Биолошки факултет
Број захтева: 33/12-1
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**:** **Марина М. Николић**

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

пријавио је докторску дисертацију под називом:

„Метаболичке карактеристике синдрома полицистичних јајника у висцералном масном ткиву и лептинска резистенција у хипоталамусу пацова третираног 5α-дихидротестостероном: улога глукокортикоида“.

из научне области: Биолошке науке.

Универзитет је дана 03.07.2014. године. својим актом под бр. 02 Број: 61206-3053/2-14 дао сагласност на предлог теме докторске дисертације која је гласила:

„Метаболичке карактеристике синдрома полицистичних јајника у висцералном масном ткиву и лептинска резистенција у хипоталамусу пацова третираног 5α-дихидротестостероном: улога глукокортикоида“.

Комисија за оцену и одбрану докторске дисертације образована је на седници одржаној 09.10.2015. год, одлуком Факултета под бр. 33/233-09.10.2015. год. у саставу:

	Име и презиме члана комисије	звање	научна област	Установа у којој је запослен
1.	др Гордана Матић	редовни професор	биохемија и молекуларна биологија	Универзитет у Београду-Биолошки факултет
2.	др Данијела Војновић Милутиновић	научни сарадник	молекуларна ендокринологија	Универзитет у Београду-Институт за биолошка истраживања "Синиша Станковић"
3.	др Ђуро Мацут	ванредни професор	ендокринологија	Универзитет у Београду - Медицински факултет

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

33/12-29.01.2016.

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:

Марине Николић, под називом:

„Метаболичке карактеристике синдрома полицистичних јајника у висцералном масном ткиву и лептинска резистенција у хипоталамусу пацова третираног 5α-дихидротестостероном: улога глукокортикоида“.

Универзитет је дана 03.07.2014. године. својим актом под бр. 02 Број: 61206-3053/2-14 дао сагласност на предлог теме докторске дисертације кандидата.

Радови и конгресна саопштења из докторске дисертације:

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

1. **Nikolić M**, Macut D, Djordjevic A, Veličković N, Nestorović N, Bursać B, Božić Antić I, Bjekić-Macut J, Matić G, Vojnović Milutinović D. Possible involvement of glucocorticoids in 5α-DHT-induced PCOS-like metabolic disturbances in the rat visceral adipose tissue. *Mol Cell Endocrinol*, 2015, 399: 22-31. **M21**

2. **Nikolić M**, Veličković N, Djordjevic A, Bursać B, Macut D, Božić Antić I, Bjekić Macut J, Matić G, Vojnović Milutinović D. 5α-dihydrotestosterone treatment induces metabolic changes associated with polycystic ovary syndrome without interfering with the hypothalamic leptin and glucocorticoid signaling. Arch Biol Sci, 2015, accepted for publication. **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 I редовној седници Наставно-научног већа Биолошког факултета Универзитета у Београду, одржаној 9.10.2015. године, прихваћен је извештај ментора др Гордане Матић и др Данијеле Војновић Милутиновић о урађеној докторској дисертацији **Марине М. Николић**, истраживача сарадника у Институту за биолошка истраживања „Синиша Станковић“, под насловом **„Метаболичке карактеристике синдрома полицистичних јајника у висцералном масном ткиву и лептинска резистенција у хипоталамусу пацова третираног 5α–дихидротестостероном: улога глукокортикоида“**, и одређена је Комисија за преглед и оцену докторске дисертације у саставу др Гордана Матић, редовни професор Биолошког факултета, др Данијела Војновић Милутиновић, научни сарадник Института за биолошка истраживања „Синиша Станковић“ и др Ђуро Мацут, ванредни професор Медицинског факултета Универзитета у Београду.

Комисија је прегледала урађену докторску дисертацију кандидата и Већу подноси следећи

И З В Е Ш Т А Ј

ОПШТИ ПОДАЦИ О ДОКТОРСКОЈ ДИСЕРТАЦИЈИ

Докторска дисертација **Марине М. Николић** под насловом **„Метаболичке карактеристике синдрома полицистичних јајника у висцералном масном ткиву и лептинска резистенција у хипоталамусу пацова третираног 5α-дихидротестостероном: улога глукокортикоида“**, написана је на 143 стране, и подељена у 8 поглавља: **Увод** (49 страна), **Циљ рада** (2 стране), **Материјал и методе** (23 стране), **Резултати** (32 стране), **Дискусија** (15 страна), **Закључци** (2 стране), **Литература** (14 страна) и **Прилози** (6 страна). Рад садржи 142 литературна цитата, 31 слику, 7 табела, Списак скраћеница, Садржај и Сажетке на српском и енглеском језику.

АНАЛИЗА ДОКТОРСКЕ ДИСЕРТАЦИЈЕ

Увод докторске дисертације садржи седам поглавља. У њему је дат сажет приказ досадашњих сазнања из литературе која су непосредно везана за предмет дисертације. У поглављу "Синдром полицистичних јајника" описани су критеријуми за постављање дијагнозе синдрома полистичних јајника (PCOS) код оболелих жена, патофизиологија синдрома, као и преглед метаболичких поремећаја који се повезују са овим синдромом. Поглавље под називом „Глукокортикоидни хормони“ даје детаљан приказ литературе о глукокортикоидним хормонима, њиховој улози у регулацији метаболичких процеса, као и молекуларним механизмима њиховог деловања. Посебан акценат је на анализи пререцепторског метаболизма глукокортикоида, као и на регулацији активности и експресије ензима 11β-хидроксистероид дехидрогеназе 1 (11β-HSD1). У оквиру поглавља „Метаболизам липида у масном ткиву: улоге глукокортикоида" описани су процеси који регулишу метаболизам липида у масном ткиву. Посебна пажња је посвећена улози

глукокортикодних хормона у висцералном масном ткиву на диференцијацију и сазревање адипоцита преко транскрипционе регулације процеса адипогенезе и липогенезе, а касније по успостављању фенотипа зрелог адипоцита и на процес липолизе. У поглављу „Глукокортикоиди и метаболичка инфламација у висцералном масном ткиву“ дат је осврт на појаву метаболичке инфламације и антиинфламаторне улоге глукокортикоида у хипертрофичном висцералном масном ткиву. У овом поглављу посебан нагласак је стављен на специфичну улогу глукокортикоидних хормона на синтезу неких проинфламаторних цитокина, као што је фактор који инхибира миграцију макрофага, као и значај њихових интеракција. У наредном поглављу под насловом „Сигнални путеви инсулина и развој инсулинске резистенције: улога глукокортикоида“ дата је анализа сигналних путева инсулина, као и молекуларни механизми развоја инсулинске резистенције, са посебним освртом на ткивно-специфичне ефекте глукокортикоида на настанак инсулинске резистенције на нивоу организма. Жене са PCOS-ом поред висцералне гојазности као главног метаболичког поремећаја, карактерише и повећан унос хране. Стога је у наредном поглављу које носи наслов „Комуникација висцералног масног ткива и централног нервног система у регулацији енергетског метаболизма“ дата детаљна анализа периферних и централних ефеката анорексигеног хормона лептина, као и молекуларних механизама развоја лептинске резистенције на нивоу хипоталамуса. У последњем поглављу под насловом „Животињски модели у изучавању PCOS-а“ дат је детаљан приказ различитих анималних модела који се користе у проучавању разних аспеката PCOS-а.

У делу **Циљ рада** описани су научни циљеви докторске дисертације. Циљ ове докторске дисертације је био да допринесе бољем разумевању улоге глукокортикоидних хормона у патогенези PCOS-а тако што би се разјаснила улога ових хормона у метаболизму липида и метаболичкој инфламацији у висцералном масном ткиву, системској и периферној осетљивости на инсулин, као и у регулацији енергетског метаболизма у хипоталамусу. Испитивањима наведених улога глукокортикоида предходила је детаљна анализа репродуктивних и метаболичких промена у коришћеном анималном моделу PCOS-а који је добијен дуготрајним третманом женки пацова 5α-дихидротестостероном (DHT).

У поглављу **Материјал и методе** наведене су хемикалије и апаратуре које су коришћене у раду, описан је третман животиња и експерименталне групе, као и све експерименталне методе и начин обраде резултата. Посебно је објашњен третман експерименталних група, који је подразумевао поткожно деловање хормонског (7,5 mg DHT) или плацебо пелета у трајању од 90 дана, а од 21. дана старости женки пацова *Wistar* соја.

У Поглављу **Резултати** изложени су резултати хормонског третмана на развој репродуктивних и метаболичких карактеристика PCOS-а код DHT-ом третираних женки пацова. Прва група резултата обухватила је добијене репродуктивне, морфолошке и метаболичке параметре добијене након третмана DHT-ом. Резултати су показали да је третман DHT-ом довео до развоја репродуктивних промена карактеристичних за PCOS, као што су анеструс и смањена маса јајника и утеруса, док је детаљна хистолошка анализа показала да се у јајницима DHT-ом третираних животиња запажа велики број атретичних фоликула. Поред наведеног, третман DHT-ом је довео до метаболичких промена као што су повећани енергетски унос, маса тела и висцерална гојазност. Хистолошка анализа висцералног масног ткива је показала да су адипоцити животиња третираних DHT-ом хипертрофирани, односно са повећаним дијаметром и површином у односу на адипоците плацебо групе. Од биохемијских параметара мерене су концентрације слободних масних киселина, триглицерида, глукозе, инсулина и лептина у плазми, концентрације

кортикостерона у плазми и висцералном масном ткиву. Добијени резултати су показали да су женке пацова третиране DHT-ом имале изражену дислипидемију која се огледала у повећаним концентрацијама триглицерида и слободних масних киселина, као и повећане нивое инсулина и лептина, док је концентрација кортикостерона у плазми била непромењена, а у висцералном масном ткиву повећана.

Улога глукокортикоидног сигналног пута у метаболичким променама у висцералном масном ткиву праћена је на нивоу експресије ензима пререцепторског метаболизма глукокортикоидних хормона, експресије и унутарћелијске дистрибуције глукокортикоидног рецептора, као и на нивоу експресија ензима укључених у липогене и липолитичке процесе, чија је експресија регулисана глукокортикоидима. Показано је да је третман DHT-ом довео до повећања експресије и активности ензима пререцепторског метаболизма глукокортикоида, 11β-HSD1, до повећања концентрације унутарћелијског кортикостерона и до активације глукокортикоидног рецептора у висцералном масном ткиву. Појачана глукокортикоидна сигнализација у висцералном масном ткиву довела је до је повећања нивоа липогених фактора, као што су протеин који се везује за елементе регулисане стеролом типа 1 (SREBP-1), микрозомални липин-1, синтаза масних киселина (FAS), фосфоенолпируват карбоксикиназа (PEPCK) и липопротеинска липаза (LPL), док је ниво липолитичког фактора липазе осетљиве на хормоне (HSL) смањен, што је све у складу са хипертрофијом адипоцита, висцералном гојазношћу и дислипидемијом.

Такође, резултати су показали да је третман DHT-ом довео до развоја метаболичке инфламације у висцералном масном ткиву, о чему сведоче повећана концентрација проинфламаторног цитокина, фактора који инхибира миграцију макрофага (MIF) и активација проинфламаторног транскрипционог регулатора једарног фактора κB (NFκB), који је довео до повећане експресије цитокина IL-1β и IL-6. Међутим, активација проинфламаторног сигналног пута код DHT-ом третираних животиња није довела до слабљења инсулинског сигналног пута на нивоу инхибиторне фосфорилације супстрата за инсулински рецептор-1 (IRS-1) и транспортера глукозе 4 (GLUT4), што указује на очувану осељивост висцералног масног ткива на деловање инсулина.

Поред горе наведених промена, праћене су промене у сигналним путевима глукокортикоида и лептина, као и ниво инфламације у хипоталамусу женки пацова третираних DHT-ом. Резултати су показали да није дошло до промена у компонентама пререцепторског метаболизма глукокортикоида и нивоа глукокортиокидног рецептора, као ни до промена нивоа учесника лептинског сигналног пута, нити до појаве инфламације у хипоталамусу третираних женки пацова.

У поглављу **Дискусија** дата је упоредна анализа оригиналних резултата ове докторске дисертације и података из литературе, која указује на значај постигнутих резултата. Ово поглавље је логично подељено на четири одељка што у потпуности доприноси разумевању тумачења резултата. Први део Дискусије односи се на добијене репродуктивне и метаболичке карактеристике PCOS-а након дуготрајног третмана младих женки пацова DHT-ом. Репродуктивне промене третираних животиња се огледају у поремећајима фоликулогенезе и овулације, присуству атретичних фоликула, одсуству жутих тела и доминацији интерстицијалних жлезда у јајницима. Добијене метаболичке промене карактеристичне за PCOS биле су повећана телесна и маса висцералног масног ткива, дислипидемија, смањена системска осетљивост на инсулин, хиперлептинемија и хиперфагија. У другом делу дато је тумачење улоге глукокортикоида у метаболичким променама у висцералном масном ткиву женки пацова третираних DHT-ом. Наиме, код животиња са PCOS-ом унутарћелијска концентрација кортикостерона је била повећана што је последица појачаног пререцепторског метаболизма глукокортикоида услед

повећане експресије 11β-HSD1. Паралелно са повећањем ткивне концентрације глукокортикоида дошло је до транслокације глукокортикоидног рецептора из цитоплазме у једро и тиме и до промене експресије његових циљних гена у висцералном масном ткиву анималног модела PCOS-а. Повећана експресија циљних гена глукокортикоида (липин-1, SREBP-1, FAS, PEPCK и LPL) је показала укљученост ових хормона у *de novo* липогенезу и настанак висцералне гојазности што је у складу са хипертрофичним растом адипоцита код животиња третираних DHT-ом. У трећем делу Дискусије посебна пажња је посвећена метаболичкој инфламацији и инсулинској осетљивости у висцералном масном ткиву женки пацова третираних DHT-ом. Наиме, код третираних животиња услед повећане активности транскрипционог регулатора NFκB присутна је локална инфламација ниског степена која се огледа у повишеним нивоима проинфламаторних цитокина IL-1β и IL-6. Поред тога, повећан је и ткивни ниво проинфламаторног цитокина MIF, што је у складу са повећаном регенерацијом глукокортикоида и активираним глукокортиокидним рецептором, с обзиром на то да ови хормони директно стимулишу експресију MIF-а. Повишени нивои свих поменутих цитокина налазе се у позитивној корелацији са величином адипоцита висцералног масног ткива. Појава локалне инфламације није праћена променама у осетљивости висцералног масног ткива на инсулин и поред њеног смањења на системском нивоу. Последњи део дискусије односи се на анализу глукокортикоидне и лептинске сигнализације у хипоталамусу женки пацова третираних DHT-ом. Резултати ове судије указују на то да је код женки пацова третман DHT-ом довео до појаве хиперфагије, односно повећаног уноса енергије, што је праћено развојем висцералне гојазности и хиперлептинемијом, али не и променама у лептинској и глукокортикоидној сигнализацији у хипоталамусу третираних животиња.

У поглављу **Закључци** сажето и јасно су изнети најважнији закључци до којих се дошло анализирањем добијених експерименталних података. Закључци се могу сумирати на следећи начин: дуготрајан третман младих женки пацова DHT-ом је довео до развоја репродуктивних и метаболичких промена који се срећу код жена са PCOS-ом. Уочена висцерална гојазност и дислипидемија код третираних животиња су последице хипертрофије адипоцита, у којој је фаворизовано нагомилавање липида. Повећање масе висцералног масног ткива прате молекуларне промене које делимично потичу од појачане глукокортиокидне сигнализације, која је метаболизам липида усмерила ка липогенези. Поред тога, висцерална гојазност и дислипидемија су праћене инфламацијом ниског интезитета, али не и смањеном инсулинском осетљивошћу у овом ткиву. С друге стране, животиње третиране DHT-ом показују смањену системску инсулинску осетљивост, која се огледа у повећаним концентрацијама инсулина потребним за одржавање нормалног нивоа глукозе у крви. Третман женки пацова DHT-ом довео је до повећаног калоријског уноса, праћеног висцералном гојазношћу и хиперлептинемијом, при чему ови метаболички поремећаји нису резултат промена и интеракција глукокортикоидне и лептинске сигнализације у хипоталамусу третираних животиња. И на крају, укупне репродуктивне и метаболичке промене добијене у овој студији на анималном моделу PCOS-а указују на адекватност примењеног третмана за изучавање разних аспеката овог синдрома код жена.

У поглављу **Литература** дата је листа коју чине 142 библиографске јединице. Наведене научне публикације се односе на области које су од значаја за урађену дисертацију и цитиране су на начин који објашњава и потврђује добијене резултате.

