estradiol. Protein samples of cardiac cell lysate were analyzed by the Western blot method using the antibody against ACE **a** and ACE2 **b**. We performed the experiments 3 times independently. Each experimental group contained 9 animals. Results are expressed as a fold of appropriate control value (ovariectomized rats on the normal diet, ND-OVX). Values are means, with their standard deviations represented by vertical bars. Representative Western blots are also shown. OVX: Ovariectomy; ND: Rats on normal diet; FRD: Rats on fructose-rich diet; E2: Estradiol treatment. $^{*}p<0.05$; $^{**}p<0.01$.

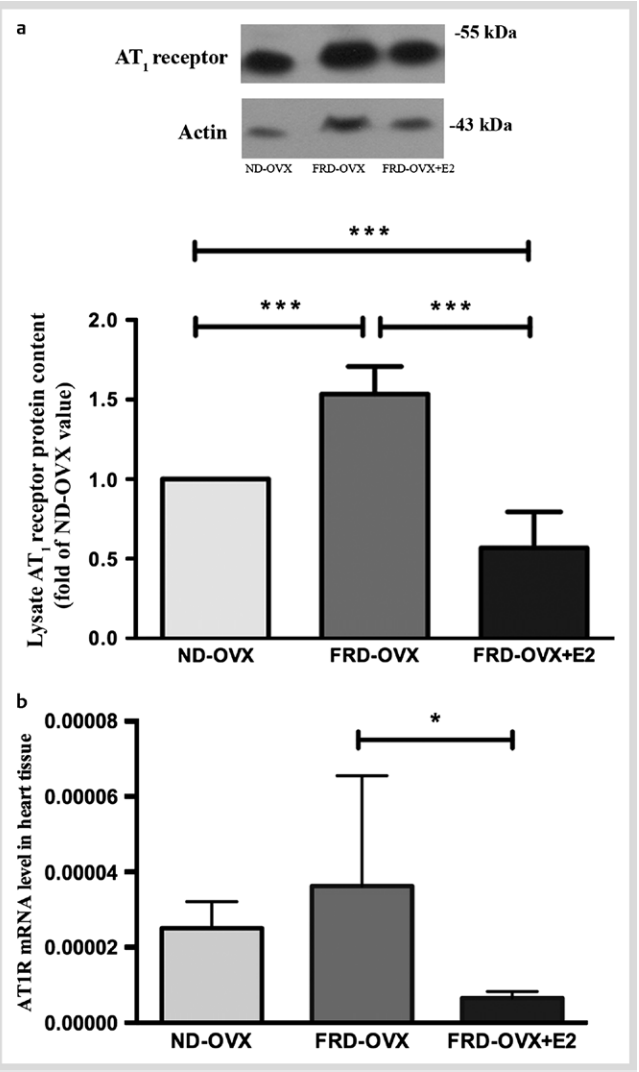

Fig. 3 AT_1 receptor protein and gene expression in ovariectomized fructose fed rats with or without the estradiol replacement. **a** Protein samples of cardiac cell lysate were analyzed by the Western blot method using the antibodies against AT_1 receptor. The results of 3 independent experiments with a total of 9 animals per each group were quantified. Results are expressed as a fold of appropriate control value (ovariectomized rats on the normal diet, ND). Values are means, with their standard deviations represented by vertical bars. Representative Western blots are also shown. **b** AT_1 receptor mRNA level in the heart of ovariectomized fructose fed rats with or without E2 replacement. Results are expressed as $2^{-\Delta Ct}$ values. Values are means for 9 animals per each experimental group, with a standard deviation represented by vertical bars. OVX: Ovariectomy; ND: Rats on normal diet; FRD: Rats on fructose-rich diet; E2: Estradiol treatment. $^{*}p<0.05$; $^{***}p<0.001$.

Discussion and Conclusion

The present study showed that FRD affects RAS components in the cardiac tissue of ovariectomized females, demonstrated by the increase of the expression of potentially deleterious molecules ACE and AT_1 receptor and decreased expression of ACE2 and AT_2 receptor. These findings coincide with the elevated plasma triglycerides, insulin and HOMA in fructose fed rats. On the other hand, the E2 replacement therapy seems to undo FRD effects on cardiac tissue RAS and metabolic parameters. It was demonstrated by the downregulation of ACE and AT_1 receptor and the reversion of expression of both potentially protective molecules in cardiac tissue, AT_2 receptor to the control level, and ACE2 significantly above the levels detected in normal-diet animals.

The local tissue RAS appears to be a key in the control of normal cardiac functions. It was previously demonstrated that FRD affects the expression of ACE in aortas and AT_1 receptor in hearts and aortas of male rats [9]. Other study also showed upregulation of AT_1 receptor mRNA and unchanged AT_2 receptor mRNA in

