JOÃO PAULO GABRIEL CAMPOREZ

**Efeitos do deidroepiandrosterona (DHEA) sobre a função
vascular e a resistência periférica à insulina em um modelo
experimental de menopausa**

Tese apresentada ao Instituto de Ciências
Biomédicas da Universidade de São Paulo, para
obtenção do Título de Doutor em Ciências
(Fisiologia Humana)

São Paulo

2012

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Área de concentração: Fisiologia Humana

Orientador:
Carla Roberta de Oliveira Carvalho

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CERTIFICADO

Certificamos que o protocolo registrado sob nº **027** nas fls. **55** do livro **02** para uso de animais em experimentação, sob a responsabilidade de Carla Roberta de Oliveira Carvalho, Coordenador(a) da Linha de pesquisa "**Menopausa e deidroepiandrosterona (DHEA): Efeitos sobre a resistência periférica à insulina e função vascular**" do qual participou(aram) o(s) alunos **João Paulo Gabriel Camporez e a pesquisadora Eliana Akamine** está de acordo com os Princípios Éticos de Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado pela **COMISSÃO DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL (CEEA)** em **24.03.08**, com validade de 3 anos.

São Paulo, 26 de março de 2008.

Prof. Dr. WOTHAN TAVARES DE LIMA
Coordenador
CEEA - ICB/USP

Profa. Dra. PATRÍCIA CASTELUCCI
Secretária
CEEA - ICB/USP

*Aos meus pais, minha madrinha e meu irmão,
não só pelo apoio incondicional durante essa
caminhada, mas por saber que sempre eu poderei
contar com vocês .*

*À Marcela, minha esposa, por tornar meus dias
muito melhores com todo seu carinho, amor e
paciência.*

*Aos meus avós Ezelino Camporez (in memorian),
Maria Fim Camporez, Alfredo Gabriel (in
memorian) e Deocacina de Araújo Gabriel (in
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“O homem erudito é um descobridor de fatos que já existem - mas o homem sábio é um criador de valores que não existem e que ele faz existir.”

Albert Einstein

RESUMO

Camporez, JPG. Efeito do deidroepiandrosterona (DHEA) sobre a função vascular e a resistência periférica à insulina em um modelo experimental de menopausa. [tese (Doutorado – Fisiologia Humana)] São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2012.

Doenças cardiovasculares associadas à complicações metabólicas, como resistência à insulina e obesidade, são menos prevalentes em mulheres jovens do que em homens na mesma idade ou mulheres na pós-menopausa. Com o envelhecimento ocorre redução da concentração plasmática de deidroepiandrosterona (DHEA) associada com o aumento da prevalência de doenças cardiovasculares e resistência à insulina. Além disso, tem sido demonstrado que a administração de DHEA leva a efeitos benéficos sobre o sistema cardiovascular, redução do acúmulo de gordura visceral, proteção contra a resistência à insulina em animais de experimentação e em humanos. Assim, o objetivo desse trabalho foi avaliar o efeito do DHEA sobre a função vascular em aorta e resistência periférica à insulina em roedores fêmeas ovariectomizadas (modelo experimental de menopausa). Para estudo da função vascular foram utilizadas Ratas Wistar com 8 semanas de idade foram ovariectomizadas (OVX) ou submetidas à cirurgia fictícia (SHAM). Após 8 semanas da cirurgia, OVX e SHAM foram tratadas com veículo ou DHEA (10 mg/kg/semana, *s.c.*) por 3 semanas. Ao final do tratamento foi realizada a medida direta da pressão arterial (PA) com animais acordados. Além disso, anéis de aorta foram isolados para avaliar resposta contrátil à fenilefrina (PHE, 10^{-10} – 10^{-4} M) e a resposta de relaxamento à acetilcolina (ACh, 10^{-11} – 10^{-5} M) e ao nitroprussiato de sódio (NPS, 10^{-11} – 10^{-5} M). A resposta à PHE e à ACh também foi estudada em anéis incubados previamente com L-NAME (inibidor da NOS, 10^{-4} M), superóxido dismutase (SOD, 150 U/mL), apocinina (inibidor da NADPH-oxidase, 10^{-4} M) ou salicilato (inibidor do NF κ B, 5×10^{-3} M). A produção tecidual de espécies reativas de oxigênio (ROS) foi avaliada *in situ* pela oxidação da diidroetidina. A expressão protéica e a fosforilação da eNOS, SOD, e citocinas pró-inflamatórias foram quantificadas por Western-blot. Foi avaliado o peso corporal, do tecido adiposo retroperitoneal e comprimento naso-anal. Para estudo da resistência à insulina foram utilizadas camundongos fêmeas ovariectomizadas com 8 semanas de idade. Esses animais foram tratados com DHEA (pellet – 10 mg liberados em 60 dias) ou veículo, e todos os animais foram mantidos com dieta hiperlipídica. Após 4 semanas,