БИБЛИОГРАФИЈА

Радови и конгресна саопштења из уже научне области:

Б1. Радови у часописима међународног значаја

1. **M21 Nikolić M**, Macut D, Djordjevic A, Veličković N, Nestorović N, Bursać B, Božić Antić I, Bjekić-Macut J, Matić G, Vojnović Milutinović D. Possible involvement of glucocorticoids in 5α-DHT-induced PCOS-like metabolic disturbances in the rat visceral adipose tissue. *Mol Cell Endocrinol,* 2015, 399: 22-31.
2. **M21** Tepavčević S, Vojnović Milutinović D, Macut D, Žakula Z, **Nikolić M**, Božić-Antić I, Romić S, Bjekić-Macut J, Matić G, Korićanac G. Dihydrotestosterone deteriorates cardiac insulin signaling and glucose transport in the rat model of polycystic ovary syndrome. *J Steroid Biochem Mol Biol*. 2014, 141:71-76.
3. **M21** Vasiljević A, Bursać B, Djordjevic A, Vojnović Milutinović D, **Nikolić M**, Matić G, Veličković N. Hepatic inflammation induced by high fructose diet is associated with altered 11βHSD1 expression in the liver of Wistar rats. *Eur J Nutr,* 2014, 53: 1393-1402.
4. **M22** Tepavčević S, Vojnović Milutinović D, Macut D, Stojiljković M, **Nikolić M**, Božić-Antić I, Ćulafić T, Bjekić-Macut J, Matić G, Korićanac G. Cardiac fatty acid uptake and metabolism in the rat model of polycystic ovary syndrome. *Endocrine*. 2015, 50(1): 193-201.
5. **M23 Nikolić M,** Veličković N, Djordjevic A, Bursać B, Macut D, Božić Antić I, Bjekić Macut J, Matić G, Vojnović Milutinović D. 5α-dihydrotestosterone treatment induces metabolic changes associated with polycystic ovary syndrome without interfering with the hypothalamic leptin and glucocorticoid signaling. *Arch Biol Sci*, 2015, accepted for publication.
6. **M23** Tepavčević S, Vojnović Milutinović D, Macut D, Stanišić J, **Nikolić M**, Božić-Antić I, Radaljević S, Bjekić-Macut J, Matić G, Korićanac G. Cardiac Nitric Oxide Synthases and Na + /K + -ATPase in the rat model of polycystic ovary syndrome induced by dihydrotestosterone. *Exp Clin Endocr Diab*. 2015, 123(05): 303-307.
7. **M23** Vojnović Milutinović D, Macut D, Božić Antić I, **Nikolić M**, Bjekić Macut J, Matić G, Nestorov J. Hypoxanthine guanine phosphoribosyltransferase is the most stable reference gene for gene expression analysis by quantitative PCR in peripheral blood mononuclear cells from women with polycystic ovary syndrome. *J Med Biochem,* 2014, 33(4): 356-363.
8. **M23** Vojnović Milutinović D, **Nikolić M**, Dinić J, Djordjevic A, Veličković N, Elaković I, Matić G, Nestorov J. Leptin and glucocorticoid signaling in the hypothalamus of female and male fructose-fed rat. *Arch Biol Sci*, 2014, 66 (2): 829-839.

Б2. Радови у часописима домаћег значаја

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

Б3. Конгресна саопштења на скуповима међународног значаја

1. **M34** Vojnović Milutinović D, **Nikolić M**, Djordjevic A, Veličković N, Bursać B, Teofilović A, Božić Antić I, Bjekić Macut J, Matić G and Macut D. Enhanced inflammation and unchanged insulin sensitivity in the visceral adipose tissue of the rat model of polycystic ovary syndrome. ESE Basic Endocrinology Course in Reproductive

Endocrinology, Edinburgh, UK, 18-20 February 2015, pp 31.

2. **M34** Djordjevic A, Bursać B, Vojnović Milutinović D, Nestorov J, **Nikolić M**, Nestorović N, Vasiljević A, Veličković N, Elaković I and Matić G. The role of hypothalamic leptin signalling and glucocorticoids in fructose diet-induced visceral adiposity of rats. ESE Basic Endocrinology Course on "Neuroendocrinology" Amsterdam, Netherlands, 15-17 January 2014, pp 12.
3. **M34** Vojnović Milutinović D, Djordjevic A, **Nikolić M,** Korićanac G, Tepavčević S, Žakula Z, Nestorović N, Božić I, Bjekić Macut J, Matić G, Macut D. Characteristics of metabolic syndrome in hyperandrogenemic female rat model of polycystic ovary syndrome. International Symphosium on Advances in PCOS, Belgrade, Serbia, 16-17 November, 2012, P2.
4. **M34** Matić G, Veličković N, Djordjevic A, Vojnović Milutinović D, Elaković I, Nestorov J, Bursać B, Vasiljević A, **Nikolić M**, Dundjerski J. Glucocorticoid signaling in the liver and adipose tissue of male and female fructose-fed rats. From Metabolism, diet and disease, Washington, USA, BMC Proceedings 2012, 6 (Suppl 3), P35.

Б4. Конгресна саопштења на скуповима домаћег значаја

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

Радови и конгресна саопштења из докторске дисертације:

Б1. Радови у часописима међународног значаја

1. **M21 Nikolić M**, Macut D, Djordjevic A, Veličković N, Nestorović N, Bursać B, Božić Antić I, Bjekić-Macut J, Matić G, Vojnović Milutinović D. Possible involvement of glucocorticoids in 5α-DHT-induced PCOS-like metabolic disturbances in the rat visceral adipose tissue. *Mol Cell Endocrinol*, 2015, 399: 22-31.
2. **M23 Nikolić M**, Veličković N, Djordjevic A, Bursać B, Macut D, Božić Antić I, Bjekić Macut J, Matić G, Vojnović Milutinović D. 5α-dihydrotestosterone treatment induces metabolic changes associated with polycystic ovary syndrome without interfering with the hypothalamic leptin and glucocorticoid signaling. Arch Biol Sci, 2015, accepted for publication.

Б2. Радови у часописима домаћег значаја

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

Б3. Конгресна саопштења на скуповима међународног значаја

1. **M34** Vojnović Milutinović D, **Nikolić M**, Djordjevic A, Veličković N, Bursać B, Teofilović A, Božić Antić I, Bjekić Macut J, Matić G and Macut D. Enhanced inflammation and unchanged insulin sensitivity in the visceral adipose tissue of the rat model of polycystic ovary syndrome. ESE Basic Endocrinology Course in Reproductive Endocrinology, Edinburgh, UK, 18-20 February 2015, pp 31.
2. **M34** Vojnović Milutinović D, Djordjevic A, **Nikolić M,** Korićanac G, Tepavčević S, Žakula Z, Nestorović N, Božić I, Bjekić Macut J, Matić G, Macut D. Characteristics of metabolic syndrome in hyperandrogenemic female rat model of polycystic ovary syndrome. International Symphosium on Advances in PCOS,

Belgrade, Serbia, 16-17 November, 2012, P2.

Б4. Конгресна саопштења на скуповима домаћег значаја

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

МИШЉЕЊЕ И ПРЕДЛОГ КОМИСИЈЕ

Докторска дисертација кандидата **Марине М. Николић**, под насловом **„Метаболичке карактеристике синдрома полицистичних јајника у висцералном масном ткиву и лептинска резистенција у хипоталамусу пацова третираног 5α-дихидротестостероном: улога глукокортикоида“** представља научни рад са јасно формулисаним циљевима заснованим на добром познавању научне проблематике, са благовремено планираним и успешно реализованим истраживачким поступком чији резултати представљају оригинални научни допринос разумевању молекуларних механизама деловања глукокортикодних хормона у патогенези PCOS-а.

У изради дисертације Марина М. Николић је показала иницијативу при дефинисању хипотезе и циљева, применила је адекватне експерименталне методе и показала висок степен самосталности при обради добијених резултата које је критички дискутовала, уз исцрпне податке из литературе. Треба нагласити да су представљени резултати објављени у врхунским међународним часописима, што потврђује да је кандидаткиња, у сарадњи са менторима, пажљиво одабрала тему истраживања, која у наредном периоду може бити успешно развијана.

На основу увида у истраживања и постигнуте резултате, Комисија закључује да су задаци постављени у циљу и програму, који су усвојени приликом прихватања теме за израду докторске дисертације, испуњени и има задовољство да предложи Наставно-научном већу Биолошког факултета, Универзитета у Београду, да прихвати позитивну оцену докторске дисертације **Марине М. Николић**, под насловом **„Метаболичке карактеристике синдрома полицистичних јајника у висцералном масном ткиву и лептинска резистенција у хипоталамусу пацова третираног 5α-дихидротестостероном: улога глукокортикоида“** и омогући кандидату јавну одбрану рада.

У Београду, 30.12.2015. године.

КОМИСИЈА:

_____

др Гордана Матић, редовни професор
Биолошког факултета Универзитета у Београду

_____

др Данијела Војновић Милутиновић, научни сарадник
Института за биолошка истраживања
„Синиша Станковић“ Универзитета у Београду

_____

др Ђуро Мацут, ванредни професор
Медицинског факултета Универзитета у Београду

Molecular and Cellular Endocrinology 399 (2015) 22–31

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

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

Possible involvement of glucocorticoids in 5α-dihydrotestosterone-induced PCOS-like metabolic disturbances in the rat visceral adipose tissue

Marina Nikolić [a], Djuro Macut [b], Ana Djordjevic [a], Nataša Veličković [a], Nataša Nestorović [c], Biljana Bursać [a], Ivana Božić Antić [b], Jelica Bjekić Macut [d], Gordana Matić [a], Danijela Vojnović Milutinović [a,*]

[a] Department of Biochemistry, Institute for Biological Research "Siniša Stanković", University of Belgrade, 142 Despot Stefan Blvd., Belgrade 11000, Serbia
[b] Clinic of Endocrinology, Diabetes and Metabolic Diseases, Clinical Center of Serbia and School of Medicine, University of Belgrade, Dr Subotića 13, Belgrade 11000, Serbia
[c] Department of Cytology, Institute for Biological Research "Siniša Stanković", University of Belgrade, 142 Despot Stefan Blvd., Belgrade 11000, Serbia
[d] CHC Bežanijska kosa, Bežanijska kosa bb, Belgrade 11000, Serbia

ARTICLE INFO

Article history:
Received 27 March 2014
Received in revised form 4 August 2014
Accepted 27 August 2014
Available online 29 August 2014

Keywords:
PCOS
5α-dihydrotestosterone
Visceral adipose tissue
Glucocorticoids
Lipogenesis

ABSTRACT

Polycystic ovary syndrome (PCOS) is a reproductive and metabolic disorder characterized by hyperandrogenism, ovulatory dysfunction, visceral obesity and insulin resistance. We hypothesized that changes in glucocorticoid metabolism and signaling in the visceral adipose tissue may contribute to disturbances of lipid metabolism in the rat model of PCOS obtained by 5α-dihydrotestosterone (DHT) treatment of prepubertal female Wistar rats. The results confirmed that DHT treatment caused anovulation, obesity and dyslipidemia. Enhanced glucocorticoid prereceptor metabolism, assessed by elevated intracellular corticosterone and increased 11 beta-hydroxysteroid dehydrogenase type 1 mRNA and protein levels, was accompanied by glucocorticoid receptor (GR) nuclear accumulation. In concert with the increased expression of GR-regulated prolipogenic genes (lipin-1, sterol regulatory element binding protein 1, fatty acid synthase, phosphoenolpyruvate carboxykinase), histological analyses revealed hypertrophic adipocytes. The results suggest that glucocorticoids influence lipid metabolism in the visceral adipose tissue in the way that may contribute to pathogenesis of metabolic disturbances associated with PCOS.

1. Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of reproductive age and is characterized by hyperandrogenism, ovulatory dysfunction and polycystic ovaries (Ehrmann, 2005; Livadas and Diamanti-Kandarakis, 2013). In addition to clinical and biochemical manifestations of these disturbances, most women with PCOS display a number of metabolic abnormalities including hyperinsulinemia, insulin resistance, dyslipidemia, and obesity (Teede et al., 2006). All of these features are hallmark components of the metabolic syndrome, making women with PCOS highly susceptible to type 2 diabetes and cardiovascular diseases (Teede et al., 2006).

The pathophysiology of PCOS is complex and multifactorial, and has been attributed to defects in various organ systems including altered steroidogenesis in thickened ovarian theca layer and adrenals, as well as exaggerated luteinizing hormone pulsatility (Catteau-Jonard and Dewailly, 2013; Norman et al., 2007). It is considered that all mentioned abnormalities form a vicious cycle in which androgen excess of ovarian and/or adrenal origin generated early in life, or even prenatally, induce neuroendocrine abnormalities, ovarian dysfunction, visceral obesity, impaired lipid metabolism and insulin resistance, which in turn further, directly or indirectly, stimulates hyperandrogenemia (Escobar-Morreale and San Millan, 2007). Visceral adipose tissue is thought to be important for the pathogenesis of PCOS, because of its association with hyperandrogenemia and its often excessive accumulation in women with PCOS (Barber and Franks, 2013; Pasquali, 2006).

Abbreviations:: 11βHSD1, 11 beta-hydroxysteroid dehydrogenase type 1; ATGL, adipose tissue triglyceride lipase; CD36, cluster of differentiation 36; CL, corpora lutea; CORT, corticosterone; DHT, 5α-dihydrotestosterone; FAS, fatty acid synthase; H6PDH, hexose-6-phosphate dehydrogenase; HSL, hormone sensitive lipase; LPL, lipoprotein lipase; NEFA, non-esterified fatty acid; PCOS, polycystic ovary syndrome; PEPCK, phosphoenolpyruvate carboxykinase; PPARγ, peroxisome proliferator-activated receptor γ; SREBP-1, sterol regulatory element binding protein 1.

* Corresponding author. Department of Biochemistry, Institute for Biological Research "Siniša Stanković", 142 Despot Stefan Blvd., Belgrade 11000, Serbia. Tel.: +381 11 2078 318; fax: +381 11 2761 433.
E-mail address: dvojnovic@ibiss.bg.ac.rs (D. Vojnović Milutinović).

http://dx.doi.org/10.1016/j.mce.2014.08.013
0303-7207/

Cushing syndrome, which is characterized by glucocorticoid excess and exhibits a cluster of metabolic abnormalities, including insulin resistance, visceral obesity and impaired lipid metabolism, suggests a possible contribution of glucocorticoids to the pathogenesis of the metabolic syndrome and PCOS-related metabolic disturbances. Glucocorticoids are major regulators of fat metabolism, and can simultaneously have anabolic (adipogenic) and catabolic (lipolytic) effects in the adipose tissue (Peckett et al., 2011). Both of these actions, although seemingly contradictory, lead to obesity and impaired lipid homeostasis and can, ultimately, induce insulin resistance (Ayala-Sumuano et al., 2013).

Unlike the Cushing syndrome, obesity seen in metabolic diseases and PCOS is not associated with increased plasma glucocorticoids (Gathercole and Stewart, 2010). However, the adipose tissue availability of these hormones may be increased locally by overstimulated intracellular regeneration of active glucocorticoids (cortisol in humans and corticosterone (CORT) in rats) (Walker and Andrew, 2006). This is performed by 11 beta-hydroxysteroid dehydrogenase type 1 (11βHSD1), a microsomal NADPH-dependent enzyme (Walker and Andrew, 2006), which is provided by hexose-6-phosphate dehydrogenase (H6PDH) (Bujalska et al., 2005). Glucocorticoid activity in visceral adipose tissue, enhanced through 11βHSD1 action, may have a role in central obesity development (Gathercole and Stewart, 2010). It was previously shown that 11βHSD1 gene expression can be enhanced by DHT in the visceral adipose tissue and that DHT can thereby influence local metabolism of CORT (Zhang et al., 2008b; Zhu et al., 2010).

At the molecular level, glucocorticoids primarily exert their effects through the intracellular glucocorticoid receptor (GR), a hormone-dependent transcription factor, undergoing a conformational change and translocation to the nucleus after hormone binding (Nicolaides et al., 2010). In this way, glucocorticoids may increase the expression of several key proadipogenic and prolipogenic factors during adipose tissue development and accumulation, particularly peroxisome proliferator-activated receptor γ (PPARγ) (Galitzky and Bouloumie, 2013), lipin-1 (Zhang et al., 2008a), sterol regulatory element binding protein 1 (SREBP-1) (Ayala-Sumuano et al., 2013) fatty acid synthase (FAS) (Wang et al., 2004) and phosphoenolpyruvate carboxykinase (PEPCK) (Gathercole and Stewart, 2010). Several enzymes including hormone sensitive lipase (HSL), adipose tissue triglyceride lipase (ATGL), lipoprotein lipase (LPL) and cluster of differentiation 36 (CD36) were previously shown to be involved in glucocorticoid induced lipolysis in the visceral adipose tissue (Campbell et al., 2011; Lee et al., 2014; Yu et al., 2010).