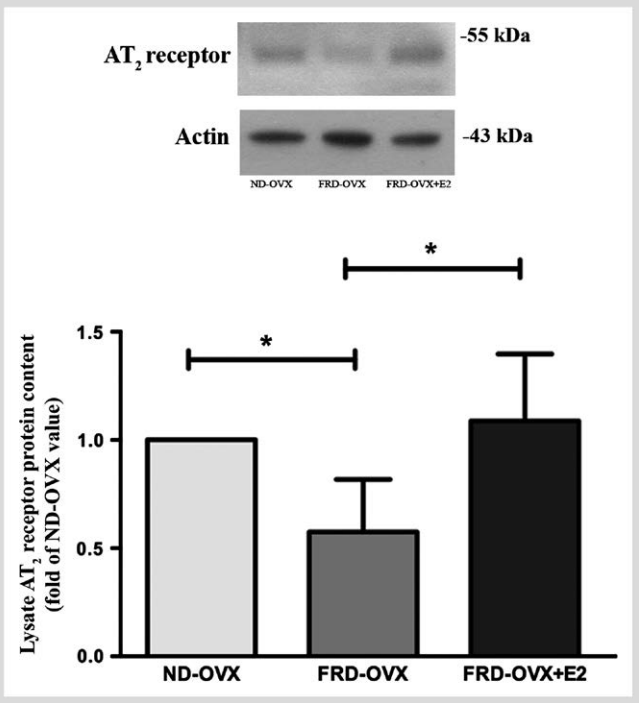

Fig. 4 AT_2 receptor protein expression in the ovariectomized fructose fed rats with or without estradiol replacement. Protein samples of cardiac cell lysate were analyzed by the Western blot method using antibody against an AT_2 receptor. We performed the experiments 3 times independently. Each experimental group contained 9 animals. Results are expressed as a fold of appropriate control value (ovariectomized rats on the normal diet, ND). Values are means, with their standard deviations represented by vertical bars. Representative Western blots are also shown. OVX: Ovariectomy; ND: Rats on normal diet; FRD: Rats on fructose-rich diet; E2: Estradiol treatment. * $p<0.05$.

aorta under the 60% FRD [20]. Prolonged AT_1 receptor activation leads to myocyte hypertrophy, which becomes a major risk factor for congestive heart failure, sudden cardiac death, and overall mortality [21]. In current experimental model of FRD regime, we did not observe cardiac hypertrophy [17] but, significant increase in AT_1 receptor and ACE expression, which we found in this study, could imply that hypertrophy would occur if the FRD regime was prolonged. An increased heart-to-body weight ratio, myocyte diameter, as well as left ventricular fibrosis and perivascular collagen type III deposition were detected previously in hearts of fructose fed male rats [22]. In addition, angiotensin (1–7) [Ang-(1–7)] treatment ameliorated cardiac hypertrophy, fibrosis, and mild hypertension and attenuated the growth-promoting pathways in the hearts of these animals. But, the main RAS molecules have not been investigated, as well as the E2 effects in fructose fed females.

In the current study, we detected early changes of the RAS molecules expression in heart of female animals, which are of the great importance since they reflect tissue changes on the subclinical level. Many experimental and clinical data showed beneficial cardiovascular effects of treatment with antagonists of the RAS. Thus, the RAS is a reasonable target for prevention of fructose-induced cardiovascular pathologies.

It was shown previously that FRD induces a cluster of abnormalities, including hypertension, hypertriglyceridemia, and glucose intolerance in addition to hyperinsulinemia [20], which is in agreement with our results. The ACE and AT_1 receptor upregulated by FRD in this study have been involved in the insulin resistance, also an early phenotype of metabolic syndrome. Namely, we reported recently that the same animals on the fructose diet developed cardiac insulin resistance [23]. ACE generates the Ang II [24,25], that through AT_1 receptor activation, leads to increased generation of reactive oxygen species (ROS). This further induces inflammation and fosters insulin resistance [26].

Moreover, RAS could influence cardiac insulin signaling and endothelial dysfunction. Namely, activation of AT_1 receptor triggers JNK and MAP-kinase pathways that may lead to increased serine phosphorylation of IRS-1 (Ser^{312} and Ser^{616}), impaired insulin-induced PI3K/Akt/eNOS signaling pathway, and finally endothelial dysfunction [27]. Importantly, AT_1 receptor-mediated MAPK activation has been shown to inhibit insulin signaling in immortalized cardiomyocytes (HL-1 cells) [28]. These mechanisms could be the focus of the future studies. Based on the reported facts we suggest that previously observed insulin resistance and impaired cardiac insulin signaling in ovariectomized fructose fed rats [17,23,29] are, at least partly, caused by activated local RAS in a heart.

Interestingly, our study showed that FRD upregulated AT_1 receptor protein level in the hearts of ovariectomized females, while there was no statistically significant change in AT_1 receptor gene expression. This may indicate the possibility of post-translational control of AT_1 receptor expression. We can speculate that FRD affects the stability of AT_1 receptor protein by protecting the receptor from post-translational degradation. On the other hand, we have demonstrated that E2 replacement therapy decreased both AT_1 receptor protein and gene expression in fructose fed ovariectomized rats. Previous studies showed that E2 can directly modulate AT_1 receptor transcription through the estrogen response elements (ERE) in the 5′-flanking region of the receptor gene [30] or it can inhibit AT_1 receptor translation [31]. We can conclude that in our experimental model E2, at least partly, downregulated AT_1 receptor protein expression by decreasing the AT_1 receptor mRNA expression.

Upregulation of ACE and AT_1 receptor protein expression in the hearts of fructose fed ovariectomized rats was diminished by E2 replacement therapy. This goes in line with the results showing that this E2 treatment restitutes control plasma insulin level [29] and suppresses negative effect of FRD on cardiac insulin resistance [17]. Thus, we can propose that E2 may achieve the protective effect on the development of insulin resistance induced by FRD and cardiac impairment, at least partly through reduced expression of ACE and AT_1 receptor.

It was previously shown that AT_1 and AT_2 receptors have the opposite role in the control of blood pressure and glucose homeostasis in fructose-induced insulin-resistant hypertensive rats [32]. It has also been shown that angiotensin II-mediated NADPH oxidase activation occurs via the action of the AT_1 receptor, whereas the AT_2 receptor appears to inhibit oxidase activation [33]. Our study demonstrated that FRD significantly down regulated the AT_2 receptor protein expression in ovariectomized females, while the E2 treatment reverted it to the level detected in rats fed a normal diet. Because AT_2 receptor shows antiproliferative, antifibrotic and blood pressure-lowering properties [34,35] we can assume that E2, by increasing AT_2 receptor expression, helps to protect the heart from the harmful effects of FRD.