metabolismo corporal e sensibilidade à insulina foram avaliados através de monitoramento na gaiola metabólica e clamp normoglicêmico-hiperinsulinêmico. As ratas OVX apresentaram aumento no peso corporal, do tecido adiposo retroperitoneal e do índice de Lee em relação as SHAM, não havendo modificação pelo tratamento com DHEA. A PA apresentou-se aumentada nas OVX, enquanto o tratamento com DHEA reduziu os valores da PA nesses animais. A resposta máxima (Emax) à PHE foi aumentada nos anéis de aorta das OVX, e a Emax à ACh foi reduzida nas OVX, sem alteração na Emax ao NPS. O tratamento com DHEA corrigiu o aumento da Emax à PHE e a redução da Emax à ACh nas OVX. A incubação prévia com SOD, apocinina ou salicilato induziu uma redução da Emax à PHE e um aumento na Emax à ACh nas OVX. Além disso, a produção de ROS estava aumentada nas aortas das OVX comparadas com as SHAM e foi corrigida pelo tratamento com DHEA. A expressão da Cu/Zn SOD foi reduzida nas aortas das OVX e corrigida pelo tratamento com DHEA. A fosforilação da eNOS foi reduzida nas aortas das OVX e o tratamento com DHEA corrigiu a mesma. As ratas OVX também apresentaram aumento da expressão da NADPH oxidase e citocinas na aorta e o DHEA reverteu esse aumento. Quanto ao estudo da resistência à insulina, não houve diferenças no peso corporal inicial e final entre os animais OVX e OVX+DHEA, embora o tratamento com DHEA reduziu a o percentual de gordura nesses animais. Além disso, o tratamento com DHEA reduziu a ingestão calórica em 20%, e não houve diferenças na gasto energético, atividade e ingestão hídrica. A sensibilidade à insulina foi aumentada em cerca de 50% nos animais tratados com DHEA, o que foi resultado do aumento da sensibilidade à insulina hepática e muscular. Tanto o conteúdo de triglicerídios (TG) quanto o de diacilglicerol hepático e muscular foi reduzido pelo tratamento com DHEA, o que pode ser o responsável pelo aumento da sensibilidade à insulina. Além disso, o tratamento com DHEA levou a uma menor ativação da PKCε no fígado e PKCθ no músculo e uma aumento da fosforilação de AKT2 tanto no fígado quanto no músculo. Nosso estudo confirma o papel do estresse oxidativo e inflamação na disfunção endotelial, e consequente aumento da PA em ratas OVX. Além disso, o tratamento com DHEA restaura a função endotelial e reverte a elevação da pressão arterial nas ratas OVX. O estudo da sensibilidade à insulina sugere que o tratamento com DHEA reduz a adiposidade em camundongos OVX através da redução da ingestão calórica, o que resulta em reduzida acumulação ectópica de lipídios e aumento da sensibilidade à insulina.

Palavras-chave: Deidroepiandrosterona. Menopausa. Ovariectomia. Disfunção endotelial. Resistência à insulina. Obesidade.

ABSTRACT

Camporez, JPG. Dehydroepiandrosterone (DHEA) effects on vascular function and peripheral insulin resistance in a menopause experimental model. [Ph.D. Thesis]. São Paulo: Instituto de Ciências Biomédicas, Universidade São Paulo; 2012.

Cardiovascular diseases associated with metabolic complications such as insulin resistance and obesity are less prevalent in women than in age-matched men or postmenopausal women. During the aging there is a reduction of plasma dehydroepiandrosterone (DHEA) associated with increased prevalence of cardiovascular disease and insulin resistance. Moreover, it has been shown that administration of DHEA leads to beneficial effects on the cardiovascular system, reduced visceral fat accumulation, and protection against insulin resistance in both animals and humans. Therefore, the objective of this study was to evaluate the effect of DHEA on vascular function in the aorta and peripheral insulin resistance in ovariectomized female rodents (experimental model of menopause). For the study of vascular function were used Wistar rats at 8 weeks of age. They were ovariectomized (OVX) or underwent sham surgery (SHAM). After 8 weeks of surgery, OVX and SHAM were treated with vehicle or DHEA (10 mg / kg / week, sc) for 3 weeks. At the end of treatment was carried out direct measurement of blood pressure (BP) in conscious animals. Furthermore, aortic rings were isolated to evaluate the contractile response to phenylephrine (PHE, 10^{-10} – 10^{-4} M) and the relaxation response to acetylcholine (ACh, 10^{-11} – 10^{-5} M) and sodium nitroprusside (NPS, 10^{-11} – 10^{-5} M). The response to PHE and ACh was also studied in rings previously incubated with L-NAME (NOS inhibitor, 10^{-4} M), superoxide dismutase (SOD, 150 U/mL), apocynin (NADPH oxidase inhibitor, 10^{-4} M) or salicylate (an inhibitor of NF κ B, 5×10^{-3} M). Tissue production of reactive oxygen species (ROS) was assessed *in situ* by the oxidation of dihydroethidium. The protein expression and phosphorylation of eNOS, SOD, and proinflammatory cytokines were quantified by Western blot. We assessed body weight and retroperitoneal adipose tissue. For the study of insulin resistance were used ovariectomized female mice at 8 weeks old. These animals were treated with DHEA (pellet - 10 mg released for 60 days) or vehicle, and all animals were fed with high-fat diet. After 4 weeks, whole-body metabolism and insulin sensitivity were assessed by metabolic cage and euglycemia-hyperinsulinemic clamp, respectively. The OVX rats showed an increase in body weight,