In light of aforementioned data, the rat model of PCOS should ideally exhibit both the reproductive and metabolic abnormalities similar to those attributed to human PCOS. In this study, we sought to confirm previous observations of Manneras et al. (2007) that such a model can be obtained by continuous release of subcutaneously administered nonaromatizable androgen, 5α-dihydrotestosterone (DHT), from pre-pubertal to adult age. Considering the apparent importance of visceral obesity and dyslipidemia in PCOS pathogenesis and possible underpinning role of glucocorticoid excess, we also investigated the potential link of prolipogenic and/or prolipolytic changes with glucocorticoid metabolism and signaling in the visceral adipose tissue of DHT-treated rats. To that end, we analyzed 11βHSD1 activity, and the expression of GR and GR-regulated prolipogenic and prolipolytic genes.

2. Materials and methods

2.1. Animals and treatment

At the 21st day after birth, female Wistar rat pups were separated from lactating dames and randomly divided in two groups differing in whether they were implanted with 90-day-continuous-release pellets containing 7.5 mg of DHT (daily dose, 83 μg; DHT group) or with pellets lacking the bioactive component (placebo group). DHT and placebo pellets were purchased from Innovative Research of America (Sarasota, FL). The dose of DHT was chosen as to induce a hyper-androgenic state mimicking that seen in women with PCOS (Fassnacht et al., 2003; Silfen et al., 2003). Each experimental group was comprised of 12 animals (n = 12), which were housed three per cage, kept in a space with controlled temperature (22 ± 2 °C) and constant humidity, and under standard 12 h/12 h light/dark cycle. All animals had *ad libitum* access to commercial chow and tap water. During the 90-day long treatment, the food intake was measured daily and body mass weekly. Energy intake was calculated as the daily calories ingested through food (food mass (g) × 11 kJ). At the end of the treatment, rats were sacrificed by decapitation in the diestrus phase of the estrous cycle. The stage of cyclicity was determined by microscopic analysis of the predominant cell type in vaginal smears obtained daily from each animal from the 10th week to the end of the treatment. All protocols were compliant with the European Communities Council Directive (86/609/EEC) for the protection of animals used for experimental and other scientific purposes, and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade (No 2-20/10), according to the guidelines of the EU registered Serbian Laboratory Animal Science Association (SLASA).

2.2. Blood sample collection and determination of plasma parameters

Blood triglyceride levels were measured at decapitation after overnight fasting, by use of the MultiCare analyzer (Biochemical Systems International, Italy). Trunk blood was collected in EDTA containing tubes and plasma was isolated by centrifugation at 1600×*g* for 10 min at room temperature, and then stored at –70 °C. Plasma level of NEFA was determined by the modified version of Duncombe's method (Duncombe, 1964). Total plasma and tissue CORT concentrations were measured by the *Corticosterone High Sensitivity* EIA kit (IDS, American Laboratory Products Co) according to the manufacturer's instructions. Absorbance at 450 nm (reference 650 nm) was read at the plate reader (Multiskan Spectrum, Thermo Electron Corporation, Finland), and CORT concentrations, determined by 4PL curve fitting analysis (ReaderFit Software, MiraiBio Group of Hitachi Solutions America, Ltd.), were presented in ng/ml for plasma samples and in ng/mg of protein for tissue samples. Intra-assay coefficient of variation (CV) was 5.9%, while inter-assay CV was 8.9%. Plasma estradiol concentration was measured by Estradiol (E2) Enzyme Immunoassay Test Kit (BioCheck Inc., CA). Intra-assay CV was 24.1%, while inter-assay CV was 26.7%.

2.3. Intraperitoneal glucose tolerance test (IPGTT)

IPGTT was performed 3 days before the end of the treatment. A glucose challenge was given intraperitoneally (2 g/kg) after overnight fasting. Blood samples were taken from the tail tip at 0, 15, 30, 60, 90 and 120 min after injection in order to determine the plasma glucose concentration. Plasma glucose concentration was measured by use of the MultiCare analyzer (Biochemical Systems International). The area under the concentration *vs.* time curve (AUC glucose 0–120 min, mmol/l *vs.* min) was calculated by the trapezoidal rule by using GraphPad Prism v5 Software (San Diego, CA, USA).

2.4. Isolation of reproductive organs

After an initial longitudinal incision and opening the abdominal cavity, the uterus was located and disconnected from the base

of the vagina, while the ovaries were removed from the end of each uterine horn.

2.5. Adipose tissue isolation and fractionation

Visceral adipose tissue was collected immediately after decapitation, washed by saline (0.9% NaCl), dried, weighed and divided in parts for Western blot, qPCR and histological analyses. The isolated visceral adipose tissue was situated within the abdominal cavity and included the omental, retroperitoneal and perirenal depots. The tissue parts intended for use in Western blot and qPCR analyses were stored in liquid nitrogen until protein and RNA isolation.

For the preparation of cytoplasmic, nuclear and microsomal fractions, visceral adipose tissue of two animals from the same experimental group was pooled. Frozen adipose tissue was homogenized (w/v = 1:1) in ice-cold homogenization buffer (20 mM Tris–HCl, pH 7.2, 10% glycerol, 50 mM NaCl, 1 mM EDTA-Na_2, 1 mM EGTA-Na_2, 2 mM DTT, protease and phosphatase inhibitors). The cell lysate was then filtered through gauze and centrifuged at 2000×*g*, 15 min, at 4 °C. The resulting supernatant and pellet were further processed to generate needed cellular fractions. For cytoplasmic fraction preparation, the supernatant was centrifuged at 10,000×*g*, 25 min, at 4 °C and then recentrifuged at 150,000×*g*, 90 min, at 4 °C. The final supernatant was used as the cytoplasmic fraction. The pellet obtained after ultracentrifugation was resuspended in the Na-pyrophosphate buffer (100 mM Na-pyrophosphate, pH 7.4) and centrifuged at 150,000×*g*, 90 min, at 4 °C. The resulting pellet was resuspended, sonicated (3 × 5 s, 1 A, 50/60 Hz) in the phosphate buffer (50 mM K-phosphate, pH 7.4, 0.1 mM EDTA-Na_2, 20% glycerol and 0.1 mM DTT) and used as the microsomal fraction. To obtain the nuclear fraction, the pellet generated by the low-speed centrifugation of cell lysate was washed twice in 1 ml of HEPES buffer (25 mM HEPES, pH 7.6, 10% glycerol, 50 mM NaCl, 1 mM EDTA-Na_2, 1 mM EGTA-Na_2, 2 mM DTT, protease and phosphatase inhibitors). This was followed by low-velocity centrifugation (4000×*g*, 10 min, 4 °C), resuspension of the resulting pellet (w/v = 1:1) in NUN buffer (25 mM HEPES, pH 7.6, 1 M urea, 300 mM NaCl, 1% Nonidet P-40, protease and phosphatase inhibitors) and incubation of the suspension on ice for 1 h with continuous agitation and frequent vortexing. The supernatant containing nuclear proteins was collected by centrifugation (20,000×*g*, 10 min, at 4 °C). All samples were stored at –70 °C until use.

2.6. Histological analysis and immunohistochemistry

Samples of visceral fat and ovaries from placebo and DHT-treated rats were fixed in 10% neutral formalin or 4% paraformaldehyde, respectively, for 24 h, dehydrated in an ethanol gradient, cleared in xylene and embedded in paraffin. The ovaries were serially sectioned at 5 μm, while the adipose tissue blocks were sectioned at 10 μm thickness. Sections of both tissues were stained with hematoxylin and eosin. Ovary sections were additionally immunohistochemically stained for cleaved caspase-3 (#9661, Cell Signaling) according to the manufacturer's instructions, using the peroxidase-antiperoxidase method. Briefly, after deparaffinization, dehydration, antigen retrieval in microwave for 10 min in 10 mM citrate buffer pH 6, and blocking of endogenous peroxidases with 3% H_2O_2 in methanol, sections were incubated with 10% goat serum for 1 h, followed by incubation with primary antibody (1:100) overnight at 4 °C. Goat anti-rabbit IgG HRP conjugate (1:200, Santa Cruz Biotechnology) served as secondary antibody. The antigen-antibody complex was visualized by incubating the sections with 3,3-diaminobenzidine (DAB; DAKO A/S, Glostrup, Denmark). Morphometric analysis of adipose tissue, i.e. determination of adipocytes diameter and area, was carried out using Adiposoft (automated software for the analysis of white adipose tissue cellularity in histological sections) (Galarraga et al., 2012). Images for analysis were acquired as previously reported (Bursac et al., 2014). Briefly, tissue boundaries were firstly recognized at low magnification. Then three high-resolution, randomly located images per section were acquired at 10 × magnification. Random locations were secured by using the newCAST software package (Visiopharm Integrator System, version 3.2.7.0, Visiopharm, Denmark) connected to a microscope (Olympus, BX-51, Olympus Corp., Tokyo, Japan) and a CCD camera (PixeLINK, Ottawa, ON, Canada). The diameter and area of the cells were measured in 100 adipocytes per section (three sections for one animal and five animals per group).

2.7. Western blot analysis

Protein concentration was determined according to Spector using bovine serum albumin as a reference (Spector, 1978). The samples were boiled in 2x Laemmli buffer for 5 min, and 40 μg (cytosolic fraction) or 80 μg (nuclear and microsomal fractions, respectively) of the proteins were resolved along with molecular mass references (10–170 kDa, Fermentas) on 10% sodium dodecyl sulphate-polyacrylamide gels using Mini-Protean II Electrophoresis Cell system (Bio-Rad Laboratories, Hercules, CA). The transfer of proteins from acrylamide gels to polyvinylidene difluoride membranes (Immobilon-FL, Millipore) was performed in 25 mM Tris buffer, pH 8.3 containing 192 mM glycine and 20% (v/v) methanol, at 135 mA overnight in Mini Trans-Blot Electrophoretic Transfer Cell system (Bio-Rad Laboratories). The membranes were blocked by phosphate-buffered saline (PBS, 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 2.7 mM KCl, 0.14 M NaCl, pH 7.2) containing 3% non-fat dry milk at room-temperature for 1 h, and then incubated overnight at 4 °C with respective primary antibodies: rabbit polyclonal anti-GR (PA1-511, Affinity Bioreagents, 1:500), rabbit polyclonal anti-11βHSD1 (ab109554, Abcam, 1:1000), rabbit polyclonal anti-H6PD (sc-67394, Santa Cruz Biotechnology, 1:1000), rabbit polyclonal anti-lipin-1 (sc-98450, Santa Cruz Biotechnology, 1:1000), rabbit polyclonal anti-SREBP-1 (sc-366, Santa Cruz Biotechnology, 1:1000), mouse monoclonal anti-PPARγ (sc-7273, Santa Cruz Biotechnology, 1:500), mouse monoclonal anti-β-actin (AC-15, Sigma-Aldrich, 1:10,000) and rabbit polyclonal anti-calnexin (ab22595, Abcam, 1:10,000) antibody. β-actin was used as an equal loading control for cytoplasmic and nuclear fractions, while calnexin served as a marker for microsomal fraction. After extensive washing (PBS containing 0.1% Tween20), all membranes were incubated with appropriate alkaline phosphatase conjugated secondary antibodies (Amersham Pharmacia Biotech, 1:20,000), and the immunopositive bands were visualized by the enhanced chemifluorescence method (ECF, GE Healthcare, USA). Quantitative analysis of immunoreactive bands was performed by ImageQuant software (GE Healthcare).

2.8. RNA isolation and reverse transcription

Total RNA was extracted from visceral adipose tissue of individual animals by TRIreagent® (AmBion Inc., Austin, TX, USA). RNA was dissolved in RNase-DNase free water (Eppendorf) and its concentration and purity were tested spectrophotometrically (OD 260/280 > 1.8 was considered satisfactory). RNA integrity was confirmed by 2% agarose gel electrophoresis. Prior to cDNA synthesis, DNA contamination was removed by DNAse I treatment (Fermentas). cDNA was synthesized out of 2 μg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions, and was stored at –70 °C until use.

2.9. Real-time PCR

TaqMan gene expression probe sets (Applied Biosystems Assays-on-Demand Gene Expression Products) were used for all gene

Table 1
Caloric intake, body mass, absolute and relative masses of visceral adipose tissue, uterus and ovary of DHT-treated and placebo rats.

	Placebo	DHT
Caloric intake (kJ/day/rat)	177.20 ± 30.10	191.30 ± 34.90**
Body mass (g)	255.10 ± 13.50	295.80 ± 40.27**
Ovary (g)	0.039 ± 0.008	0.024 ± 0.015**
Ovary-to-body ratio (×100)	0.015 ± 0.003	0.009 ± 0.006**
Uterus (g)	0.44 ± 0.08	0.33 ± 0.14*
Uterus-to-body ratio (×100)	0.17 ± 0.02	0.11 ± 0.05**
Mass of visceral adipose tissue (g)	9.34 ± 2.29	13.02 ± 2.48**
Visceral adipose tissue-to body ratio (×100)	3.60 ± 0.92	4.31 ± 0.48*

The data are presented as means ± SD (n = 12 animals per group). Comparisons between DHT-treated and placebo rats were made by unpaired Student's *t*-test. Asterisks indicate significant differences; *P < 0.05, **P < 0.01.

Table 2
Biochemical and hormonal parameters in plasma samples and CORT level in adipose tissue of DHT-treated and placebo rats.

	Placebo	DHT
Triglycerides (mmol/l)	1.09 ± 0.17	1.59 ± 0.34***
NEFA (mmol/l)	0.46 ± 0.10	0.59 ± 0.14**
Estradiol (pg/ml)	4.74 ± 0.40	4.50 ± 0.43
Plasma CORT (ng/ml)	91.94 ± 46.37	116.50 ± 32.73
Visceral adipose tissue CORT (ng/mg)	1.67 ± 1.47	4.08 ± 1.85*
IPGTT glucose AUC	924.20 ± 187.60	965.50 ± 166.10

The data are presented as means ± SD (n = 12 animals per group). Comparisons between DHT-treated and placebo rats were made by unpaired Student's *t*-test. Asterisks indicate significant differences; *P < 0.05, **P < 0.01, ***P < 0.001.

expression experiments. All probes were 6-carboxyfluorescein-labeled and were as follows: GR (Rn00561369_m1), 11βHSD1 (Rn00567167_m1), PEPCK (Rn01529014_m1), LIPE (Rn00563444_m1), ATGL (PNPLA2, Rn01479969_m1), FAS (Rn01463550_m1), LPL (Rn00561482_m1) and CD36 (Rn02115479_g1). To identify the most suitable reference gene for quantitative normalization of gene expression in visceral adipose tissue of DHT-treated and placebo rats, the expressional stability of four potential reference genes: hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1, Rn01527840_m1*), b-actin (BA, Rn00667869_m1*), b2-microglobulin (B2M, Rn00560865_m1*) and TATA box binding protein (TBP, Rn01455646_m1*) was analyzed by TaqMan real-time RT PCR method, using GeNorm and NormFinder software packages. Both softwares identified HPRT1 as the most stable and therefore suitable reference gene for gene expression analyses in the adipose tissue of DHT-treated and placebo rats.

Real-time PCR was performed on ABI Prism 7000 Sequence Detection System (Applied Biosystems) in a total volume of 25 µl containing 1 × TaqMan Universal Master Mix with AmpErase UNG, 1 × Assay Mix (Applied Biosystems) and cDNA template (20 ng) at cycle conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. No template control containing nuclease-free water instead of cDNA template was used in each run. All reactions were run in triplicate. Relative quantification of target genes was achieved by the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The obtained results were analyzed by Sequence Detection Software version 1.2.3 for 7000 System SDS Software RQ Study Application (Applied Biosystems) with a confidence level of 95 % (P ≤ 0.05).

2.10. Statistical analyses

The morphological, biochemical, hormonal and histological parameters are given as means ± SD. The real-time PCR and Western blot data are presented as means ± SEM. Student's unpaired *t*-test was used to compare means between groups (DHT-treated rats *vs.* placebo rats). Results were considered statistically significant at P < 0.05. Statistical analyses were performed by using GraphPad Prism v5 Software.

3. Results

3.1. Reproductive characteristics of DHT-treated animals

All DHT-treated rats were acyclic, in diestrus phase of estrous cycle with leukocytes dominating in vaginal smears. The placebo rats preserved normal cyclicity with 4–5 days long estrous cycle. Absolute masses of ovary and uterus and their relative ratios to total body mass were significantly decreased (P < 0.05) in DHT-treated group compared to the placebo group (Table 1). As shown in Table 2 total estradiol plasma levels were unchanged in DHT-treated animals compared to placebos. In agreement with estrous cycles, ovaries of placebo group contained several generations of *corpora lutea* (CL, Fig. 1A), healthy follicles at all stages of folliculogenesis, and rare atretic follicles at various stages of degeneration (not shown). In regressive CL, apoptotic, cleaved caspase-3 immunoreactive granulosa lutein cells were present (Fig. 1A – inset). Ovaries of DHT-treated rats were smaller in size and CL were absent (Fig. 1B). Light microscopic analysis showed that a majority of follicles were atretic, ranging from early to late stages of degeneration. Secondary interstitial glands were abundant, reaching the very periphery of the ovarian cortex, which is a clear sign of dominating atretic processes (Fig. 1B). Follicles in earlier stage of atresia contained cleaved caspase-3 positive granulosa cells, while secondary interstitial glands did not, since granulosa cells that underwent programmed cell death are cleared at this stage (Fig. 1B).