Furthermore, our study showed that FRD decreased the ACE2 expression while the E2 replacement induced the ACE2 expression and reversed it to physiological level. The ACE2 cleaves the AngII into the inactive Ang-(1–7). It is highly expressed in a heart. It was shown that the mice carrying ACE, but not ACE2

gene, developed cardiovascular disease and impaired heart function. Targeted disruption of ACE2 in mice lead to severe impairment in myocardial contractility and increased AngII level [36]. Also, a new molecule of the RAS with vasodilating, antifibrotic, antihypertensive, and central effects alamandine is generated by catalytic action of ACE2 [37]. Increased expression of ACE2 seems to be an additional mechanism by which E2 exerts its protective effect against FRD.

It is known that, when overactivated, the AngII/AT_1 receptor axis is essentially involved in the cardiovascular pathology, such as hypertension and related cardiac hypertrophy, as well as the vascular remodeling in hypertension and diabetes [38]. But, complexity has been added to the RAS through the discovery of several axes, which counteract the AngII/AT_1 receptor axis and act tissue protective. The main axis so far is AngII/AT_2 receptor. The others are Ang-(1–7)/Mas receptor, and alamandine/Mas related, G-protein-coupled receptor. Current study was focused on the main AngII receptors, the AT_1 and AT_2 receptors, and 2 enzymes, one of which produces AngII and the one that removes it, ACE and ACE2. Further studies should include the other molecules from complex RAS cascade.

In conclusion, FRD induced the increase in the expression of ACE and AT_1 receptor and the decrease in the expression of ACE2 and AT_2 receptor in heart tissue. The E2 replacement abolishes the effect of FRD on upregulation of RAS components that have profibrotic, proliferative and proinflammatory actions in states of prolonged activation, and enhances the expression of potentially protective RAS molecules in the heart. Obtained results could be of utmost importance in further research of phenotypes like insulin resistance, metabolic syndrome development, and following cardiovascular pathology in females.

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Conflict of Interest

The authors declare that they have no conflict of interest in the authorship or publication of this contribution.

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Original Article

Oestradiol Treatment Counteracts the Effect of Fructose-Rich Diet on Matrix Metalloproteinase 9 Expression and NFκB Activation

(fructose-rich diet / matrix metalloproteinase 9 / NFκB / oestradiol, heart / rat)

M. BUNDALO[1], M. ZIVKOVIC[1], T. CULAFIC[2], M. STOJILJKOVIC[2], G. KORICANAC[2], A. STANKOVIC[1]

[1]Laboratory for Radiobiology and Molecular Genetics, [2]Laboratory for Molecular Biology and Endocrinology, Vinca Institute of Nuclear Sciences, University of Belgrade, Belgrade, Serbia

Abstract. Fructose-rich diet induces metabolic changes similar to those observed in metabolic syndrome. Among other matrix metalloproteinases, MMP-9 has an important role in adverse cardiac remodelling and might have a role in the development of cardiovascular disorders associated with metabolic syndrome. The changes of MMP-9 expression could be mediated via the NFκB pathway. In this study we investigated the effect of fructose-rich diet on MMP-9 expression in the heart of male and female rats, along with the effect of fructose-rich diet and oestradiol on MMP-9 expression in ovariectomized females. We further assessed the effect of fructose-rich diet and oestradiol on NFκB activation, measured as the level of p65 phosphorylation at Ser 276. The results showed that the diet regime did not affect the heart mass. Higher MMP-9 gene expression was found in cardiac tissue of male rats fed the fructose-rich diet than in females on the same diet regime. In ovariectomized females, fructose-rich diet upregulated MMP-9 protein and mRNA expression in the heart, as well as phosphorylation of the p65 subunit of NFκB at Ser 276. Oestradiol replacement therapy reverted these changes in the heart of ovariectomized females. This study has shown that oestradiol could revert the early molecular changes in MMP-9 expression induced by fructose-rich diet that occurred before cardiac hypertrophy development by decreasing phosphorylation of the NFκB p65 subunit at Ser 276.

Introduction

Metabolic syndrome is described as a pro-inflammatory condition associated with increased risk for diabetes, accelerated atherosclerosis and increased incidence of cardiovascular diseases (Isomaa et al., 2001; Grundy et al., 2004; Paoletti et al., 2006). It combines several risk factors for cardiovascular disease occurrence: hypertension, insulin resistance, hyperinsulinaemia, dyslipidaemia and abdominal obesity (Eckel et al., 2005; Grundy, 2007). Numerous experiments on animals and human demonstrated that fructose-rich diet (FRD) might induce metabolic changes similar to those observed in metabolic syndrome (Dai and McNeil, 1995; Sharabi et al., 2007; Orron-Herman et al., 2008; Axelsen et al., 2010; Dekker et al., 2010; Tappy and Le, 2010).

Recently, it was suggested that matrix metalloproteinases (MMPs) might have a role in the development of cardiovascular disorders associated with metabolic syndrome (Cicero et al., 2007; Miksztowich et al., 2008). MMPs are calcium dependent, zinc-containing endopeptidases, which are involved in extracellular matrix (ECM) remodelling (Bode and Maskos, 2003; Visse and Nagase, 2003). Knowing that adverse ECM remodelling of the myocardium and vasculature leads to the development of cardiovascular disorders, it is not surprising that MMPs represent an important biological components in the myocardium (Spinale, 2002; Lopes et al., 2004; Newby, 2005).