retroperitoneal adipose tissue and the Lee index compared to SHAM rats, without modification by DHEA treatment. The BP presented increased in OVX, while treatment with DHEA reduced BP values in these animals. The maximum response (R_{max}) to PHE was increased in aortic rings from OVX, and R_{max} to ACh was reduced in OVX, with no change in R_{max} to NPS. The DHEA treatment corrected the increased R_{max} to PHE and the reduction of R_{max} to ACh in OVX rats. The incubation with SOD, apocynin or salicylate reduced the R_{max} to PHE and increased the R_{max} to ACh in OVX rats. In addition, ROS production was increased in the aortas from OVX compared to SHAM and it was corrected by DHEA treatment. The expression of Cu/Zn SOD was reduced in aortas from OVX and corrected by DHEA treatment. Phosphorylation of eNOS was reduced in the aortas from OVX and treatment with DHEA corrected it. The OVX rats also showed increased expression of NADPH oxidase and cytokines in aorta and DHEA reversed this increase. Regarding the study of insulin resistance, there was no difference in initial and final body weight between OVX and OVX+DHEA mice, although DHEA treatment reduced the percentage of fat in these animals. Furthermore, DHEA treatment reduced caloric intake by 20%, and there was no difference in energy expenditure, activity and water intake. Insulin sensitivity was increased by 50% in DHEA-treated mice, which was accounted by increased hepatic and muscular insulin sensitivity. Both triglycerides (TG) and diacylglycerol (DAG) content in liver and muscle was reduced by DHEA treatment, which may be responsible for the increased insulin sensitivity. Furthermore, DHEA treatment led to a decreased activation of PKC ϵ in the liver and PKC θ in muscle and increased phosphorylation of AKT2 in both liver and muscle. Our study confirms the role of oxidative stress and inflammation in endothelial dysfunction and consequent increase in BP in OVX rats. Furthermore, DHEA treatment restores endothelial function and reverses the BP elevation in OVX rats. The study of insulin sensitivity suggests that DHEA treatment reduces adiposity in OVX mice by reducing caloric intake, resulting in reduced ectopic lipid accumulation and increased insulin sensitivity.

Keywords: Dehydroepiandrosterone. Menopause. Ovariectomy. Endothelial dysfunction. Insulin resistance. Obesity.

Lista de Abreviaturas

2-DG:	2-deoxy-glicose
ACh:	Acetilcolina
AGL:	Ácidos graxos livres
AKT:	<i>RAC-alpha serine/threonine-protein kinase</i>
AKT2:	Isoforma 2 da <i>RAC-alpha serine/threonine-protein kinase</i>
DAG:	Diacilglicerol
DHE:	dihidroetidina
DHEA:	Deidroepiandrosterona
DHEA-S:	Deidroepiandrosterona sulfatado
DM2:	Diabete Melito tipo 2
EGP:	Produção endógena de glicose
eNOS:	Sintase endotelial de óxido nítrico
ER α :	Isoforma alfa do receptor para estrogênios
ER β :	Isoforma beta do receptor para estrogênios
EROS:	Espécies reativas de oxigênio
FSH:	Hormônio folículo estimulante
GIR:	Ritmo de infusão de glicose
GLUT-4:	Isoforma 4 do transportador para glicose
IL-1 β :	Interleucina 1 beta
IL-6:	Interleucina 6
LH:	Hormônio luteinizante
NO:	Óxido nítrico
NPS:	Nitroprussiato de sódio
OVX:	Animais que sofreram ovariectomia
PI3-K:	Fosfatidilinositol 3 cinase
PKC ϵ :	Proteína cinase C – isoforma epsilon
PKC θ :	Proteína cinase C – isoforma theta
RER:	Quociente respiratório
Rmax:	Resposta máxima