3.2. Physiological and biochemical characteristics of DHT-treated animals

As presented in Table 1, DHT-treated rats had a statistically significant increase in caloric intake (P < 0.01), which was accompanied with increased body mass, absolute (P < 0.01) and relative to body visceral adipose tissue mass (P < 0.05), compared to placebos. Total CORT plasma levels were unchanged in DHT-treated animals compared to placebos, whereas visceral adipose tissue CORT level was significantly increased (P < 0.05) (Table 2). As shown in Table 2, the IPGTT AUC values were not significantly different between the experimental groups, which is in accordance with unchanged circulating glucocorticoids. DHT-treated rats also displayed significantly increased plasma triglyceride (P < 0.001) and NEFA (P < 0.01) levels compared to the placebo animals (Table 2).

3.3. Histological and morphometric analysis of visceral adipose tissue

The gross anatomy images of rats from each experimental group (Fig. 2A) showed a remarkable difference between DHT-treated and placebo animals in the visceral adipose tissue size, which was confirmed by histological and morphometric analyses of visceral adipocytes. Namely, histological analysis revealed hypertrophic adipocytes in DHT-treated rats (Fig. 2B). Further morphometric analysis showed that adipocytes of DHT-treated rats had enlarged diameter and area (Fig. 2C, P < 0.01) compared to adipocytes of placebo animals.

3.4. Glucocorticoid prereceptor metabolism and glucocorticoid receptor intracellular redistribution

To analyze the glucocorticoid prereceptor metabolism in the adipose tissue, Western blot and qPCR analyses of the key enzymes involved in the intracellular conversion of 11-dehydrocorticosterone

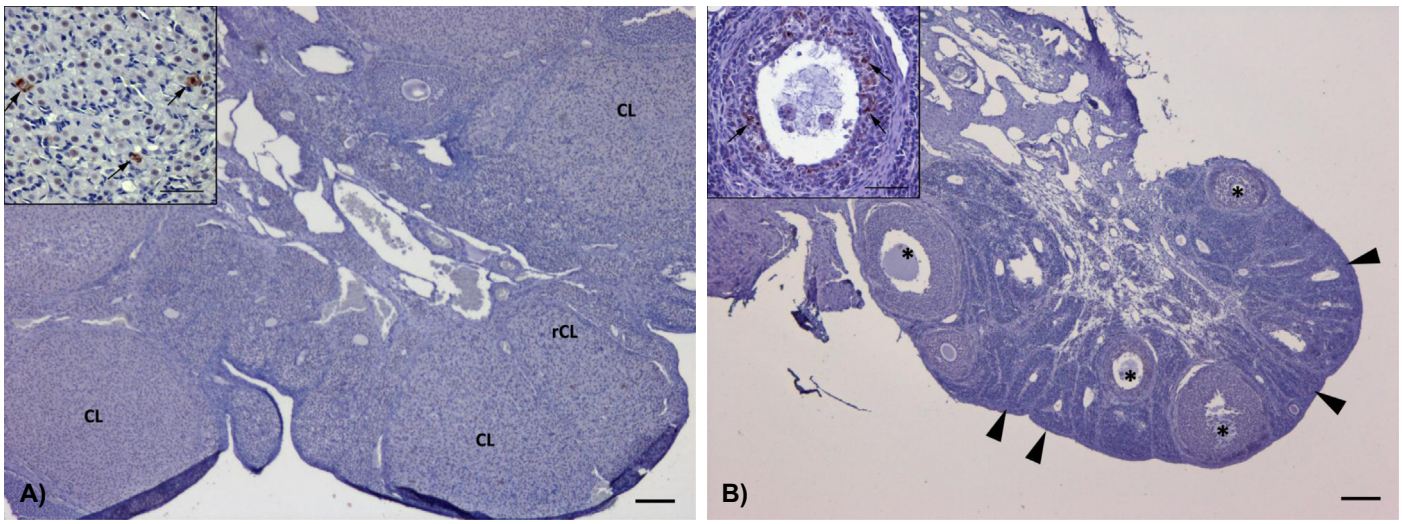

Fig. 1. Immunohistochemical localization of cleaved caspase-3 in ovaries of placebo and DHT-treated rats. (A) Numerous healthy *corpora lutea* (CL) and rare regressive CL (rCL) are present in the ovaries of placebo group. In rCL, cleaved caspase-3 immunoreactive granulosa lutein cells can be seen (inset – arrows). (B) Ovaries of DHT-treated females are smaller in size with no CL and numerous secondary interstitial glands (arrowheads). Atretic follicles (*) with cleaved caspase-3 positive granulosa cells are also present (inset – arrows). Scale bar = 100 μm, inset = 50 μm.

to CORT was performed. 11βHSD1 mRNA and protein levels were significantly increased ($P < 0.01$, Fig. 3A and C), whereas those of its coenzyme, H6PDH (Fig. 3B and D), remained unchanged in the adipose tissue microsomal fraction of DHT-treated as compared to placebo animals.

Analysis of the GR protein level and intracellular redistribution was performed by Western blot in both cytoplasmic and nuclear fractions of visceral adipose tissue. As shown in Fig. 4, a statistically significant decrease ($P < 0.05$) of the GR protein level in the cytoplasmic fraction, and its concomitant increase ($P < 0.05$) in the nuclear fraction of the visceral adipose tissue was observed after DHT treatment.

3.5. Expression of GR-regulated prolipogenic and prolipolytic genes

Visceral adipose tissue nuclear fraction was analyzed by Western blot in order to assess DHT-related changes in the levels of proadipogenic proteins PPARγ and SREBP-1. The comparisons of DHT-treated and placebo group showed a lack of change in the nuclear PPARγ protein level (Fig. 5A) and a statistically significant increase in the nuclear level of SREBP-1 ($P < 0.05$, Fig. 5B) after DHT treatment.

In the endoplasmic reticulum, lipin-1 has an enzymatic phosphatidate phosphatase (PAP-1) activity, while in the nucleus it acts as a transcriptional co-regulator of fatty acid oxidation genes. Because of this dual role, we analyzed the lipin-1 intracellular distribution in the visceral adipose tissue by performing Western blot analysis of its protein level in the microsomal and nuclear fractions. The level of lipin-1 protein in the nuclear fraction remained unchanged (Fig. 5C), while a significant increase of this protein was observed in the microsomal fraction ($P < 0.05$, Fig. 5D) after DHT treatment.

The analysis of prolipogenic FAS and PEPCK gene expression by qPCR showed significantly increased levels of both mRNAs in DHT-treated animals compared to placebos ($P < 0.05$, Table 3). The mRNA level of the lipolytic enzyme HSL was significantly decreased ($P < 0.05$, Table 3) whereas the level of ATGL mRNA remained unchanged, and that of LPL mRNA was increased ($P < 0.05$, Table 3). Transcription of fatty acid translocase CD36 gene was downregulated in the visceral adipose tissue after DHT treatment.

4. Discussion

The results of the present study showed that DHT-treated female rats present with both the reproductive and metabolic characteristics of PCOS, thereby providing an animal model suitable for biochemical and molecular studies of these disturbances in PCOS. Long-term treatment of young rats with DHT led to the arrested estrous cycle, decreased weight of ovaries and uteri and the appearance of anovulatory ovaries. The effects of DHT treatment on visceral adipose tissue included enhanced glucocorticoid prereceptor metabolism and signaling paralleled with the induction of lipogenic factors: SREBP-1, microsomal lipin-1, FAS and PEPCK. These molecular alterations were accompanied by adipocyte hypertrophy, visceral obesity, elevated plasma NEFA and triglyceride concentrations.

In our study of DHT-induced androgen excess, PCOS characteristics were confirmed at several levels. Namely, treated females were arrested in diestrus phase of the estrous cycle, while plasma estradiol levels remained unchanged. We also observed the decreased weight of both ovaries and uteri. Histological analysis of ovaries of DHT-treated rats revealed that normal processes of folliculogenesis and ovulation were disturbed, with total absence of ovulation and promotion of degenerative processes as evidenced by the presence of cleaved caspase-3 in granulosa cells of early atretic follicles. This is confirmed by the absence of CL and predomination of interstitial glands within the ovary.

The observed reproductive characteristics of PCOS were accompanied by a significant increase in both total body mass and the mass of visceral adipose tissue of DHT treated rats. It was previously shown that increased adipose tissue mass can be a consequence of either adipocyte hypertrophy or hyperplasia (Smith et al., 2006) and our histological analysis showed that DHT treatment led to dominantly hypertrophic morphology of visceral adipocytes, which is in accordance with previous findings on DHT-treated mice (van Houten et al., 2012). Apart from these histological changes, significant increases in triglyceride and NEFA levels in the plasma of DHT-treated animals were observed, which is consistent with the notion that dyslipidemia is the most common metabolic abnormality in PCOS (Macut et al., 2008).

Considering the already established link between local glucocorticoid excess and visceral obesity (Gathercole and Stewart, 2010),

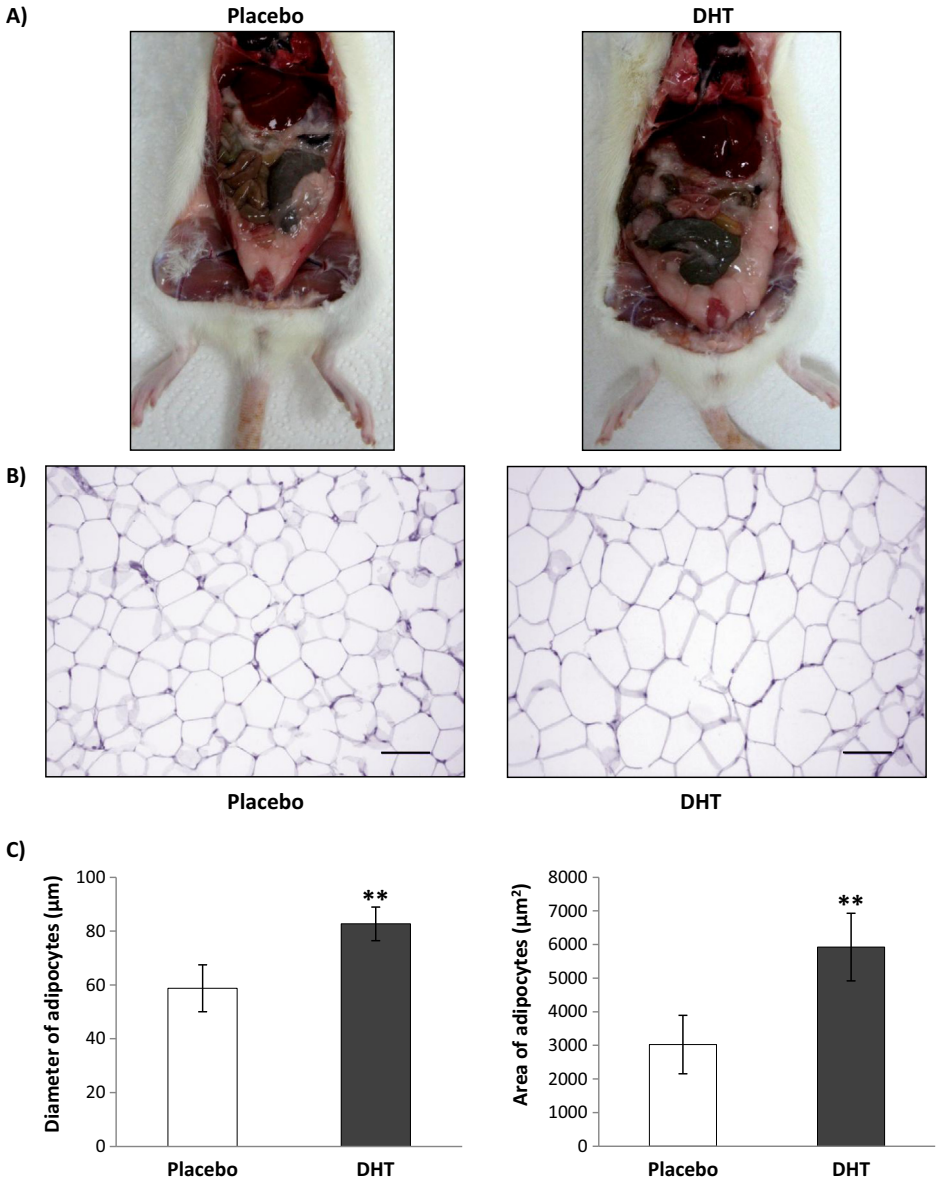

Fig. 2. Histological and morphometric analysis of visceral adipose tissue. Images of gross anatomy of DHT and placebo rats with visible visceral fats (A) and representative micrographs of hematoxylin-eosin-stained sections of visceral adipose tissue of placebo and DHT-treated rats (B). Scale bar = 100 µm. Morphometric analysis of adipocyte cell diameter and area (C) in placebo and DHT-treated rats**.** In each group, the diameter and area of the cells were measured in a total of 100 adipocytes per section (three sections per tissue part per animal and five animals per group). Data are presented as mean ± SD. DHT – rats treated with DHT. Comparisons between DHT-treated and placebo groups were made by unpaired Student's *t*-test. Significant difference between the groups is indicated as $^{**}P < 0.01$.

the aim of this study was to examine whether a similar link can be observed in DHT-treated rats. We assessed glucocorticoid prereceptor metabolism by determining mRNA and protein levels of 11βHSD1 and H6PDH enzymes, combined with tissue CORT quantification, as a measure of these enzymes activity. In spite of unchanged plasma CORT concentrations, we found locally elevated CORT within the adipose tissue of DHT-treated rats, which was probably related to enhanced 11βHSD1 activity, judged by its increased mRNA and protein levels. The level of H6PDH, providing NADPH as a cofactor for 11βHSD1 activity, was unchanged in the adipose tissue of DHT-treated rats. We presume, however, that the elevation in adipose tissue CORT can still be attributed to the sustained activity of 11βHSD1, independently of H6PDH activity, owing to the greater availability of NADPH compared to $NADPH^+$ leading to predominant reductase reaction *in vivo* (Czegle et al., 2012). Apart from analyzing the prereceptor metabolism of glucocorticoids, we also assessed the level of GR expression and its intracellular redistribution (Nicolaides et al., 2010). After DHT treatment, cytoplasmic GR level in the adipose tissue was decreased, while its statistically significant increase was observed in the nuclear fraction, suggesting that the excessive CORT found in visceral adipose tissue after treatment drives GR activation and consequent changes in the expression of GR target genes.

As already known, glucocorticoids can modulate both lipogenesis and lipolysis in the adipose tissue and the most interesting aspect of this modulation is that these hormones can concomitantly affect both of these processes in adipocytes (Wang et al., 2012). One of the GR regulated prolipogenic genes analyzed in this study is lipin-1 which has a dual role at the molecular level, acting as a transcriptional co-regulator of fatty acid oxidation genes in the nucleus, and as a phosphatidate phosphatase (PAP-1) enzyme responsible for diacylglycerol synthesis in the endoplasmic reticulum (Reue and

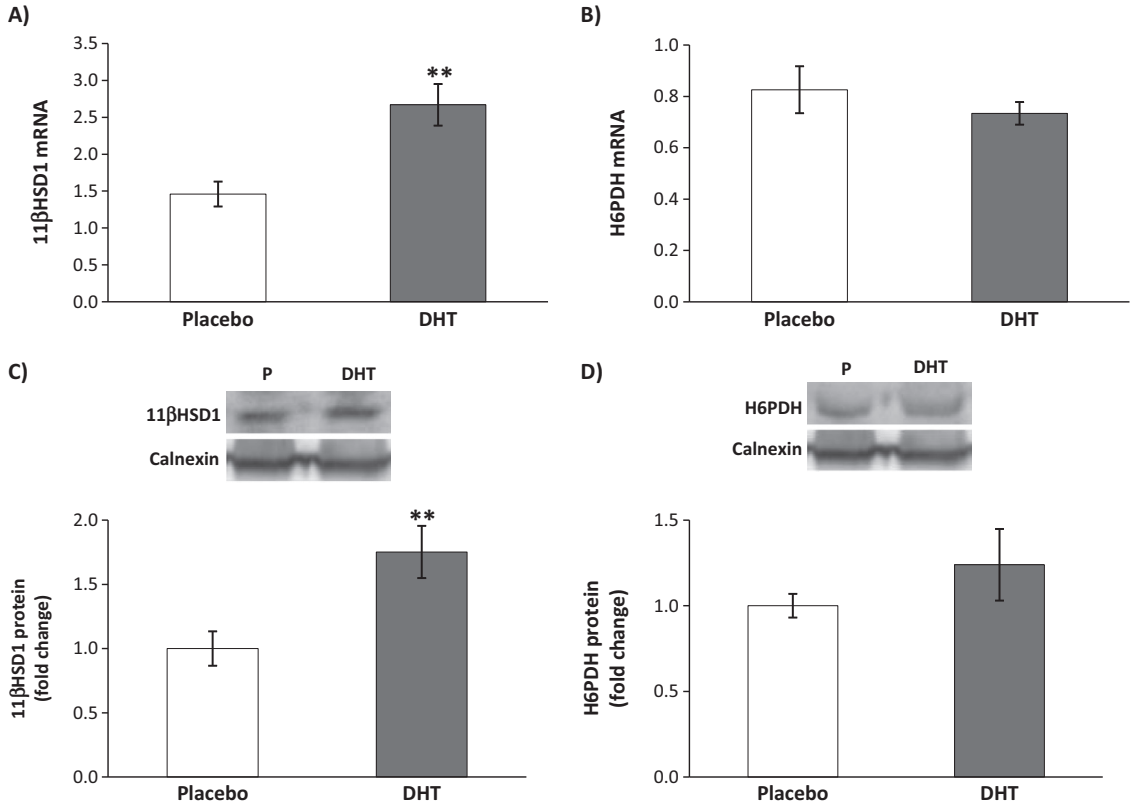

Fig. 3. Prereceptor glucocorticoid metabolism in the visceral adipose tissue of DHT-treated rats. Relative quantification of 11βHSD1 (A) and H6PDH (B) mRNA levels in the visceral adipose tissue of placebo and DHT-treated rats. Representative Western blots and relative quantification of 11βHSD1 (C) and H6PDH (D) protein levels in the microsomal fractions of adipocytes of placebo and DHT-treated rats. Lower parts of the blots were probed with calnexin antibody as an equal loading control. Data are presented as mean ± SEM (12 animals per experimental group). P – placebo rats, DHT – rats treated with DHT. Comparisons between DHT-treated and placebo groups were made by unpaired Student's *t*-test. Significant difference between the groups is indicated as $^{**}P < 0.01$.