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Corresponding author: Aleksandra Stankovic, Vinca Institute of Nuclear Sciences, University of Belgrade, Laboratory for Radiobiology and Molecular Genetics, PO Box 522, 11000 Belgrade, Serbia. Phone: (+ 381) 11 3408 566; Fax: (+ 381) 11 644 7485; e-mail: alexas@vinca.rs

Abbreviations: ACE – angiotensin-converting enzyme, AT1R – angiotensin type 1 receptor, BCA – bicinchoninic acid, cDNA – complementary DNA, E2 – 17β-oestradiol, ECM – extracellular matrix, FRD – fructose-rich diet, IκB – inhibitor κB, IL – interleukin, MMP – matrix metalloproteinase, ND – normal diet, NFκB – nuclear factor κ-light-chain-enhancer of activated B cells, OVX – ovariectomized, PVDF – polyvinylidene fluoride, Q-PCR – quantitative real-time PCR, rRNA – ribosomal RNA, SDS – sodium dodecyl sulphate, SHHF – spontaneously hypertensive heart failure, TNF-α – tumour necrosis factor α.

MMP-9 or gelatinase B, which is an inducible enzyme tearing down type IV collagen (Gioia et al., 2009), has been shown to play an important role in the pathogenesis of a wide spectrum of cardiovascular disorders (Dollery et al., 1995; Lindsay et al., 2002). An elevated level of MMP-9 represents one of the risk factors for development of cardiovascular diseases and myocardial infarction (Ferroni et al., 2003). An increasing circulating MMP-9 level and activity was found in patients with metabolic syndrome (Cicero et al., 2007; Goncalves et al, 2009), hyperglycaemia (Lee et al., 2005), and type 2 diabetes mellitus (Uemura et al., 2001). Previous studies have shown that FRD enhanced MMP-9 activity in rat smooth muscle cells (Lu et al., 2013), as well as the MMP-9 protein level in aortic tissue of ApoE-KO mice (Cannizzo et al., 2012). As far as we know, there are no experimental studies investigating the effects of FRD on MMP-9 expression in the heart. Because MMP-9 expression shows gender differences with higher levels detected in male rats (Woodrum et al., 2005), we considered interesting to examine whether FRD affects MMP-9 expression in a gender-specific manner and to evaluate the influence of female sex hormones on MMP-9 expression in the context of fructose-rich diet.

The changes of MMP-9 expression could be mediated via the nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) pathway (Guo et al., 2008). NFκB is activated through phosphorylation of the p65 subunit at Ser 276 (Kim et al., 2012). Therefore, we hypothesized that phosphorylation of the p65 subunit at Ser 276 could be implicated in the induction of MMP-9 expression.

The purpose of this study was to investigate the effect of FRD on MMP-9 expression in the heart of male and female rats and to evaluate the effect of oestrogen on MMP-9 expression in fructose-fed rats. We further assessed the effect of FRD and 17β-oestradiol (E2) on NFκB activation, measured as the level of p65 phosphorylation at Ser 276.

Material and Methods

Chemicals

We purchased fructose from API-PEK (Becej, Serbia). Anti-NFκB p65 (phospho S276) and anti-MMP-9 antibodies were obtained from Abcam (Cambridge, UK). Secondary anti-rabbit antibody was the product of Santa Cruz Biotechnology (Santa Cruz, CA). 17β-oestradiol (E2) was purchased from Sigma-Aldrich Corporation (St.Louis, MO).

Animals

This research was approved by the official Vinca Institute's Ethical Committee for Experimental Animals and conformed to the Directive 2010/63/EU of the European Parliament. Twenty-one-day-old male and female Wistar rats were separated from their mothers. Rats were bred at Animal facility of Vinca institute of Nuclear Sciences. Male rats were divided into a normal diet (ND-male) and a fructose-fed group (FRD-male). Females were divided into an intact and an ovariectomized group. Intact females were further divided into a normal diet (ND-female) and a fructose-rich diet (FRD-female) group. Ovariectomized females were divided into a normal diet group (ND-OVX), a fructose/rich diet group (FRD-OVX) and animals fed the fructose-rich diet and subjected to oestradiol replacement therapy (FRD-OVX+E2). Animals held on normal diet had free access to the tap water and normal laboratory chow. Animals fed the fructose-rich diet also had standard laboratory chow, while 10 % (w/v) fructose solution replaced the tap water. This diet regime lasted for nine weeks. In the ovariectomized group, ovariectomy was performed two weeks before sacrifice under ketamine (40 mg/kg, intraperitoneally) – xylazine (5 mg/kg, intraperitonelly) anaesthesia. E2 replacement therapy (40 μg/kg, subcutaneously, every second day to achieve concentration near the physiological level) started a day after ovariectomy and continued until the day before sacrifice (Sales et al., 2010). This duration of E2 replacement therapy was shown to allow E2 to achieve long-term genomic effects, but is also sufficient for E2 to influence non-genomic signalling mechanisms (Koricanac et al., 2009). In order to avoid the effects of injection stress, ND-OVX and FRD-OVX animals were injected with vehicle (linseed oil). Each experimental group contained nine animals (N = 9), which means that a total of 63 animals participated in the experiment.

Measurement of body mass and mass of heart

Body mass was measured at the end of the experiment. Heart mass was determined immediately after killing the animals. The hearts were excised, washed in saline and dried before the measurement.