SHAM:	Animais que sofreram cirurgia fictícia
SHR:	Ratos espontaneamente hipertensos
SOD:	Superóxido dismutase
TG:	Triglicerídeos
TNF- α :	Fator de necrose tumoral alfa

Lista de Ilustrações

- Figura 1** – Reatividade vascular para fenilefrina, acetilcolina e nitroprussiato de sódio. pg 32
- Figura 2** – Espécies reativas de oxigênio em aortas. pg 35
- Figura 3** – Expressão protéica da Cu/Zn-SOD, Mn-SOD e subunidades da NADPH oxidase. pg 36
- Figura 4** – Fosforilação da enzima eNOS em aortas. pg 37
- Figura 5** – Expressão de citocinas pro-inflamatórias em aorta. pg 38
- Figura 6** – Reatividade vascular para fenilefrina e acetilcolina em animais tratados com inibidor de aromatase (formestane). pg 39
- Figura 7** – Caracterização metabólica dos camundongos fêmeas OVX. pg 41
- Figura 8** – Comportamento alimentar e físico dos camundongos fêmeas OVX. pg 42
- Figura 9** – Caracterização *in vivo* da sensibilidade à insulina dos camundongos fêmeas OVX. pg 43
- Figura 10** – Captação de glicose e 2-deoxy-glicose (2-DG) estimulada por insulina. pg 44
- Figura 11** – Valores de produção endógena de glicose. pg 45
- Figura 12** – Valores de ácidos graxos livres dos camundongos fêmeas OVX. pg 46
- Figura 13** – Perfil do conteúdo tecidual lipídico – triglicerídeos (TG). pg 47
- Figura 14** – Perfil do conteúdo tecidual lipídico – diacilglicerol (DAG). pg 47
- Figura 15** – Perfil do conteúdo tecidual lipídico – ceramidas. pg 47
- Figura 16** – Ativação das novas PKCs no fígado e músculo. pg 48
- Figura 17** – Fosforilação da enzima AKT2. pg 49

Lista de Tabelas

Tabela 1 – Dados zoomórficos das ratas após 11 semanas de cirurgia. pg 30

Tabela 2 – Perfil hormonal de estradiol, progesterona, testosterona, LH, FSH e DHEA ao final de 11 semanas. pg 31

Tabela 3 – Pressão arterial sistólica (PAS), pressão arterial diastólica (PAD) e frequência cardíaca das ratas ao final de 11 semanas. pg 31

Tabela 4 – Efeito da remoção do endotélio (E-), L-NAME, SOD, Apo e salicilato na resposta máxima (Rmax) à fenilefrina. pg 33

Tabela 5 – Efeito da incubação com SOD, Apo e salicilato na resposta máxima (Rmax) à ACh. pg 34

Tabela 6 – Dados zoomórficos e perfil lipídico e hormonal dos animais após 4 semanas da cirurgia. pg 40

SUMÁRIO

1 INTRODUÇÃO.....	19
1.1 Menopausa.....	19
1.2 Deidroepiandrosterona (DHEA).....	20
1.3 Ojetivos.....	22
2 MATERIAL E METÓDOS.....	23
2.1 Material.....	23
2.2 Animais e desenho experimental.....	23
2.3 Caracterização das ratas.....	24
2.4 Avaliação da pressão arterial.....	25
2.5 Estudo <i>in vitro</i> da reatividade de anéis de aorta torácica isolada.....	25
2.6 Determinação da produção de espécies reativas de oxigênio na aorta.....	26
2.7 Avaliação <i>in vivo</i> da massa gordurosa e monitoramento metabólico dos camundongos.....	26
2.8 Avaliação <i>in vivo</i> da sensibilidade à insulina nos camundongos.....	26
2.9 Mensuração do conteúdo tecidual de lipídeos.....	27
2.10 Western blot.....	28
2.11 Análise estatística.....	29
3 RESULTADOS.....	30
3.1 Estudo da função vascular.....	30
3.2 Estudo da resistência periférica à insulina.....	40
4 DISCUSSÃO.....	50
4.1 Estudo da função vascular.....	50
4.2 Estudo da resistência periférica à insulina.....	56
5 CONSIDERAÇÕES FINAIS.....	61
REFERÊNCIAS.....	62
ANEXO A Composição corporal dos animais dos camundongos fêmeas SHAM e OVX.....	73
ANEXO B Concentração plasmática de E2 e DHEA.....	73
ANEXO C Medida <i>in vivo</i> da sensibilidade à insulina.....	74

ANEXO D Certificado do curso de radioproteção.....	75
ANEXO E Aprovação do comitê de ética da Universidade de Yale.....	76
ANEXO F Certificado do curso de radioproteção da Universidade de Yale.....	77
ANEXO G Artigos publicados e em fase de submissão durante o doutorado.....	78