Brindley, 2008). Our results demonstrated its significant increase in the microsomal fraction and a lack of its change in the nuclear fraction after DHT treatment, which implies the prevailing role of lipin-1 in stimulation of lipogenesis in the visceral adipose tissue of DHT-treated animals.

Yet another prolipogenic protein under expressional control of the glucocorticoids is SREBP-1 (Ayala-Sumuano et al., 2013). We observed a significant increase in the nuclear level of this protein in visceral adipose tissue after DHT treatment, which is consistent with the observed hypertrophic state of the adipocytes. SREBP-1 is known

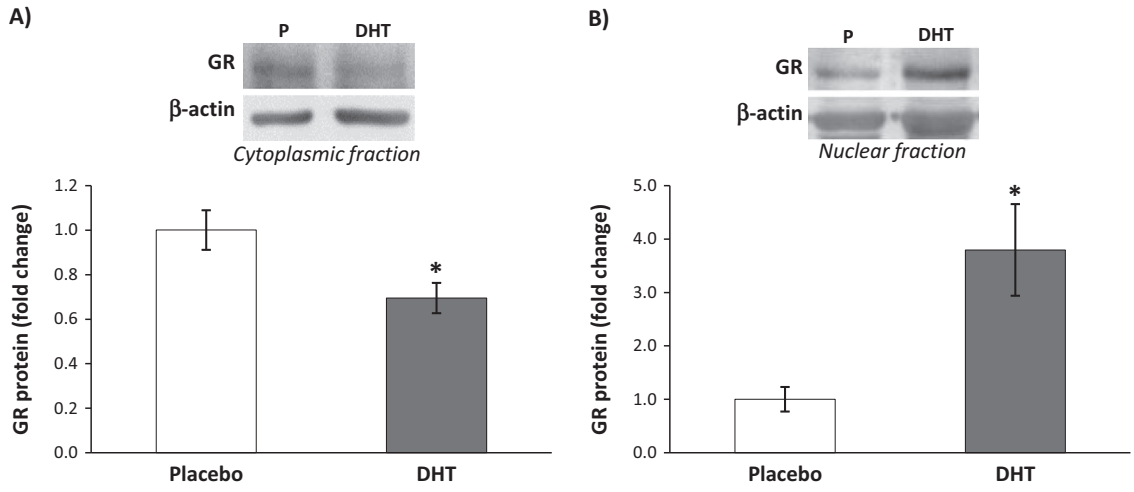

Fig. 4. GR protein level in the cytoplasmic and nuclear fractions of visceral adipose tissue of DHT-treated rats. Representative Western blots and relative quantification of GR protein levels in cytoplasmic (A) and nuclear fractions (B) of visceral adipose tissue of placebo and DHT-treated rats. Lower parts of the blots for both cellular fractions were probed with β-actin antibody as an equal loading control. Data are presented as mean ± SEM (12 animals per experimental group). P – placebo rats, DHT – rats treated with DHT. Comparisons between placebo and DHT-treated groups were made by unpaired Student's *t*-test. Significant difference between the groups is indicated as $^{*}P < 0.05$.

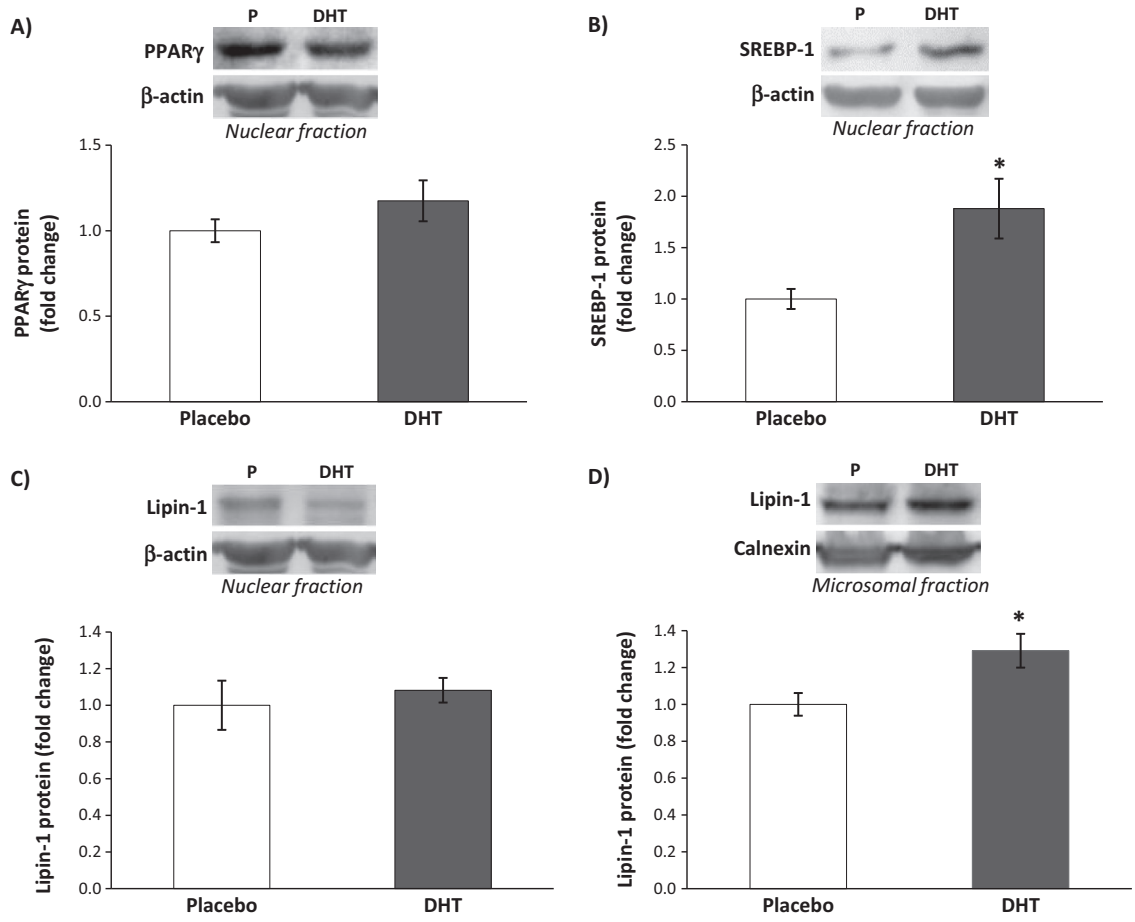

Fig. 5. PPARγ, SREBP-1 and lipin-1 protein levels in the visceral adipose tissue of DHT-treated rats. Representative Western blots and relative quantification of PPARγ (A), SREBP-1 (B) and lipin-1 (C) protein levels in the nuclear fraction and lipin-1 protein level in the microsomal fraction (D) of visceral adipose tissue of placebo and DHT-treated rats. Lower parts of the blots for the nuclear fraction were probed with β-actin antibody as an equal loading control, whereas calnexin antibody was used accordingly for the microsomal fraction. Data are presented as mean ± SEM (12 animals per experimental group). P – placebo rats, DHT – rats treated with DHT. Comparisons between placebo and DHT-treated groups were made by unpaired Student's *t*-test. Significant difference between the groups is indicated as *P < 0.05.

for its critical role in lipogenesis through direct transcriptional stimulation of genes involved in lipid synthesis and accumulation, such as FAS and acetyl-CoA carboxylase (Mandard and Kersten, 2006). In line with elevated nuclear SREBP-1, we have also observed the elevation of FAS mRNA level in the adipose tissue of DHT-treated rats.

Another GR regulated gene involved in lipogenesis is PEPCK, whose product is a key enzyme of glyceroneogenesis in the adipose tissue (Gathercole and Stewart, 2010). We found a significant increase of PEPCK mRNA level in the visceral adipose tissue of DHT-treated rats. Considering a set of previously reported data implying that the ectopic expression of PEPCK increases adipocyte lipid accumulation and NEFA re-esterification (Franckhauser et al., 2002; Lee et al., 2014), it can be presumed that PEPCK up-regulation in the visceral adipose tissue of our PCOS model is set to facilitate lipogenesis.

We also analyzed the level of PPARγ, a transcriptional factor involved in lipid metabolism (Fajas et al., 2001). PPARγ is an important regulator of adipogenesis, since it is required for the beginning of the adipocyte differentiation process through proliferation and hyperplasia (Drolet et al., 2008; Farmer, 2006). Thus, the finding of unchanged PPARγ expression in the adipose tissue of DHT-treated rats is in line with histological data indicating the hypertrophic state of adipocytes without visible hyperplasia in these animals.

These results imply that glucocorticoids are likely involved in lipogenesis and development of visceral obesity in DHT teated rats. However, it is noteworthy that DHT treatment itself can regulate the expression of all aforementioned molecules (Bolduc et al., 2004; McInnes et al., 2006; Zhang et al., 2008a) apart from lipin-1, which

Table 3
Relative quantification of mRNA level of lipogenic and lipolytic genes in the visceral adipose tissue of placebo and DHT-treated rats.

	Placebo	DHT
FAS	1.30 ± 0.26	4.83 ± 1.09*
PEPCK	1.79 ± 0.17	2.24 ± 0.12*
ATGL	0.88 ± 0.06	0.55 ± 0.14
HSL	1.20 ± 0.11	0.92 ± 0.07*
LPL	1.37 ± 0.12	2.08 ± 0.28*
CD36	0.83 ± 0.19	0.70 ± 0.14*

The data are presented as means ± SEM (n = 12 animals per group) of the triplicate analysis of RNA samples. Comparisons between DHT-treated and placebo rats were made by unpaired Student's *t*-test. A value of P < 0.05 was considered statistically significant. Asterisks indicate significant differences; *P < 0.05. ATGL, adipose tissue triglyceride lipase; CD36, cluster of differentiation 36; FAS, fatty acid synthase; HSL, hormone sensitive lipase; LPL, lipoprotein lipase; PEPCK, phosphoenolpyruvate carboxykinase.

implies a possible interplay between glucocorticoids and androgens in the regulation of lipid metabolism in our model. Although androgens have an important role in adipose lipid metabolism (O'Reilly et al., 2014), we suggest that the lipogenesis observed in our model is at least to some extent influenced by the enhanced glucocorticoid signaling, based on the implications of current research suggesting a possibility of glucocorticoids inactivating DHT and thereby overriding its effects in the adipose tissue (Hartig et al., 2012; Veilleux et al., 2012).

As mentioned before, glucocorticoids can affect both lipogenesis and lipolysis in the adipocytes and thus it was of interest to analyze the expression of several lipolytic enzymes, such as ATGL, HSL and LPL, as well as fatty acid translocase CD36 in the visceral adipose tissue. In DHT-treated rats, HSL mRNA level was significantly decreased, while that of ATGL remained unchanged. Interestingly, we also found elevated LPL mRNA level, resulting in enhanced NEFA release in the circulation. It was previously shown that facilitation of NEFA uptake is mediated by scavenger receptor CD36 (Goldberg et al., 2009), whose expression was downregulated after DHT-treatment. It could be proposed that LPL-mediated NEFA release is not supported by adequate uptake of fatty acids by CD36, resulting in increased circulating NEFA level.

The results of this study led us to several conclusions. Firstly, DHT treatment of female rats in prepubertal age induces a plethora of reproductive and metabolic disturbances compatible with those seen in human PCOS, making this animal model suitable for research on pathogenesis of this syndrome. The most important metabolic abnormalities induced by DHT treatment are dyslipidemia and visceral obesity that stems from adipocyte hypertrophy. These metabolic disturbances can at least partly derive from enhanced glucocorticoid signaling in the visceral adipose tissue, leading to intensified lipogenesis through the induction of prolipogenic factors, SREBP-1, FAS and lipin-1.

However, since some molecular mechanisms of lipid metabolism regulation of androgens and glucocorticoids overlap, it should be noted that these effects can result from a complicated interplay between androgens and glucocorticoids in the course of fat accumulation and that additional research is required in order to make a definite distinction between the effects of the two hormones in the studied model of PCOS. However, considering the aforementioned possibility of glucocorticoids inactivating DHT and thereby overriding its effects in the adipose tissue, the involvement of pronounced glucocorticoid signaling in the visceral adipose tissue accumulation and lipid metabolism disturbances should not be neglected.

Acknowledgement

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Grant III41009. The authors are thankful to Dr. Verica Milošević for assistance in histological analysis.