Tissue homogenization and Western blot

Heart tissue was homogenized with an Ultra-turrax homogenizer in homogenization buffer (pH = 7.4; containing: 10 mM Tris, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, protease inhibitors – 2 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin and phosphatase inhibitors – 1 mM Na_3VO_4 and 10 mM NaF) and homogenates were centrifuged at 600 *g* for 20 min. The supernatants were further ultracentrifuged at 100,000 *g* for 60 min. To determine the protein concentration we used the BCA method. After boiling in Laemmli sample buffer, supernatants were used for Western blot analysis. Equal amounts of proteins (100 μg/lane) were fractioned by 10% SDS polyacrylamide gels and transferred on PVDF membranes. Membranes were blocked with 5% bovine serum albumin for 2 h at room temperature, after which they were incubated with primary antibodies MMP-9 (ab7299, dilution 1 : 500) and NFκB p65 (Phospho S276) (ab30632, dilution 1 : 1000) overnight at 4 °C. Membranes were subsequently washed and incubated with peroxidase-labelled secondary anti-rabbit antibody for two hours at room temperature. Signals were detected with ECL reagents.

All Western blot experiments were performed in triplicates. To assess the equal protein loading, we used β-actin as a loading control. We optimized the protein quantity, primary and secondary antibody concentrations, and conditions of signal development in order to avoid ECL signal saturation. Films were scanned and intensities were determined using ImageJ software (NIH, Bethesda, MD).

RNA isolation and expression by real-time polymerase chain reaction

Total RNA was extracted from the heart tissue using Trizol reagent (Ambion Inc., Austin, TX) according to the protocol recommended by the manufacturer. The quantity of RNA was assessed spectrophotometrically (NanoDrop® ND-1000, Thermo Scientific, Rockford, IL). In order to eliminate the possible contamination with genomic DNA, purified RNA was treated with DNAse I. First strand cDNAs were generated from 2 μg of total pure RNA and First Strand cDNA Synthesis kit, with oligodT18 primers, according to manufacturer's instructions (Fermentas, Vilnius, Lithuania). Quantitative real-time PCR (Q-PCR) amplification was performed in duplicate using the ABI Real-time 7500 system (ABI, Foster City, CA). The relative levels of specific MMP-9 mRNA in heart were assessed by amplification in a total volume of 25 μl by pre-developed TaqMan® Gene Expression Assays Rn00579162_m1. 18s rRNA was used as an internal reference (Gene Expression Assays ID Hs99999901_s1). Cycling parameters were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of a denaturation step at 95 °C for 15 s, and an annealing step at 60 °C for 60 s. In order to process the data and compare the differences in relative gene expression between groups we used the $2^{-\Delta Ct}$ method. The calculated C_t values for MMP-9 in response to various treatments were normalized to the respective C_t values for 18S rRNA.

Statistical analysis

Data were analysed using the GraphPad Prism 5 statistical package (San Diego, CA). All data are expressed as mean ± standard deviation, for nine animals per each experimental group. Values of continuous variables with skewed distribution were compared by the nonparametric Mann-Whitney U test, which was used to examine the statistical significance among experimental groups. The differences in relative gene expression between groups were calculated using $2^{-\Delta Ct}$ methods (Livak and Schmittgen, 2001). Values of $P < 0.05$ were considered statistically significant.

Results

Body mass between female and male rats was significantly different in both, ND and FRD group ($P < 0.001$, ND Female *vs*. ND Male; $P < 0.05$, FRD Female *vs*. FRD Male) (Table 1). In males, FRD did not alter the body mass, while in females FRD increased the body mass ($P < 0.05$, ND Female *vs*. FRD Female). The mass of the heart was not affected by the diet regime regardless of sex. Males that were fed normal diet had significantly higher mass of the heart than females on the same diet regime ($P < 0.01$, ND Female *vs*. ND Male). The heart-to-body ratio did not differ among the groups.

To examine the effect of FRD on *MMP9* gene expression we performed quantitative RT-PCR analysis. The results showed higher *MMP9* gene expression in FRD-male compared to the FRD-female group ($P < 0.01$, FRD Female vs. FRD Male) (Fig. 1a). FRD did not change *MMP9* gene expression in females. In males, FRD upregulated *MMP9* expression, but this showed not to be statistically significant. The fact that the *MMP9* gene showed higher expression in FRD-males compared to FRD-females made us to focus on the role of oestradiol in the regulation of *MMP9* expression in the heart of fructose-fed rats.

We therefore compared the effects of FRD and subsequent oestradiol treatment on ovariectomized female rats. In ovariectomized females, FRD upregulated *MMP9* gene expression ($P < 0.01$, ND-OVX *vs*. FRD-OVX). In contrast, E2 replacement therapy significantly down-regulated *MMP9* gene expression in fructose-fed ovariectomized animals ($P < 0.01$, FRD-OVX *vs.* FRD-OVX+E2) and decreased it even below the level detected in ND ovariectomized females ($P < 0.01$, ND-OVX *vs*. FRD-OVX) (Fig. 1b).

As presented in Table 2, neither FRD nor E2 replacement therapy affected body mass or the mass of the heart. This showed that heart hypertrophy had not yet occurred.

To (semi)quantitatively assay the MMP-9 protein level in the context of FRD and subsequent E2 replacement therapy, we performed Western blot analysis. These results are presented in Fig. 2. Similarly as for the mRNA level, FRD significantly increased the MMP-9

Table 1. Body mass and the mass of the heart of intact females and males

	ND Female	FRD Female	ND Male	FRD Male
Body mass (g)	263.20 ± 30.26	279.50 ± 20.52*	338.90 ± 40.45$^{\#}$	311.30 ± 37.58$^{\$}$
Mass of the heart (g)	0.83 ± 0.13	0.83 ± 0.08	1.05 ± 0.14**	0.94 ± 0.16
Heart to body ratio ($\times 10^{-3}$)	3.17 ± 0.16	3.04 ± 0.21	3.11 ± 0.19	3.00 ± 0.26

Values are expressed as means ± standard deviations for a total of 9 rats in each experimental group. Mann-Whitney U test was used to compare the values of skewed continuous variables between groups; in all tests, P values < 0.05 were considered statistically significant. ND – normal diet, FRD – fructose-rich diet. $^{*}P < 0.05$ ND Female vs. FRD Female, $^{\#}P < 0.001$ ND Female vs. ND Male, $^{\$}P < 0.05$ FRD Female vs. FRD Male, $^{**}P < 0.01$ ND Male vs. ND Female.