1 INTRODUÇÃO

1.1 Menopausa

Com o envelhecimento natural os ovários perdem a responsividade às gonadotrofinas, hormônio luteinizante (LH) e hormônio folículo estimulante (FSH), reduzindo sua função, e então desaparecendo com os ciclos menstruais (menopausa). Esse fenômeno está associado, e provavelmente causado, pelo declínio do número dos folículos primordiais nos ovários. Dessa forma, os ovários não produzem mais progesterona e estrogênios em quantidades significantes. Gradualmente, o útero e a vagina se tornam atroficos. As concentrações de LH e FSH se elevam devido a falta de *feedback* negativo dos estrogênios e progesterona. Esse fenômeno da menopausa ocorre nas mulheres entre as idades de 45 e 55 anos (Ganong, 2003)

Essa privação dos hormônios sexuais femininos que ocorre na menopausa, principalmente dos estrogênios, está associada ao desenvolvimento de doenças cardiovasculares e metabólicas, tanto em mulheres (Mercurio et al., 2004) quanto em modelos experimentais (Folwarczna et al., 2007; Ito et al., 2006).

A hipertensão é mais prevalente em homens do que em mulheres na pré-menopausa, porém após a menopausa, o aumento da pressão arterial e a prevalência de hipertensão são maiores nas mulheres do que nos homens da mesma idade (Burl et al., 1995). A menopausa causada por ovariectomia em mulheres normotensas férteis de meia idade causou aumento nos valores médios da pressão arterial sistólica e diastólica (Mercurio et al., 2004). A ovariectomia também aumenta a pressão arterial em ratas Wistar normotensas (Mendonça et al., 2007) e em camundongos fêmeas (Xue et al., 2005). Além disso, a menopausa causada por ovariectomia ou envelhecimento em ratas espontaneamente hipertensas (SHR) agravou a hipertensão (Fortepiani et al., 2003; Recklhoff e Fortepiani, 2004). Ainda, quando comparado o efeito hipertensivo da angiotensina II (Xue et al., 2005, 2007) e o efeito hipertensivo da inibição da síntese de óxido nítrico (NO) (Sáinz et al., 2004) observa-se um aumento maior da pressão arterial em machos intactos e em fêmeas ovariectomizadas do que em fêmeas intactas. Em SHR, as fêmeas possuem menor pressão arterial do que os machos, além de possuírem uma menor relação de receptores de angiotensina tipo 1/tipo 2 (AT1_R/AT2_R) do que os machos, em aorta, rins e vasos do mesentério (Silva-Antonialli et al., 2004), sendo que essa relação foi invertida pela ovariectomia.

Esse efeito hipertensivo da menopausa pode ser decorrente, principalmente, da deficiência de estrogênios. Em mulheres que sofreram ovariectomia, a terapia de reposição

hormonal com estrogênio reduziu os valores da pressão arterial, semelhante aos valores precedentes à menopausa (Mercuro et al., 2004). Em um estudo epidemiológico, foi demonstrado que, após a menopausa, o risco de elevação da pressão arterial está associado com excesso de peso e sedentarismo e que a terapia de reposição hormonal com estrogênio reduz esse risco (Gruppo di Studio Progetto Menopausa Italia, 2006).

A ausência/deficiência dos estrogênios no sexo feminino não causa apenas efeitos deletérios sobre o sistema cardiovascular. Alguns estudos descrevem aumento do peso após a ovariectomia em roedores (Chen e Heiman, 2001; Diemenet et al., 2006; Kimura et al., 2002) e resistência à insulina associada a esse aumento do peso (Lemieux et al., 2003). Camundongos fêmeas ovariectomizadas ficam mais sensíveis a indução de resistência à insulina por dieta hiperlipídica do que os animais intactos (Riant et al., 2009) e a reposição hormonal com estradiol reverte esses efeitos. Ademais, em mulheres na menopausa, a incidência de diabetes melito tipo 2 (DM2) é maior nas mulheres que não fazem uso de terapia de reposição hormonal (Margolis et al., 2004). Assim, observa-se que a ovariectomia/menopausa causa diversas alterações metabólicas, que estão inter-relacionadas, como a hipertensão, obesidade e resistência à insulina.

Entretanto, embora diversos estudos descrevem efeitos protetores/benéficos da reposição hormonal com estrogênios (Dean et al., 2005; Garcia et al., 2005; Giménez et al., 2006; Nuedling et al., 2003), em mulheres ainda não está claro se há benefícios da reposição hormonal, podendo ocorrer aumento do risco de trombo-embolismo e câncer (Prentice et al., 2009). Dessa forma, tratamentos alternativos na menopausa, ao invés da reposição hormonal clássica com estrogênios, seria de grande importância para prevenção/tratamento de eventos cardiovasculares, obesidade, resistência à insulina e câncer.