References

Ayala-Sumuano, J.T., Velez-delValle, C., Beltran-Langarica, A., Marsch-Moreno, M., Hernandez-Mosqueira, C., Kuri-Harcuch, W., 2013. Glucocorticoid paradoxically recruits adipose progenitors and impairs lipid homeostasis and glucose transport in mature adipocytes. Sci. Rep. 3, 2573.
Barber, T.M., Franks, S., 2013. Adipocyte biology in polycystic ovary syndrome. Mol. Cell. Endocrinol. 373, 68–76.
Bolduc, C., Larose, M., Yoshioka, M., Ye, P., Belleau, P., Labrie, C., et al., 2004. Effects of dihydrotestosterone on adipose tissue measured by serial analysis of gene expression. J. Mol. Endocrinol. 33, 429–444.
Bujalska, I.J., Draper, N., Michailidou, Z., Tomlinson, J.W., White, P.C., Chapman, K.E., et al., 2005. Hexose-6-phosphate dehydrogenase confers oxo-reductase activity upon 11 beta-hydroxysteroid dehydrogenase type 1. J. Mol. Endocrinol. 34, 675–684.
Bursac, B.N., Vasiljevic, A.D., Nestorovic, N.M., Velickovic, N.A., Vojnovic Milutinovic, D.D., Matic, G.M., et al., 2014. High-fructose diet leads to visceral adiposity and hypothalamic leptin resistance in male rats – do glucocorticoids play a role? J. Nutr. Biochem. 25, 446–455.
Campbell, J.E., Peckett, A.J., D'Souza, A.M., Hawke, T.J., Riddell, M.C., 2011. Adipogenic and lipolytic effects of chronic glucocorticoid exposure. Am. J. Physiol. Cell Physiol. 300, C198–C209.
Catteau-Jonard, S., Dewailly, D., 2013. Pathophysiology of polycystic ovary syndrome: the role of hyperandrogenism. Front. Horm. Res. 40, 22–27.
Czegle, I., Csala, M., Mandl, J., Benedetti, A., Karadi, I., Banhegyi, G., 2012. G6PT-H6PDH-11betaHSD1 triad in the liver and its implication in the pathomechanism of the metabolic syndrome. World J. Hepatol. 4, 129–138.
Drolet, R., Richard, C., Sniderman, A.D., Mailloux, J., Fortier, M., Huot, C., et al., 2008. Hypertrophy and hyperplasia of abdominal adipose tissues in women. Int. J. Obes. (Lond) 32, 283–291.
Duncombe, W.G., 1964. The colorimetric micro-determination of non-esterified fatty acids in plasma. Clin. Chim. Acta 9, 122–125.
Ehrmann, D.A., 2005. Polycystic ovary syndrome. N. Engl. J. Med. 352, 1223–1236.
Escobar-Morreale, H.F., San Millan, J.L., 2007. Abdominal adiposity and the polycystic ovary syndrome. Trends Endocrinol. Metab. 18, 266–272.
Fajas, L., Debril, M.B., Auwerx, J., 2001. PPAR gamma: an essential role in metabolic control. Nutr. Metab. Cardiovasc. Dis. 11, 64–69.
Farmer, S.R., 2006. Transcriptional control of adipocyte formation. Cell Metab. 4, 263–273.
Fassnacht, M., Schlenz, N., Schneider, S.B., Wudy, S.A., Allolio, B., Arlt, W., 2003. Beyond adrenal and ovarian androgen generation: increased peripheral 5 alpha-reductase activity in women with polycystic ovary syndrome. J. Clin. Endocrinol. Metab. 88, 2760–2766.
Franckhauser, S., Munoz, S., Pujol, A., Casellas, A., Riu, E., Otaegui, P., et al., 2002. Increased fatty acid re-esterification by PEPCK overexpression in adipose tissue leads to obesity without insulin resistance. Diabetes 51, 624–630.
Galarraga, M., Campion, J., Munoz-Barrutia, A., Boque, N., Moreno, H., Martinez, J.A., et al., 2012. Adiposoft: automated software for the analysis of white adipose tissue cellularity in histological sections. J. Lipid Res. 53, 2791–2796.
Galitzky, J., Bouloumie, A., 2013. Human visceral-fat-specific glucocorticoid tuning of adipogenesis. Cell Metab. 18, 3–5.
Gathercole, L.L., Stewart, P.M., 2010. Targeting the pre-receptor metabolism of cortisol as a novel therapy in obesity and diabetes. J. Steroid Biochem. Mol. Biol. 122, 21–27.
Goldberg, I.J., Eckel, R.H., Abumrad, N.A., 2009. Regulation of fatty acid uptake into tissues: lipoprotein lipase- and CD36-mediated pathways. J. Lipid Res. 50 (Suppl.), S86–S90.
Hartig, S.M., He, B., Newberg, J.Y., Ochsner, S.A., Loose, D.S., Lanz, R.B., et al., 2012. Feed-forward inhibition of androgen receptor activity by glucocorticoid action in human adipocytes. Chem. Biol. 19, 1126–1141.
Lee, M.J., Pramyothin, P., Karastergiou, K., Fried, S.K., 2014. Deconstructing the roles of glucocorticoids in adipose tissue biology and the development of central obesity. Biochim. Biophys. Acta 1842, 473–481.
Livadas, S., Diamanti-Kandarakis, E., 2013. Polycystic ovary syndrome: definitions, phenotypes and diagnostic approach. Front. Horm. Res. 40, 1–21.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T). Method. Methods. 25, 402–408.
Macut, D., Panidis, D., Glisic, B., Spanos, N., Petakov, M., Bjekic, J., et al., 2008. Lipid and lipoprotein profile in women with polycystic ovary syndrome. Can. J. Physiol. Pharmacol. 86, 199–204.
Mandard, S., Kersten, S., 2006. Regulation of lipogenic genes in obesity, In: Brigelius-Flohe, R., Joost, H.G., (Eds.), Nutritional Genomics: Impact on Health and Disease. Wiley-VCH Verlag GmbH & Co., Weinheim, pp. 208–219.
Manneras, L., Cajander, S., Holmang, A., Seleskovic, Z., Lystig, T., Lonn, M., et al., 2007. A new rat model exhibiting both ovarian and metabolic characteristics of polycystic ovary syndrome. Endocrinology 148, 3781–3791.
McInnes, K.J., Corbould, A., Simpson, E.R., Jones, M.E., 2006. Regulation of adenosine 5′,monophosphate-activated protein kinase and lipogenesis by androgens contributes to visceral obesity in an estrogen-deficient state. Endocrinology 147, 5907–5913.
Nicolaides, N.C., Galata, Z., Kino, T., Chrousos, G.P., Charmandari, E., 2010. The human glucocorticoid receptor: molecular basis of biologic function. Steroids 75, 1–12.
Norman, R.J., Dewailly, D., Legro, R.S., Hickey, T.E., 2007. Polycystic ovary syndrome. Lancet 370, 685–697.
O'Reilly, M.W., House, P.J., Tomlinson, J.W., 2014. Understanding androgen action in adipose tissue. J. Steroid Biochem. Mol. Biol. 143C, 277–284.
Pasquali, R., 2006. Obesity and androgens: facts and perspectives. Fertil. Steril. 85, 1319–1340.
Peckett, A.J., Wright, D.C., Riddell, M.C., 2011. The effects of glucocorticoids on adipose tissue lipid metabolism. Metabolism 60, 1500–1510.
Reue, K., Brindley, D.N., 2008. Thematic review series: glycerolipids. Multiple roles for lipins/phosphatidate phosphatase enzymes in lipid metabolism. J. Lipid Res. 49, 2493–2503.
Silfen, M.E., Denburg, M.R., Manibo, A.M., Lobo, R.A., Jaffe, R., Ferin, M., et al., 2003. Early endocrine, metabolic, and sonographic characteristics of polycystic ovary syndrome (PCOS): comparison between nonobese and obese adolescents. J. Clin. Endocrinol. Metab. 88, 4682–4688.

Smith, J., Al-Amri, M., Dorairaj, P., Sniderman, A., 2006. The adipocyte life cycle hypothesis. Clin. Sci. (Lond.) 110, 1–9.
Spector, T., 1978. Refinement of the coomassie blue method of protein quantitation. A simple and linear spectrophotometric assay for less than or equal to 0.5 to 50 microgram of protein. Anal. Biochem. 86, 142–146.
Teede, H.J., Hutchison, S., Zoungas, S., Meyer, C., 2006. Insulin resistance, the metabolic syndrome, diabetes, and cardiovascular disease risk in women with PCOS. Endocrine 30, 45–53.
van Houten, E.L., Kramer, P., McLuskey, A., Karels, B., Themmen, A.P., Visser, J.A., 2012. Reproductive and metabolic phenotype of a mouse model of PCOS. Endocrinology 153, 2861–2869.
Veilleux, A., Cote, J.A., Blouin, K., Nadeau, M., Pelletier, M., Marceau, P., et al., 2012. Glucocorticoid-induced androgen inactivation by aldo-keto reductase 1C2 promotes adipogenesis in human preadipocytes. Am. J. Physiol. Endocrinol. Metab. 302, E941–E949.
Walker, B.R., Andrew, R., 2006. Tissue production of cortisol by 11beta-hydroxysteroid dehydrogenase type 1 and metabolic disease. Ann. N. Y. Acad. Sci. 1083, 165–184.
Wang, J.C., Gray, N.E., Kuo, T., Harris, C.A., 2012. Regulation of triglyceride metabolism by glucocorticoid receptor. Cell Biosci. 2, 19.
Wang, Y., Jones Voy, B., Urs, S., Kim, S., Soltani-Bejnood, M., Quigley, N., et al., 2004. The human fatty acid synthase gene and de novo lipogenesis are coordinately regulated in human adipose tissue. J. Nutr. 134, 1032–1038.
Yu, C.Y., Mayba, O., Lee, J.V., Tran, J., Harris, C., Speed, T.P., et al., 2010. Genome-wide analysis of glucocorticoid receptor binding regions in adipocytes reveal gene network involved in triglyceride homeostasis. PLoS ONE 5, e15188.
Zhang, P., O'Loughlin, L., Brindley, D.N., Reue, K., 2008a. Regulation of lipin-1 gene expression by glucocorticoids during adipogenesis. J. Lipid Res. 49, 1519–1528.
Zhang, Y., Calvo, E., Martel, C., Luu-The, V., Labrie, F., Tchernof, A., 2008b. Response of the adipose tissue transcriptome to dihydrotestosterone in mice. Physiol. Genomics 35, 254–261.
Zhu, L., Hou, M., Sun, B., Buren, J., Zhang, L., Yi, J., et al., 2010. Testosterone stimulates adipose tissue 11beta-hydroxysteroid dehydrogenase type 1 expression in a depot-specific manner in children. J. Clin. Endocrinol. Metab. 95, 3300–3308.

5α-DIHYDROTESTOSTERONE TREATMENT INDUCES METABOLIC CHANGES ASSOCIATED WITH POLYCYSTIC OVARY SYNDROME WITHOUT INTERFERING WITH HYPOTHALAMIC LEPTIN AND GLUCOCORTICOID SIGNALING

Marina Nikolić[1], Nataša Veličković[1], Ana Djordjevic[1], Biljana Bursać[1], Djuro Macut[2], Ivana Božić Antić[2], Jelica Bjekić Macut[3], Gordana Matić[1] and Danijela Vojnović Milutinović[1,*]

[1]*Department of Biochemistry, Institute for Biological Research "Siniša Stanković", University of Belgrade*, 142 Despot Stefan Blvd., 11000 Belgrade, Serbia
[2]*Institute of Endocrinology, Diabetes and Metabolic Diseases, Clinical Center of Serbia and School of Medicine, University of Belgrade*, Dr Subotića 13, 11000 Belgrade, Serbia
[3]*CHC Bežanijska kosa*, Bežanijska kosa bb, 11080 Belgrade, Serbia

***Corresponding author:** dvojnovic@ibiss.bg.ac.rs

Received: December 14, 2015; **Revised:** December 23, 2015; **Accepted:** December 28, 2015

Abstract: Polycystic ovary syndrome (PCOS) is the most common endocrinopathy in women of reproductive age. It is a heterogenous disorder, with hyperandrogenism, chronic anovulation and polycystic ovaries as basic characteristics, and associated metabolic syndrome features. Increased secretion of leptin and leptin resistance are common consequences of obesity. Leptin is a hormone with anorexigenic effects in the hypothalamus. Its function in the regulation of energy intake and consumption is antagonized by glucocorticoids. By modulating leptin signaling and inflammatory processes in the hypothalamus, glucocorticoids can contribute to the development of metabolic disturbances associated with central energy disbalance. The aim of the study was to examine the relationship between hypothalamic leptin, glucocorticoid and inflammatory signaling in the development of metabolic disturbances associated with PCOS. The study was conducted on an animal model of PCOS generated by a continual, 90-day treatment of female rats with 5α-dihydrotestosterone (DHT). The model exhibited all key reproductive and metabolic features of the syndrome. mRNA and/or protein levels of the key components of hypothalamic glucocorticoid, leptin and inflammatory pathways, presumably contributing to energy disbalance in DHT-treated female rats, were measured. The results indicated that DHT treatment led to the development of hyperphagia and hyperleptinemia as metabolic features associated with PCOS. However, these metabolic disturbances could not be ascribed to changes in hypothalamic leptin, glucocorticoid or inflammatory signaling pathways in DHT-treated rats.

Keywords: DHT; hypothalamus; leptin; glucocorticoids; inflammation

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common female endocrinopathy affecting 4-8% of women of reproductive age [1-3]. It is a heterogenous endocrinological disorder involving both reproductive and metabolic abnormalities, most importantly hyperandrogenemia, chronic anovulation and polycystic ovaries. Features of the metabolic syndrome, notably visceral obesity, dyslipidemia, low glucose tolerance and insulin resistance are frequently associated with PCOS [3, 4]. It has been suggested that hyperandrogenemia, as one of the key features of PCOS, can affect food intake by increasing food craving, and thereby induce weight gain in women [5].

Obesity, most frequently the central type, is very common in PCOS, with hyperandrogenemia particularly stimulating the propagation of visceral adipose tissue [6, 7]. The most important influences of obesity on the genesis and self-propagation of PCOS include the stimulation of hyperinsulinemia and insulin resistance, chronic low-grade inflammation and general lipotoxicity [8, 9].

Adipose tissue influences central food intake and energy expenditure control through the secretion of adipokines, among which is leptin [9, 10]. Leptin blood concentration is positively correlated with adipocyte size and general adiposity. Its secretion is stimulated by insulin and glucocorticoids, and inhibited by androgens [11, 12]. Leptin passes the blood-brain barrier (BBB) and, after binding to the long form of leptin receptor (ObRb) in the hypothalamus, exerts its anorexigenic effects through the transcriptional activation/repression of a number of genes, including those coding for neuropeptide Y (NPY), Agouti-related peptide (AgRP) and proopiomelanocortin (POMC) [13].

Leptin resistance is a state in which leptin is not able to perform its functions, in spite of its high levels in the circulation [14]. It is frequently a consequence of visceral obesity [14], and can induce food consumption abnormalities, which intensify the effects of obesity on the pathophysiology of PCOS [15]. Leptin resistance can arise at different stages of the leptin transport through the BBB, or the hypothalamic ObRb signaling: most importantly the stimulated downregulation of ObRb [14, 16, 17] and/or expression of the suppressor of the cytokine signaling 3 (SOCS-3) gene, which leads to the increased negative feedback regulation of the leptin signal transduction [14, 18, 19]. Furthermore, an important role in the genesis of leptin resistance can be attributed to the local inflammatory mediator tumor necrosis factor α (TNFα), interleukin 6 (IL-6), IκB kinase (IKKβ)/nuclear factor κB (NFκB) and the proinflammatory actions of free fatty acids (FFA) [13, 14, 18, 19].

Glucocorticoid hormones can modulate the leptin signaling pathway at the level of signal transducer and activator of transcription 3 (STAT3) and SOCS-3 gene expressions [20]. The levels of bioactive forms of glucocorticoids can be regulated locally through the action of 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1)/hexoso-6-phosphate dehydrogenase (H6PDH) enzymatic system, which converts the inactive forms of these hormones to the active ones [21, 22]. Increased activation of glucocorticoids is considered important for the genesis of metabolic disorders, including those linked to PCOS [21-24]. Glucocorticoids are also well-known as anti-inflammatory molecules generally suppressing the expression and modifying the activities of proinflammatory cytokines, such as IL-6 and TNF-α, and transcription regulators, such as NFκB [25, 26], aforementioned as possible mediators of leptin resistance in the hypothalamus.

Our previous work has shown the possibility of involvement of glucocorticoid genomic effects, generally exerted after binding to and activating the glucocorticoid receptor (GR), in metabolic disturbances in the rat model of PCOS generated by 5α-dihydrotestosterone (DHT) treatment [27]. More specifically, we have found changes in visceral adipose tissue lipid metabolism leading to hypertrophic visceral obesity [27]. Considering the positive correlation of obesity and adipocyte size with leptin secretion, the importance of leptin in the hypothalamic regulation of energy intake and its aforementioned interactions with glucocorticoid signaling in the hypothalamus, we investigated the link between hypothalamic leptin, glucocorticoid and inflammatory signaling changes, and energy intake disturbances in female rats subjected to long-term DHT treatment.

MATERIALS AND METHODS

Animals and treatment

At the 21st day after birth, female Wistar rat pups were separated from lactating dames and randomly divided into two groups. The first group was implanted with 90-day-continuous-

release pellets containing 7.5 mg of DHT (daily dose, 83 μg; DHT group), and the second was treated with pellets lacking the bioactive component (Placebo group). DHT and placebo pellets were purchased from Innovative Research of America (Sarasota, FL, USA). The dose of DHT was chosen to induce the hyperandrogenic state corresponding to that seen in women with PCOS [28, 29]. Each experimental group was comprised of 12 animals (n=12), which were housed three per cage, kept in a space with controlled temperature (22±2°C) and constant humidity, and under a standard 12 h/12 h light/dark cycle. All animals had *ad libitum* access to commercial chow and tap water. During the 90-day treatment, food intake was measured daily and body mass weekly. Energy intake was calculated as daily calories ingested through food (food mass (g) × 11 kJ). At the end of the treatment, rats were killed by decapitation in the diestrus phase of the estrous cycle. The stage of cyclicity was determined by microscopic analysis of the predominant cell type in vaginal smears obtained daily from each animal from the 10^{th} week to the end of the treatment. All protocols were compliant with the European Communities Council Directive (86/609/EEC) for the protection of animals used for experimental and other scientific purposes, and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade (No 2-20/10), according to the guidelines of the EU-registered Serbian Laboratory Animal Science Association (SLASA).

Tissue and blood sample collection and determination of plasma parameters

Immediately after the experimental animals were killed by rapid decapitation, visceral adipose tissue and hypothalami were isolated, weighed and frozen in liquid nitrogen for storage until further use. Trunk blood was collected at decapitation in EDTA-containing tubes and the blood triglyceride concentration was measured on site by MultiCare strips (Biochemical Systems International, Arezzo, Italy). Plasma was isolated by centrifugation at 1600 × g for 15 min at room temperature, and then stored at -70°C. The plasma level of FFA was determined using a modified version of Duncombe's method [30]. Total plasma leptin concentrations were measured by the Rat Leptin ELISA Kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Absorbance at 450 nm (reference 590 nm) was read using a plate reader (Multiskan Spectrum, Thermo Electron Corporation, Waltham, MA, USA, and plasma leptin concentrations, determined by 4PL curve fitting analysis (ReaderFit Software, MiraiBio Group of Hitachi Solutions America, Ltd., San Bruno, CA, USA), were presented in ng/mL. The intra-assay coefficient of variation (CV) was 5.9%, while inter-assay CV was 8.9%.