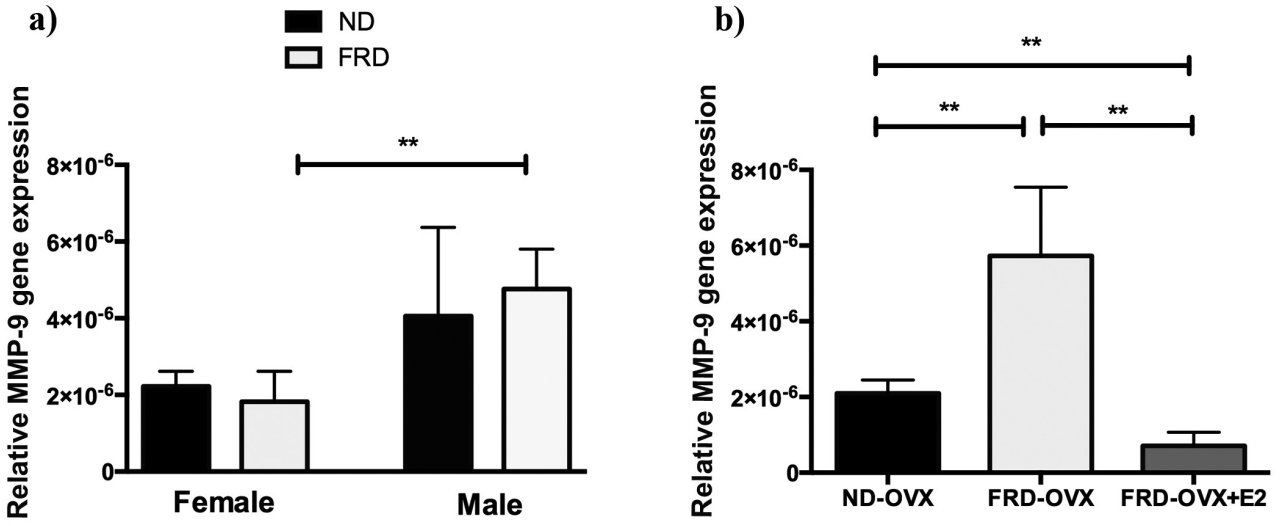

Fig. 1. Relative *MMP9* gene expression.
The cDNAs from heart tissue specimens were used as templates in RT-qPCR for relative quantification of MMP-9 mRNA expression. For each specimen, the expression level of MMP-9 mRNA was normalized to the housekeeping gene 18S rRNA. Relative expression of MMP-9, normalized against the housekeeping gene, was calculated using the comparative C_t method. (**a**) Relative *MMP9* gene expression in male and female rats fed a standard laboratory food and fructose-rich diet. Males on fructose-rich diet showed higher *MMP9* gene expression than females on the same diet. ND – normal diet, FRD – fructose-rich diet, ** $P < 0.001$. (**b**) Relative *MMP9* gene expression in ovariectomized fructose-fed rats with or without oestradiol replacement. OVX – ovariectomy, E2 – oestradiol treatment, ** $P < 0.001$.

protein level in the heart of ovariectomized rats ($P < 0.001$, ND-OVX *vs*. FRD-OVX). In contrast, oestradiol treatment significantly decreased MMP-9 protein expression compared to both, FRD-OVX ($P < 0.01$, FRD-OVX *vs*. FRD-OVX+E2) and ND-OVX animals ($P < 0.001$, ND-OVX *vs*. FRD-OVX+E2).

Having in mind that NFκB regulates MMP-9 expression, we investigated the effect of FRD and E2 (in the context of FRD) on activation of this transcription factor. As it was presented in Fig. 3, FRD increased phosphorylation of the p65 subunit at Ser 276 ($P < 0.01$, ND-OVX *vs*. FRD-OVX), thereby activating NFκB, while E2 replacement therapy reduced p65 phosphorylation ($P < 0.05$, FRD-OVX *vs*. FRD-OVX+E2) and decreased it to the level below that detected in ND-OVX females ($P < 0.01$, ND-OVX *vs*. FRD-OVX+E2).

Discussion

The major finding of this study is that FRD increases MMP-9 expression in ovariectomized female rats even before cardiac structural changes have been detected. This change in MMP-9 expression is oestrogen dependent and is in line with increased phosphorylation of the p65 subunit of NFκB transcription factor, which occurs during the FRD regime.

The results showed that 9-week treatment with 10% fructose solution did not cause any changes in the mass of the heart. This indicated that heart hypertrophy had not yet developed. Other studies demonstrated that either increased concentration of the consumed fructose or prolonged period of the diet regime leads to development of cardiac hypertrophy, excessive collagen deposition and increased stiffness of the left ventricle (Kobayashi et al., 1993; Kamide et al., 2002; Patel et al., 2009).

Previously, we have reported that 10% fructose-rich diet regime in the duration of 9 weeks leads to development of cardiac insulin resistance (Zakula et al., 2011; Romic et al., 2013). We also reported that FRD significantly elevated blood pressure in male rats, while in females we did not observe any differences in blood pressure as a consequence of FRD (Koricanac et al., 2013).