1.2 Deidroepiandrosterona (DHEA)

Deidroepiandrosterona (DHEA) e sua forma sulfatada (DHEA-S) são produzidos e secretados pelo córtex adrenal e são os hormônios esteróides de maior concentração em humanos. Próximo à segunda década de vida, as concentrações desses hormônios aumentam e chegam a seus valores máximos, apresentando diferenças entre os sexos e sendo maiores nos homens (Greenspan e Gardner, 2006; Migeon et al., 1957). A partir da quarta década da vida, a concentração de DHEA começa a declinar, chegando a concentrações mínimas por volta dos 80 anos de idade (Bélanger et al., 1994).

Esse declínio do DHEA e DHEA-S plasmático relatado para a idade tem sido relacionado com o desenvolvimento de diversas doenças crônicas associadas ao envelhecimento, como a resistência à insulina (Schriock et al., 1988), obesidade (Macewen e Kurzman, 1991; Nestler et al., 1988), doenças cardiovasculares (Barrett-Connor et al., 1986), câncer (Schwartz et al., 1986), redução da defesa imunológica (Casson et al., 1993), depressão e redução da sensação de bem estar (Morales et al., 1994).

Em ratos e camundongos, a administração de DHEA reduz o acúmulo de gordura visceral induzida por dieta hiperlipídica (Cleary e Zisk, 1986; Hansen et al., 1997; Mohan et al., 1990; Yen et al., 1977). Outro efeito benéfico do DHEA observado é a redução da resistência à insulina que aparece com o envelhecimento (Han et al., 1998) e melhora da sinalização da insulina em tecido muscular e hepático (Campbell et al., 2004). Ainda em ratos envelhecidos, a administração de dose única de DHEA aumenta a secreção de insulina estimulada por glicose em ilhotas pancreáticas isoladas (Medina et al., 2006). Além disso, redução da resistência à ação da insulina acompanhada de redução da gordura subcutânea abdominal foi verificada em mulheres que receberam creme contendo DHEA por 12 meses (Diamond et al., 1996). Ademais, administração diária de 50 mg de DHEA por 6 meses a homens com idade entre 65-78 anos reduziu a gordura subcutânea e visceral abdominal, além de aumentar a resposta de secreção de insulina frente a sobrecarga de glicose e melhora da sensibilidade à insulina (Villareal e Holloszy, 2004).

Efeitos benéficos do DHEA sobre o sistema cardiovascular também foram descritos. Em modelos de ratos obesos e diabéticos, a administração de DHEA reduziu o estresse oxidativo no coração, através da redução da atividade do NF κ B, receptores de produtos finais de glicação e receptores de TNF- α (Aragno et al., 2006), e melhorou a função vascular e neural (Yorek et al., 2002). Em células endoteliais, DHEA inibe a resposta inflamatória induzida por TNF- α (Gutiérrez et al., 2007) e protege estas células contra apoptose, pela via da PI3-K/AKT (Liu et al., 2007). Também pela via da PI3-K/AKT, DHEA estimula a produção de NO por essas células (Formoso et al., 2006) via receptor de membrana (Liu e Dillon, 2002). Além disso, em humanos, baixas concentrações de DHEA e DHEA-S podem predizer doença cardíaca isquêmica (Feldman et al., 2001) e aumentar o risco de diversas outras doenças cardiovasculares (Tchernof e Labrie, 2004).

1.3 Objetivos

Em resumo, a menopausa, seja fisiológica ou cirúrgica, tanto em humanos quanto em modelos experimentais, aumenta o risco de hipertensão arterial e disfunção endotelial, devido em parte, pela perda do efeito protetor dos estrogênios. Além disso, a menopausa também está associada à elevação do peso e à obesidade, situações que são relacionadas à resistência à insulina e esta, por sua vez, com o DM2 e o envelhecimento.

DHEA é um hormônio esteróide e sua queda durante o envelhecimento tem sido relacionada à obesidade, resistência à insulina e doenças cardiovasculares. A utilização de DHEA como suplemento alimentar, tanto em humanos quanto em modelos experimentais, foi relacionado à redução da obesidade, da resistência à insulina e do risco de doenças cardiovasculares. Dessa forma, como a reposição hormonal com estrogênios leva a um aumento do risco de eventos cardiovasculares e câncer em mulheres na menopausa (Prentice et al., 2009), a utilização de DHEA surge como uma possível terapia alternativa ao estrogênio.

Assim, o objetivo desse trabalho foi avaliar o efeito do DHEA sobre a função vascular em aorta e resistência periférica à insulina em roedores fêmeas ovariectomizadas (modelo experimental de menopausa).