Preparation of hypothalamic whole cell extracts

Hypothalami were homogenized in 4 vol. (w/v) of radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet NP40, 0.1% SDS, 2 mM DTT, 1 mM EDTA-Na2, 0.15 mM spermine, 0.15 mM spermidine, protease and phosphatase inhibitors) using a glass/teflon (Potter-Elvehjem) homogenizer. The homogenates were sonicated on ice (3 x 10 s at 10 MHz, Hielscher Ultrasound Processor, Hielscher Ultrasonics GmbH, Teltow, Germany) and incubated for 60 min at 0^{o}C prior to 20-min centrifugation at 14000 x g. The resulting supernatants were stored at -70^{o}C. Protein content was determined according to Spector [31].

SDS-PAGE and immunoblotting

Proteins were resolved on 7.5% SDS-polyacrylamide gels using Mini-Protean II Electrophoresis Cell (Bio-Rad Laboratories, Hercules, CA, USA). Transfer of proteins from acrylamide gels to PVDF membranes (Immobilon-FL, Millipore Billerica, MA, USA) was performed in 25 mM Tris buffer, pH 8.3, containing 192 mM glycine and 20% (v/v) methanol, at 135 mA overnight in Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked by phosphate-buffered saline (PBS, 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 2.7 mM KCl, 0.14 M NaCl, pH 7.2)

containing 3% non-fat dry milk for 90 min at room temperature. After extensive washing (PBS containing 0.1% Tween20), membranes were incubated overnight at 4ºC with respective primary antibodies: rabbit polyclonal anti-leptin receptor (ab5593, Abcam, Cambridge, UK), rabbit polyclonal anti-GR (PA1-511, Thermo Scientific, Waltham, MA, USA), rabbit polyclonal anti-11β-HSD1 (ab109554, Abcam, Cambridge, UK), rabbit polyclonal anti-H6PD (sc-67394, Santa Cruz Biotechnology, Dallas, TX, USA), rabbit polyclonal anti-NFκB (sc-372, Santa Cruz Biotechnology, Dallas, TX, USA) and mouse monoclonal anti-β-actin (AC-15, Sigma-Aldrich, Saint Louis, MO, USA), which was used as an equal loading control. After thorough washing, all membranes were incubated with alkaline phosphatase conjugated secondary antibodies (Amersham Pharmacia Biotech, Little Chalfont, UK, 1:20000). The immunoreactive proteins were visualized by an enhanced chemifluorescence method (ECF, Amersham Pharmacia Biotech, Little Chalfont, UK) and quantitative analysis was performed by Image-Quant software (GE Healthcare, Little Chalfont, UK).

RNA isolation and reverse transcription

Total hypothalamic RNA was isolated using TRI Reagent® (AmBion, Waltham, MA, USA). RNA was dissolved in RNase-DNase free water (Eppendorf, Hamburg, Germany) and its concentration and purity were tested spectrophotometrically (OD 260/280>1.8 was considered satisfactory). RNA integrity was confirmed by 2% agarose gel electrophoresis. RNase inhibitor (Applied Biosystems, Foster City, CA, USA) was added and the samples were frozen at -70^{o}C until use. Prior to cDNA synthesis, DNA contamination was removed by DNase I treatment (Fermentas, Waltham, MA, USA). cDNA was synthesized from 2 μg of RNA. Reverse transcription was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions, and cDNA was stored at -70^{o}C until use.

Quantitative real-time PCR

Quantification of ObRb, GR, NPY, SOCS-3, TNFα and IL-6 mRNA levels in the hypothalamus was performed by TaqMan® Real Time PCR. Primers and probes for GR, ObRb, NPY, SOCS-3, TNFα and IL-6 (Rn00567167_m1, Rn00561369_m1, Rn01410145_m1 and Rn00585674_s1, Rn01525859_g1, Rn01410330_m1, respectively) were obtained from Applied Biosystems Assay-on-Demand Gene Expression Products. HPRT1 (Rn01527840_m1) was used as a previously validated endogenous control. Quantitative real time PCR (qPCR) was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City CA, USA) in a total volume of 25 μL containing 1 × TaqMan® Universal Master Mix with AmpErase UNG, 1 × Assay Mix (Applied Biosystems, Foster City CA, USA) and the cDNA template (20 ng of RNA converted to cDNA) as follows: at 50^{o}C for 2 min and 95^{o}C for 10 min, followed by 50 cycles at 95^{o}C for 15 s and 60^{o}C for 90 s. No template control was used in any run. All reactions were run in triplicate. Relative quantification of target genes was performed using the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The obtained results were analyzed by Sequence Detection Software version 1.2.3 for 7000 System SDS Software RQ Study Application (Applied Biosystems, Foster City CA, USA) with a confidence level of 95% ($p \leq 0.05$).

Statistical analyses

Statistical analyses were performed using Prism software 5.00 (GraphPad, San Diego, CA, USA). The results are expressed as means±SD for biochemical and hormonal parameters, and as means±SEM for data from Western blot analysis and qPCR. Values were considered statistically significant when the p value was less than 0.05.

RESULTS

Morphological and basic metabolic parameters of DHT-treated female rats

The energy intake of DHT-treated rats was significantly increased when compared placebo-treated rats and was accompanied by an increase in visceral adipose tissue mass (Table 1, $p<0.05$).

Blood triglyceride, FFA and leptin levels

Statistically significant increases in blood triglyceride level (***, $p<0.001$), as well as in the plasma levels of FFA (*, $p<0.05$) and leptin (**, $p<0.01$) were observed after DHT-treatment in comparison to the Placebo group (Figs. 1 and 2).

Leptin signaling alterations in DHT-treated animals

The effects of DHT treatment on the expression of hypothalamic ObRb, SOCS-3 and NPY were examined by qPCR and Western blot. Subsequent Student t-test analyses showed the lack of significant changes in both relative protein and mRNA levels of ObRb, as well as in the relative amounts of SOCS-3 and NPY mRNAs in the hypothalami of DHT-treated rats (Fig. 3).

Glucocorticoid signaling in the hypothalami of DHT-treated rats

Changes in glucocorticoid signaling in the hypothalami of female rats after DHT treatment were examined by semiquantitative Western blot analyses of glucocorticoid prereceptor metabolism, qPCR analysis of relative GR mRNA levels and Western blot analysis of relative GR protein levels in the hypothalamic whole cell extracts. The results obtained are shown in Fig. 4, and imply an unchanged glucocorticoid regeneration and signaling in the hypothalamus of the DHT-treated rats.

Local inflammation in the hypothalami of DHT-treated rats

The relative local expression of several cytokines potentially involved in the changes of hypothalamic leptin signaling was also studied by qPCR and Western blot analyses. Succeeding statistical evaluations showed that neither IL-6 and TNFα mRNA, nor relative NFκB protein levels were significantly influenced by DHT treatment (Table 2, Fig. 5).

DISCUSSION

This study was performed on a hyperandrogenemic rat model of PCOS, obtained by the continual subcutaneous administration of a nonaromatizable form of testosterone, DHT, from the beginning of puberty up to adulthood. The model exhibited the main reproductive and metabolic features of PCOS [27]. The obtained results confirmed the existence of visceral adiposity, dyslipidemia and hyperphagia with increased energy intake in DHT-treated female rats (Table 1, Fig. 1). Significant hypertrophy of visceral fat adipocytes was also previously observed in the same animals [27]. Taking this fact and the noticed metabolic changes into account, the presence of blood hyperleptinemia and possible hypothalamic leptin resistance seems to be a reasonable assumption [11, 12, 14]. A significant elevation in blood leptin concentration was indeed observed in the DHT-treated females (Fig. 2). Namely, hyperleptinemia is often linked with hypothalamic leptin resistance, especially in obese animals. The state of leptin resistance can include signal transduction impediments after hormone binding to ObRb [17], involving the increased expression of the SOCS-3 gene in response to leptin and/or increased negative regulation of ObRb receptor gene expression [14, 18, 19]. However, in spite of the observed hyperleptinemia, the results of qPCR and Western blot analyses did not indicate these changes in the leptin signaling pathway (Fig. 3A, B and C).

One of the most important effector molecules in leptin control of energy balance is NPY [13], whose expression is influenced by glucocorticoids and insulin [32]. The effect of glucocorticoids on the NPY secretion in the hypothalamus is notably opposite to that of leptin and insulin [33] and involves the NPY gene transcriptional control [32, 34]. Therefore, in this study the changes in the corresponding hypothalamic mRNA levels were analyzed, but our

results did not show a statistically significant increase in NPY gene expression after DHT treatment (Fig. 3D).

The analyses of local glucocorticoid signaling were also performed, taking into account the aforementioned crosstalk between glucocorticoid and leptin signaling at the levels of NPY, ObRb and SOCS-3 gene expressions in central energy-intake regulation [20, 32, 33]. Analyses of the levels of 11β-HSD1 and H6PDH enzymes, the hypothalamic proteins involved in the regeneration of biologically active glucocorticoids [34], revealed that glucocorticoid prereceptor metabolism, as well as hypothalamic GR mRNA and protein levels were unaltered by DHT treatment (Fig. 4). Together, the observed results imply an unaffected glucocorticoid signaling, and therefore the absence of the potential effects of glucocorticoids on the leptin-signaling components and NPY expression in the hypothalamus of the chosen PCOS model.

An important role in the metabolic disturbances linked to the energy intake disbalance can be assigned to local inflammatory processes in the hypothalamus. Local inflammatory consequences can arise due to the overactivity of the IKKβ/NFkB system, or the increase in TNFα and IL-6 proinflammatory mediators [13, 14, 18, 19]. However, the analyses performed in this study did not show changes in TNFα and IL-6 mRNA levels (Table 2), which is in accordance with the unaltered NFκB protein level in the hypothalamic whole-cell extract of DHT-treated rats (Fig. 5).

The results of the present study point to a disturbed energy balance after DHT treatment, as illustrated by visceral adiposity, dyslipidemia and an increased energy intake, with adjoining hyperleptinemia. At the same time, the performed molecular analyses did not confirm the relation of the listed metabolic changes with either hypothalamic leptin, glucocorticoid or inflammatory signaling changes in the chosen PCOS model. Therefore, some additional aspects of leptin resistance, such as FFA-influenced STAT3 regulation, or the hyperleptinemia-stimulated inhibitory phosphorylations of ObRb, should be analyzed as potential markers of disturbed energy balance control in DHT-treated rats.

Acknowledgments: This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Grant III41009.

Authors' contributions: Animal handling and sample collecting were carried out by MN, BB and DVM. The theoretical analysis was carried out by IBA, JBM, DM and GM. Experiments were carried out by MN and DVM. All authors analyzed and discussed the results. The manuscript was written by MN and revised by ADJ, NV, DVM and GM. All authors read the manuscript and agreed with its content.

Conflict of interest disclosure: The authors declare no conflicts of interest.

REFERENCES

1. Rotterdam Eshre Asrm-Sponsored Pcos Consensus Workshop Group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. Fertil Steril. 2004;81(1):19-25.
2. Conway G, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Franks S, Gambineri A, Kelestimur F, Macut D, Micic D, Pasquali R, Pfeifer M, Pignatelli D, Pugeat M, Yildiz BO. The polycystic ovary syndrome: a position statement from the European Society of Endocrinology. Eur J Endocrinol. 2014;171(4):P1-29.
3. Sirmans SM, Pate KA. Epidemiology, diagnosis, and management of polycystic ovary syndrome. Clin Epidemiol. 2013;6:1-13.
4. Diamanti-Kandarakis E, Papavassiliou AG, Kandarakis SA, Chrousos GP. Pathophysiology and types of dyslipidemia in PCOS. Trends Endocrinol Metab. 2007;18(7):280-5.

5. Kanaya N, Vonderfecht S, Chen S. Androgen (dihydrotestosterone)-mediated regulation of food intake and obesity in female mice. J Steroid Biochem Mol Biol. 2013;138:100-6.
6. Barber TM, Franks S. Adipocyte biology in polycystic ovary syndrome. Mol Cell Endocrinol. 2013;373(1-2):68-76.
7. Rodriguez-Cuenca S, Monjo M, Proenza AM, Roca P. Depot differences in steroid receptor expression in adipose tissue: possible role of the local steroid milieu. Am J Physiol Endocrinol Metab. 2005;288(1):E200-7.
8. Diamanti-Kandarakis E. Polycystic ovarian syndrome: pathophysiology, molecular aspects and clinical implications. Expert Rev Mol Med. 2008;10:e3.
9. Tchernof A, Despres JP. Pathophysiology of human visceral obesity: an update. Physiol Rev. 2013;93(1):359-404.
10. Lee MJ, Wu Y, Fried SK. Adipose tissue heterogeneity: implication of depot differences in adipose tissue for obesity complications. Mol Aspects Med. 2013;34(1):1-11.
11. Ahima RS, Osei SY. Molecular regulation of eating behavior: new insights and prospects for therapeutic strategies. Trends Mol Med. 2001;7(5):205-13.
12. Ribiere C, Plut C. Nutritional regulation of leptin signaling. Curr Hypertens Rep. 2005;7(1):11-6.
13. Pan H, Guo J, Su Z. Advances in understanding the interrelations between leptin resistance and obesity. Physiol Behav. 2014;130:157-69.
14. Myers MG, Cowley MA, Munzberg H. Mechanisms of leptin action and leptin resistance. Annu Rev Physiol. 2008;70:537-56.
15. Rojas J, Chavez M, Olivar L, Rojas M, Morillo J, Mejias J, Calvo M, Bermudez V. Polycystic ovary syndrome, insulin resistance, and obesity: navigating the pathophysiologic labyrinth. Int J Reprod Med. 2014;2014:719050.
16. Morris DL, Rui L. Recent advances in understanding leptin signaling and leptin resistance. Am J Physiol Endocrinol Metab. 2009;297(6):E1247-59.
17. Yang R, Barouch LA. Leptin signaling and obesity: cardiovascular consequences. Circ Res. 2007;101(6):545-59.
18. Munzberg H, Bjornholm M, Bates SH, Myers MG, Jr. Leptin receptor action and mechanisms of leptin resistance. Cell Mol Life Sci. 2005;62(6):642-52.
19. Wauman J, Tavernier J. Leptin receptor signaling: pathways to leptin resistance. Front Biosci (Landmark Ed). 2011;16:2771-93.
20. La Fleur SE. The effects of glucocorticoids on feeding behavior in rats. Physiol Behav. 2006;89(1):110-4.
21. Gathercole LL, Stewart PM. Targeting the pre-receptor metabolism of cortisol as a novel therapy in obesity and diabetes. J Steroid Biochem Mol Biol. 2010;122(1-3):21-7.
22. Wang M. The role of glucocorticoid action in the pathophysiology of the Metabolic Syndrome. Nutr Metab (Lond). 2005;2(1):3.
23. Czegle I, Csala M, Mandl J, Benedetti A, Karadi I, Banhegyi G. G6PT-H6PDH-11β-HSD1 triad in the liver and its implication in the pathomechanism of the metabolic syndrome. World J Hepatol. 2012;4(4):129-38.
24. Mlinar B, Marc J, Jensterle M, Bokal EV, Jerin A, Pfeifer M. Expression of 11β-hydroxysteroid dehydrogenase type 1 in visceral and subcutaneous adipose tissues of patients with polycystic ovary syndrome is associated with adiposity. J Steroid Biochem Mol Biol. 2011;123(3-5):127-32.
25. Oakley RH, Cidlowski JA. The biology of the glucocorticoid receptor: new signaling mechanisms in health and disease. J Allergy Clin Immunol. 2013;132(5):1033-44.
26. Staab CA, Maser E. 11β-hydroxysteroid dehydrogenase type 1 is an important regulator at the interface of obesity and inflammation. J Steroid Biochem Mol Biol. 2010;119(1-2):56-72.