Table 2. Body mass and the mass of the heart of ovariectomized rats

	ND-OVX	FRD-OVX	FRD-OVX+E2
Body mass (g)	293.20 ± 23.09	286.70 ± 23.87	280.60 ± 18.15
Mass of the heart (g)	0.85 ± 0.08	0.84 ± 0.10	0.84 ± 0.07
Heart to body ratio ($\times 10^{-3}$)	2.92 ± 0.13	3.00 ±0.14	2.98 ± 0.15

Data presented are means ± standard deviations for 9 rats per each experimental group. Mann-Whitney U test was used to compare the values of skewed continuous variables between groups; in all tests, P values < 0.05 were considered statistically significant. None of the comparisons showed statistical significance. FRD and E2 replacement therapy did not affect body mass and the mass of the heart. ND – normal diet, FRD – fructose-rich diet, OVX – ovariectomized, E2 – oestradiol treatment.

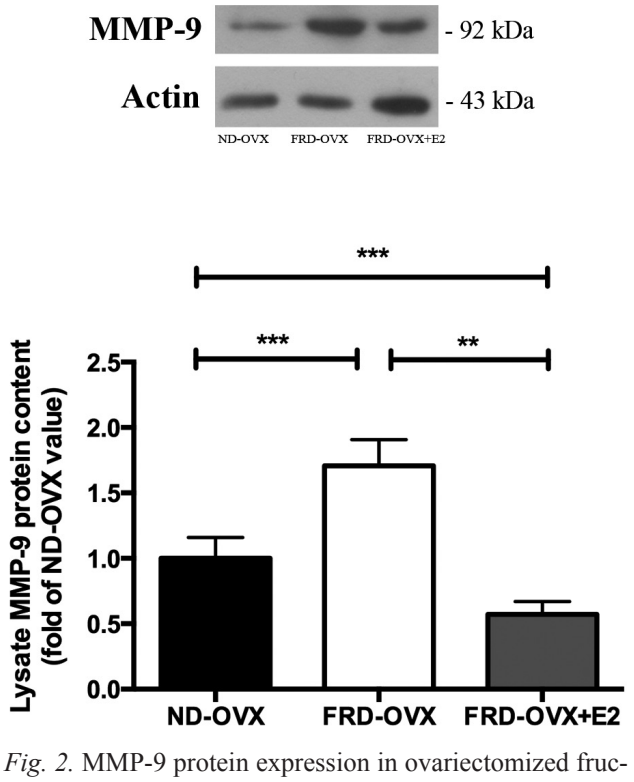

Fig. 2. MMP-9 protein expression in ovariectomized fructose-fed rats, rats with or without oestradiol treatment. Fructose-rich diet increased MMP-9 protein expression, while oestradiol replacement therapy reverted this change and decreased MMP-9 expression even below the level detected in animals on normal diet. ND – normal diet, FRD – fructose-rich diet, OVX – ovariectomy, E2 – oestradiol treatment, **P < 0.001, ***P < 0.0001.

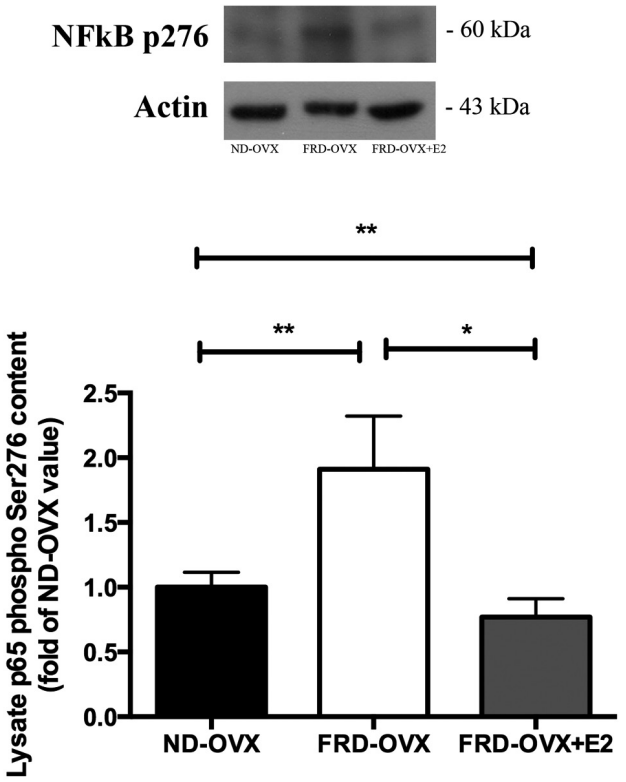

Fig. 3. Effects of fructose-rich diet and oestradiol on phosphorylation of NFκB p65 subunit at Ser 276. Fructose-rich diet increased phosphorylation of p65 subunit at Ser 276, thereby activating NFκB, while E2 replacement therapy reduced p65 phosphorylation. ND – normal diet, FRD – fructose-rich diet, OVX – ovariectomy, E2 – oestradiol treatment, *P < 0.05, **P < 0.001.

Adverse ECM remodelling in the myocardium finally results in mortality and morbidity associated with various cardiovascular diseases including hypertension, myocardial infarction, cardiomyopathy, and finally heart failure (Cohn et al., 2000). MMPs are proposed to lead toward LV dilatation and heart failure. The MMP-9 protein level and activity were significantly elevated in the spontaneously hypertensive heart failure (SHHF) rat model (Li et al., 2000). The results of other studies also indicate that MMP-9 has an important role in the cardiac remodelling associated with hypertension (Tayebjee et al., 2004; Chiao et al., 2012). MMP-9 directly promotes myofibrilar transformation (Jiang et al., 2013), which is important in the development of cardiac fibrosis and scar formation (Porter and Turner, 2009).