2 MATERIAL E MÉTODOS

2.1 Material

Os reagentes e os aparelhos para eletroforese em gel de sódio dodecil sulfato de poliacrilamida (SDS-PAGE) foram da BioRad (Bio-Rad Laboratories, Inc., Richmond, CA, EUA). Metano hidroximetilamina (TRIS), fenilmetilsulfonilfluoreto (PSMF), aprotinina, ditioneitol (DTT), DHEA, albumina e poli-lisina foram fornecidos pela Sigma Chemical Co. (Sigma-Aldrich Co. LLC, St. Louis, MO, EUA) e insulina regular pela Lilly (Lilly USA, LLC, EUA). A membrana de nitrocelulose, e os kits para detecção por quimioluminescência foram fornecidos pela Amersham (GE Healthcare, EUA). D-glicose anidra e PEG (polietileno glicol + tampão borato) foram fornecidos pelo Labsynth (Labsynth, Diadema, S.P., Brasil). Os anticorpos anti-eNOS e anti-peNOS foram da Cell Signaling Technology® (Cell Signaling Technology, Inc, EUA), anti-Cu/Zn SOD e IL-6 foram da Abcam (Abcam plc, EUA), anti-gp91phox foi da Upstate Biotechnology (EUA), anti-p22phox foi da Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc, EUA), anti-TNF- α e IL-1 β foram da Biolegend (BioLegend, Inc, EUA).

2.2 Animais e desenho experimental

Ratas Wistar foram divididas em quatro grupos para o estudo da função vascular:

- a) grupo 1 – controle (SHAM);
- b) grupo 2 – controle + DHEA (SHAM+DHEA);
- c) grupo 3 – ovariectomizada (OVX);
- d) grupo 4 – ovariectomizada + DHEA (OVX+DHEA);

A ovariectomia foi realizada nas ratas com 8 semanas de vida. As ratas foram anestesiadas com uma mistura de cetamina e xilazina (90 e 5 mg/kg, respectivamente, i.p.). Após incisão abdominal, os ovários foram clampeados e removidos. Depois a pele foi suturada antes de os animais acordarem. O grupo SHAM sofreu o mesmo procedimento, porém, os ovários não foram retirados.

Após 8 semanas da cirurgia, as ratas OVX+DHEA foram tratadas por 21 dias com DHEA (10 mg/kg de peso corporal, s.c.) diluído em óleo de soja no 1º, 7º e 14º dias, tratamento adaptado de publicações recentes do nosso grupo (Campbel et al., 2004; Medina et

al., 2006). As ratas foram utilizadas para experimento no 21º dia. As ratas SHAM e OVX foram tratadas com volume semelhante de veículo.

Esse procedimento foi aprovado pelo comitê de ética do Instituto de Ciências Biomédicas (ICB) da Universidade de São Paulo (USP), registrado sob o número 027 nas folhas 55 do livro 02.

Para estudo da resistência à insulina foi utilizado camundongos fêmeas C57BL/6. Os experimentos foram realizados na Universidade de Yale (EUA), no laboratório sob coordenação do prof. Dr. Gerald I. Shulman. Todos os experimentos realizados foram aprovados e conduzidos de acordo com as diretrizes do Comitê Institucional de Uso e Cuidados com Animais da Escola de Medicina da Universidade de Yale (Institutional Animal Care and Use Committee guidelines of Yale University School of Medicine – Anexo E).

Com 8 semanas de idade, camundongos fêmeas C57BL/6 foram anestesiadas com injeção intraperitoneal da mistura contendo ketamina, xilazina e acepromazina contendo 64,9, 3,2 e 0,78 mg kg⁻¹, respectivamente, e submetidas a ovariectomia bilateral (OVX). Os animais foram aleatoriamente divididos em 2 grupos: controle (OVX) e tratados com DHEA (OVX+DHEA). Nos animais do grupo OVX+DHEA foi implantado pellet na região interescapular, subcutaneamente, contendo DHEA – 10 mg liberados por 60 dias (Innovative Research of America, Sarasota, FL, USA); os controles receberam um pellet de parafina considerado como placebo. Os pellets foram implantados no mesmo dia da ovariectomia. Durante todo o estudo os animais foram mantidos em sala com temperatura constante, com ciclo claro:escuro de 12:12, com livre acesso a água e dieta hiperlipídica (DHL), contendo 60% das calorias provenientes de gordura animal (D12492, Research Diets, Inc, NJ, EUA).

2.3 Caracterização das ratas

No final do período de 11 semanas, peso, gordura retroperitoneal, peri-uterina e o peso do útero foram avaliados das ratas. Após restrição alimentar de 8 h, amostras de sangue foram obtidas para determinação de glicose. Dosagem de estradiol, progesterona, DHEA, LH, FSH, e testosterona foram realizadas por radioimunoensaio, no laboratório do Prof. Celso Rodrigues Franci – USP/Ribeirão Preto.