27. Nikolić M, Macut D, Djordjevic A, Veličković N, Nestorović N, Bursać B, Antić IB, Macut JB, Matić G, Vojnović Milutinović D. Possible involvement of glucocorticoids in 5α-dihydrotestosterone-induced PCOS-like metabolic disturbances in the rat visceral adipose tissue. Mol Cell Endocrinol. 2015;399:22-31.
28. Fassnacht M, Schlenz N, Schneider SB, Wudy SA, Allolio B, Arlt W. Beyond adrenal and ovarian androgen generation: Increased peripheral 5α-reductase activity in women with polycystic ovary syndrome. J Clin Endocrinol Metab. 2003;88(6):2760-6.
29. Silfen ME, Denburg MR, Manibo AM, Lobo RA, Jaffe R, Ferin M, Levine LS, Oberfield SE. Early endocrine, metabolic, and sonographic characteristics of polycystic ovary syndrome (PCOS): comparison between nonobese and obese adolescents. J Clin Endocrinol Metab. 2003;88(10):4682-8.
30. Duncombe WG. The Colorimetric Micro-Determination of Non-Esterified Fatty Acids in Plasma. Clin Chim Acta. 1964;9:122-5.
31. Spector T. Refinement of the coomassie blue method of protein quantitation. A simple and linear spectrophotometric assay for less than or equal to 0.5 to 50 microgram of protein. Anal Biochem. 1978;86(1):142-6.
32. Patel R, Williams-Dautovich J, Cummins CL. Minireview: new molecular mediators of glucocorticoid receptor activity in metabolic tissues. Mol Endocrinol. 2014;28(7):999-1011.
33. Morton GJ, Cummings DE, Baskin DG, Barsh GS, Schwartz MW. Central nervous system control of food intake and body weight. Nature. 2006;443(7109):289-95.
34. Geer EB, Islam J, Buettner C. Mechanisms of glucocorticoid-induced insulin resistance: focus on adipose tissue function and lipid metabolism. Endocrinol Metab Clin North Am. 2014;43(1):75-102.

Table 1. Energy intake and visceral adipose tissue mass of DHT-treated and placebo rats.

	Placebo	**DHT**
Energy intake (kJ/day/rat)	177.20±30.10	191.30±34.90 **
Visceral adipose tissue mass (g)	9.34±2.29	13.02±2.48 **

Data were analyzed by Student t-test and represent the mean values ± SD of 12 animals per group. A value of $p<0.05$ was considered statistically significant (******, $p<0.01$).

Table 2. IL-6 and TNFα mRNA levels in the hypothalamus of DHT-treated and placebo rats.

	Placebo	**DHT**
TNFα	1.00±0.06	1.16±0.09
IL-6	1.00±0.14	0.78±0.10

Data were analyzed by Student t-test and represent the mean values ± SD of 12 animals per group. A value of $p<0.05$ was considered statistically significant.

Figure Legends

Fig. 1. Blood triglyceride and plasma FFA levels. The levels of blood triglyceride (A) and plasma FFA (B) in DHT-treated (DHT) and Placebo group. Data represent the mean values ± SD of 12 animals per each group. Group comparisons were done by Student t-test. A value of $p<0.05$ was considered statistically significant (*****, $p<0.05$; *******, $p<0.001$).

Fig. 2. Plasma leptin levels. Data represent the mean values ± SD of 12 animals per each group, DHT-treated (DHT) and Placebo. Group comparisons were done by Student t-test. A value of $p<0.05$ was considered statistically significant (******, $p<0.01$).

Fig. 3. The levels of ObRb, SOCS-3 and NPY gene expressions. Representative Western blot and relative levels of ObRb protein in the hypothalamic whole cell extract of placebo (P) and DHT-treated rats (DHT) (A). Respective relative hypothalamic levels of ObRb (B), SOCS-3 (C) and NPY (D) mRNA in placebo and DHT-treated rats. Blots were probed with β-actin antibody as an equal loading control. Data are presented as mean ± SEM and were analyzed by Student t-test.

Fig. 4. Hypothalamic levels of 11β-HSD1, H6PDH and GR. Representative Western blots and respective relative levels of 11β-HSD1 (A), H6PDH (B) and GR (C) proteins in the hypothalamic whole cell extract of placebo (P) and DHT-treated rats (DHT). Relative hypothalamic levels of GR mRNA in placebo and DHT-treated rats (D). Blots were probed with β-actin antibody as an equal loading control. Data are presented as mean ± SEM and were analyzed by Student t-test.

Fig. 5. NFκB protein level. A representative Western blot and relative level of NFκB protein in the hypothalamic whole cell extract of placebo (P) and DHT-treated rats (DHT). Blots were probed with β-actin antibody as an equal loading control. Data are presented as mean ± SEM and were analyzed by Student t-test.

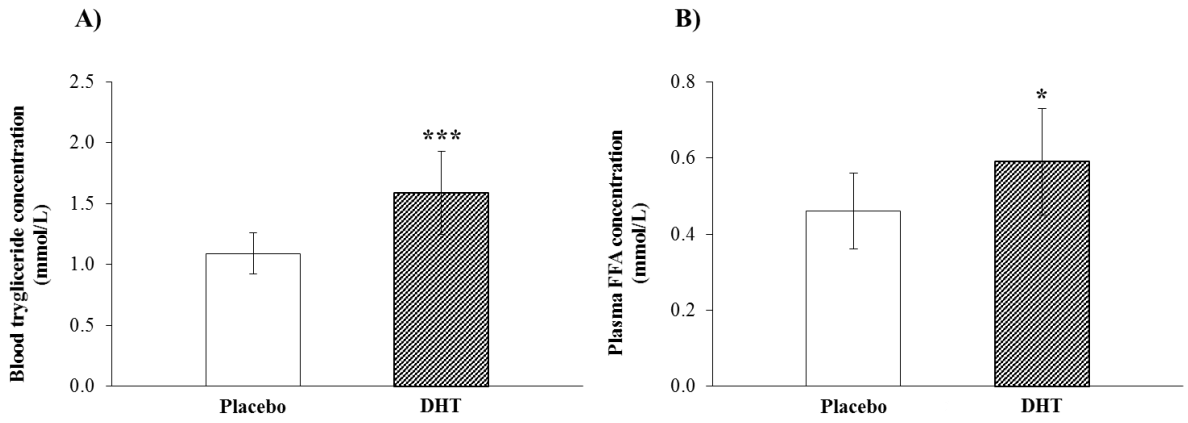
A)
Blood tryglicerid concentration (mmol/L)
2.5
2.0
1.5
1.0
0.5
0.0
***
Placebo
DHT
B)
Plasma FFA concentration (mmol/L)
0.8
0.6
0.4
0.2
0.0
*
Placebo
DHT

Fig. 1.

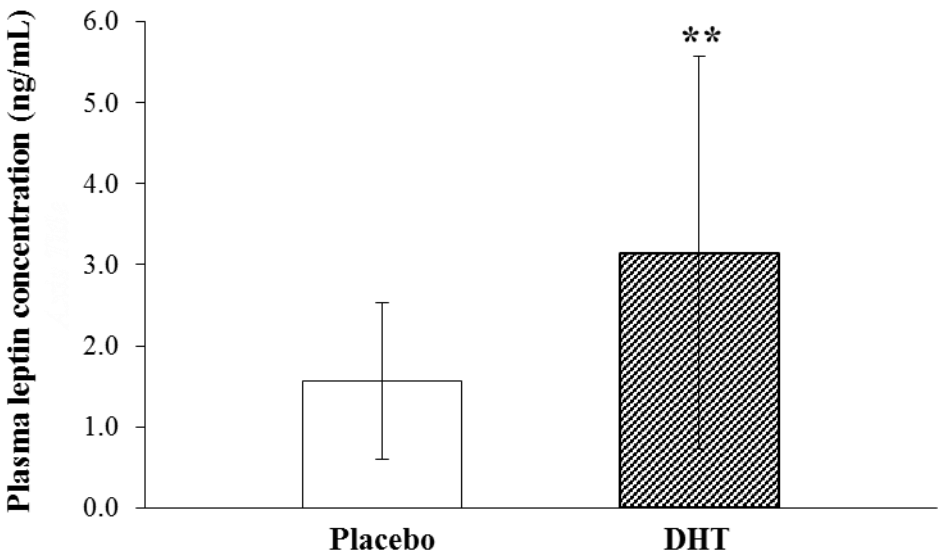
Plasma leptin concentration (ng/mL)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
**
Placebo
DHT

Fig. 2.

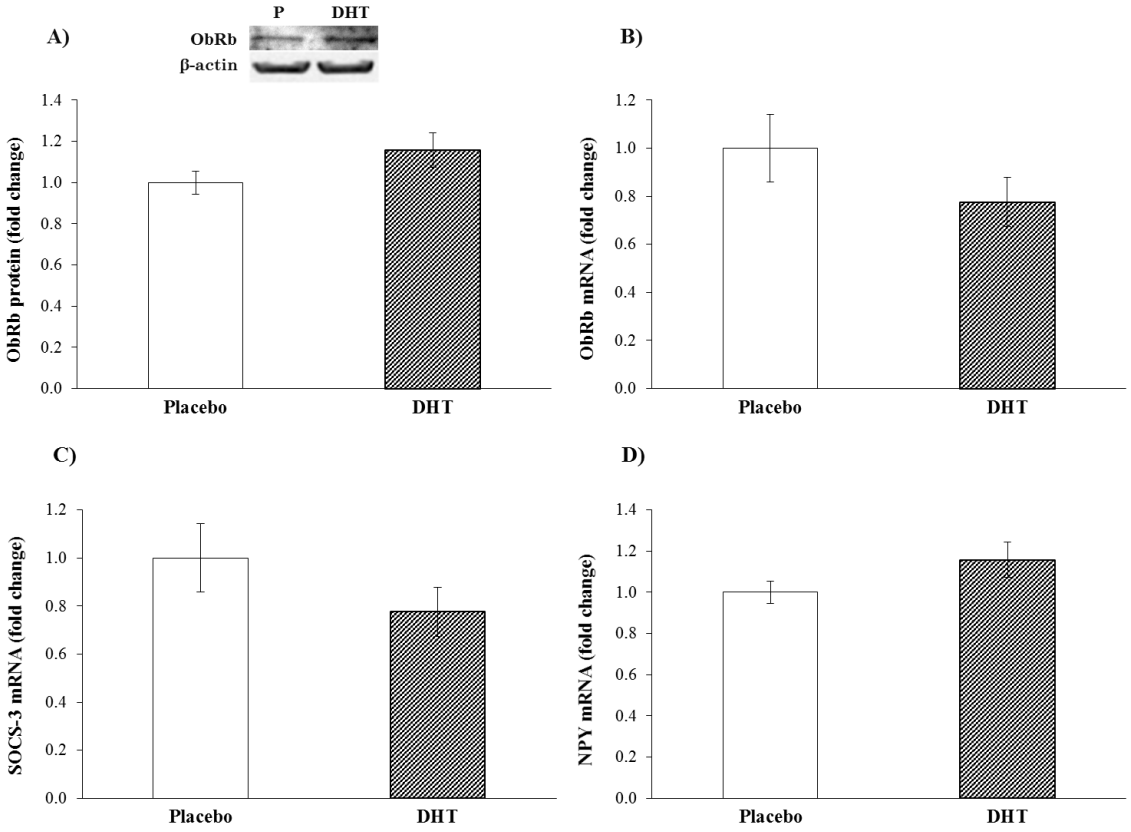
A)
P
DHT
ObRb
β-actin
ObRb protein (fold change)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Placebo
DHT
B)
ObRb mRNA (fold change)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Placebo
DHT
C)
SOCS-3 mRNA (fold change)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Placebo
DHT
D)
NPY mRNA (fold change)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Placebo
DHT

Fig. 3.

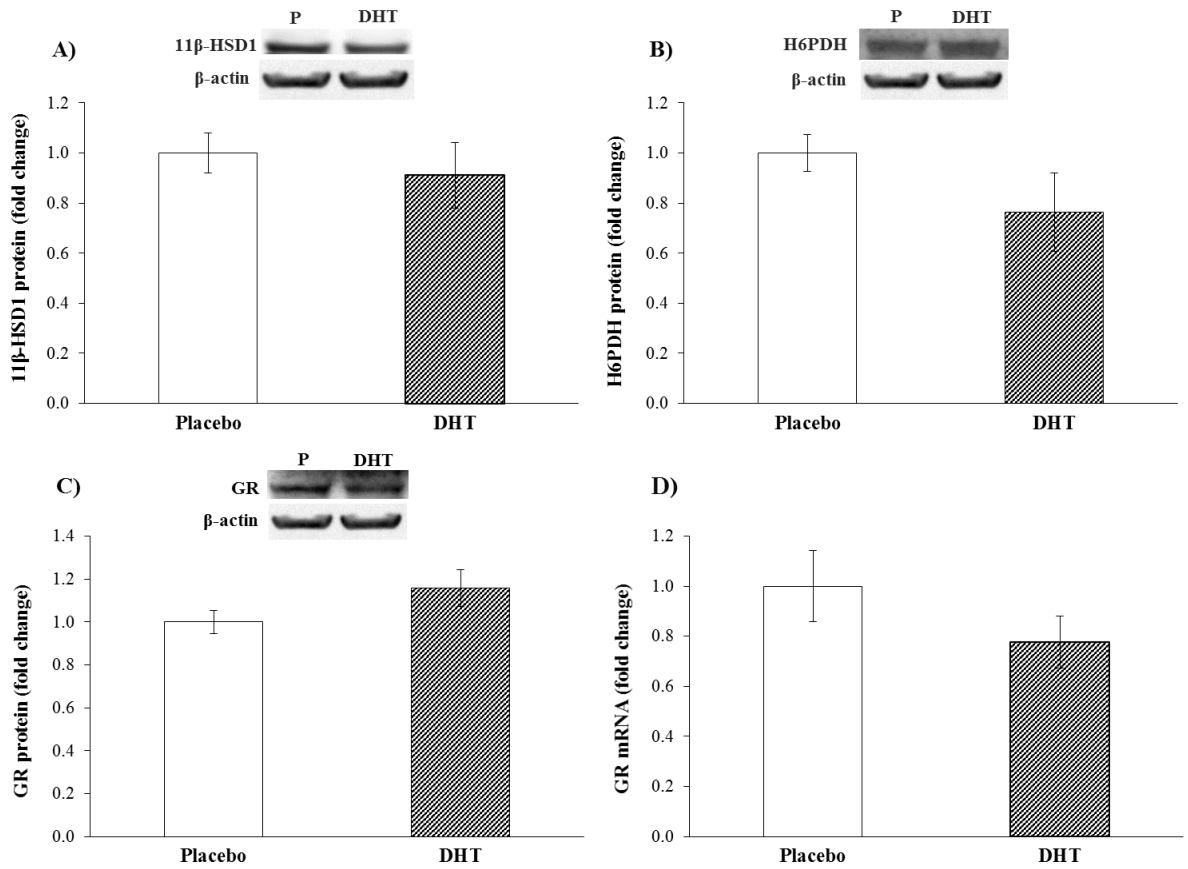
A)
P
DHT
11β-HSD1
β-actin
11β-HSD1 protein (fold change)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Placebo
DHT
B)
P
DHT
H6PDH
β-actin
H6PDH protein (fold change)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Placebo
DHT
C)
P
DHT
GR
β-actin
GR protein (fold change)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Placebo
DHT
D)
GR mRNA (fold change)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Placebo
DHT

Fig. 4.

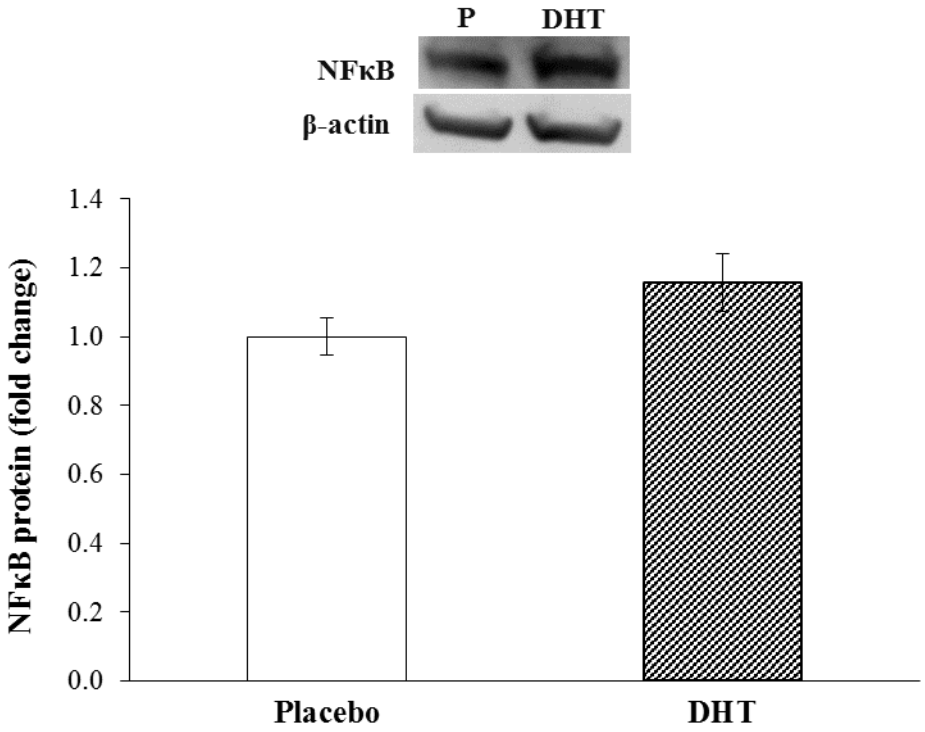
P
DHT
NFκB
β-actin
NFκB protein (fold change)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Placebo
DHT

Fig. 5.