In presented study we detected elevated *MMP9* gene expression in FRD-male rats compared to FRD-females. Further, we found that FRD upregulated *MMP9* gene expression in ovariectomized females. A previous study showed increased MMP-9 activity in intimal smooth muscle cells of fructose-fed rats (Lu et al., 2013). Because E2 could reduce MMP-9 expression (Vegeto et al., 2001), we have further investigated whether E2 could revert changes in *MMP9* gene expression induced by FRD. Indeed, we showed that E2 replacement was able to revert these changes. This confirms that E2 is able to protect rats from changes in the *MMP9* gene expression that are consequences of FRD. As a further confirmation of this protective effect of E2 in the context of FRD, beside results obtained at the gene level, we have also studied the MMP-9 changes at protein level. Changes in the MMP-9 protein level were in the same direction as those observed for gene expression, except that E2 decreased the MMP-9 protein level even below the level detected in ND-OVX animals. MMP-9 is a key contributor to adverse myocardial remodelling. Because the cellular source of MMP-9 appears to be predominantly infiltrating inflammatory cells (Mukheriee et al., 2006), it is not surprising that inhibition of MMP-9 expression or activity reduced myocardial inflammation and remodelling (Spinale, 2007). Based on the aforementioned findings, we can conclude that FRD causes changes in MMP-9 expression in the rat heart even before structural changes have occurred, and that this early molecular changes are reversible with E2. This suggests that E2 could protect the heart from possible harmful effects of FRD.

Angiotensin II was shown to promote cardiovascular remodelling through the increase of MMP-9 expression via AT1R, while telmisartan (AT1R blocker) and captopril (angiotensin-converting enzyme inhibitor) suppress MMP-9 expression and activities (Okada et al., 2008,

2009, 2010). Several studies have confirmed that NFκB is absolutely required and the most important transcription factor for MMP-9 production (Bond et al., 1998, 2001; Moon et al., 2004). In various cell types, angiotensin II activates NFκB (Marui et al., 1993; Kranzhofer et al., 1999), and MMP-9 expression is mediated via this signalling pathway (Guo et al., 2008). NFκB activation could occur through phosphorylation and subsequent proteolytic degradation of inhibitory protein IκB (Ruiz-Ortega et al., 2006), but it was shown that there is weak degradation of IκB in response to angiotensin II, particularly in induction of MMP expression (Browatzaki et al., 2005). Alternatively, angiotensin II could activate NFκB through phosphorylation of the p65 subunit at Ser 276 via AT1R (Kim et al., 2012).

Our previous study (Bundalo et al., 2015) demonstrated that FRD increased AT1R and ACE expression in ovariectomized rats and that oestradiol was able to revert these changes. Interestingly, in this study we demonstrated that the pattern of changes in MMP-9 expression due to FRD and oestradiol treatment is similar to those previously reported for AT1R and ACE expression (Bundalo et al., 2015). Considering previously mentioned facts (Marui et al., 1993; Kranzhofer et al., 1999; Bond et al., 2001; Moon et al., 2004; Okada et al., 2008, 2009, 2010; Guo et al., 2008), we found interesting to investigate changes in p65 phosphorylation at Ser 276 under the influence of FRD, and subsequent oestradiol treatment in the context of FRD. The results showed that FRD increased p65 phosphorylation, thereby activating NFκB, while E2 replacement therapy restored the initial level of phosphorylation in p65. A previous study implied that E2 inhibits NFκB activation by enhancing the inhibitor kBα (IkBα) kinase level and/or by stabilization of IκBα (by decreasing its phosphorylation) (Wen et al., 2004; Xing et al., 2012). This is the first study demonstrating that E2 decreases phosphorylation of the p65 subunit of NFκB at Ser 276. NFκB plays a fundamental role in pathogenesis of insulin resistance and type 2 diabetes mellitus. Taking into account that NFκB, beside MMP-9, also controls many pro-inflammatory genes including cytokines (such as TNF-α, IL-6 and IL-8), adhesion molecules and chemokines (Cai et al., 2005; Shoelson et al., 2006; Wei et al., 2008), it is obvious that E2, by decreasing NFκB activation, displays its protective effect on the development of FRD-induced heart disorders. This effect of E2 on NFκB activation is in accordance with our previous study that demonstrates that E2 shows a protective effect on the development of cardiac insulin resistance in fructose-fed rats (Romic et al., 2013).

The pattern of changes in p65 phosphorylation is similar to that obtained for MMP-9 under the same treatment. Our experiment demonstrated that early molecular changes in FRD-treated rats include elevated p65 phosphorylation at Ser 276, possibly as a result of increased AT1R expression. This phosphorylation activates NFκB, which consequently induces enhanced MMP-9 expression. Further studies are required to unequivocally confirm this signalling pathway. Should this diet regime be prolonged, the changes at the molecular level might promote adverse cardiovascular remodelling, which could result in development of various cardiovascular disorders. Obviously, this is the key point where changes at the molecular level have occurred but cardiac impairment has not yet emerged. An interesting finding was that the increase in MMP-9 expression and NFκB activation that occurred as a result of FRD was reversible with E2. This implicates that E2 shows a protective effect on the rat cardiac tissue by reducing the possibility of adverse ECM remodelling in the myocardium, which may be a result of the harmful effect of FRD.

Acknowledgment

The authors declare no conflict of interest.

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