2.4 Avaliação da pressão arterial

Após 11 semanas da cirurgia, ratas foram anestesiadas com uma mistura de cetamina, xilazina e acepromazina (64,9 – 3,2 – 0,78 mg/kg, respectivamente, i.p.). Um catéter de polietileno (PE-50), preenchido com salina heparinizada (100 U/mL), foi introduzido na carótida direita e exteriorizado na região mediana escapular. Após 24 horas, o cateter foi conectado a um transdutor de pressão (modelo DT-100; Utah Medical Products, Inc, Midvale, Utah, EUA), que estava acoplado a um amplificador (Bridge Amp; ADInstruments Pty Ltda, Castle Hill, Austrália) e a um sistema de aquisição de dados (Power Lab 4/30; ADInstruments Pty Ltda, Castle Hill, Austrália). Os registros de pressão arterial e frequência cardíaca em animais conscientes foram gravados utilizando-se um software (LabChart Software; ADInstruments Pty Ltda, Castle Hill, Austrália). A frequência cardíaca foi determinada pelo intervalo entre batimentos.

2.5 Estudo *in vitro* da reatividade de anéis de aorta torácica isolada

As aortas torácicas foram removidas e rapidamente imersas em solução nutriente Krebs-Henseleit modificada e seccionadas em anéis transversais de 4 mm. Em um grupo de anéis, o endotélio foi removido mecanicamente com uma haste fina de algodão. Os anéis com e sem endotélio foram suspensos por um par de ganchos de aço inoxidável, sendo que um gancho foi fixado à base da cuba de vidro para estudo de órgão isolado e o outro conectado a um transdutor de sinal (ML T001 transdutor isométrico de tensão, Power Lab/8S, ADInstruments Pty Ltda, Australia) acoplado a um computador. As cubas de vidro foram preenchidas com 5 ml de solução de Krebs-Henseleit modificada, que permaneceu gaseificada com mistura de 95% de O₂ e 5% de CO₂ e aquecida à temperatura de 37±0,5° C durante todo o protocolo experimental. As preparações permaneceram sob tensão de 1,0 g por um período de 60 minutos para estabilização, com trocas de solução nutriente e ajuste de tensão a cada 20 minutos, quando foram iniciadas as curvas concentração-efeito para fenilefrina (agonista α -adrenérgico, 10⁻¹⁰–10⁻⁴M), acetilcolina (ACh, 10⁻¹¹–10⁻⁵M) e nitroprussiato de sódio (NPS, 10⁻¹¹–10⁻⁵M). Em anéis com endotélio intacto, as curvas concentração-efeito também foram realizadas após incubação com L-NAME (inibidor da NOS, 10⁻⁴M), superóxido dismutase (SOD, 150 U/mL), apocinina (inibidor da NADPH-oxidase, 10⁻⁴M) ou salicilato (inibidor do NF κ B, 5 x 10⁻³M).

Os resultados de contração estão expressos como gramas por miligramas de peso seco

dos respectivos anéis. Os resultados de relaxamento estão expressos como porcentagem de contração residual em relação ao valor de pré-contracção à fenilefrina.

Os efeitos do L-NAME, SOD, apocinina e salicilato sobre a resposta à fenilefrina e ACh também foram expressos como a resposta máxima (Rmax) das curvas.

2.6 Determinação da produção de espécies reativas de oxigênio na aorta

A geração de espécies reativas na aorta foi avaliada utilizando-se o composto dihidroetidina (DHE), que quando oxidado dá origem a dois produtos fluorescentes, etídeo (E) e 2-hidróxi etídeo (EHO), que têm afinidade pelo DNA nuclear. Os anéis de aorta foram retirados como no item anterior e incluídos em meio de congelamento. Foram feitos cortes transversais de 10 µm em criostato, que foram colocados em lâminas silanizadas. Os cortes de aorta foram incubados com 50 µL de DHE (5 µM/ 30 min, a 37° C) em câmara úmida e protegidos da luz. Após este período, as lâminas foram observadas em microscópio óptico (ZEISS) equipado com filtro para rodamina e câmera fotográfica, utilizando-se uma objetiva para fluorescência de 40X. A localização da geração de espécies reativas foi realizada visualmente em três áreas diferentes de cada corte de aorta, onde foi observada regiões da aorta onde existiam núcleos marcados com fluorescência vermelha proveniente dos produtos de oxidação da DHE.

2.7 Avaliação *in vivo* da massa gordurosa e monitoramento metabólico dos camundongos

Após 4 semanas da cirurgia, e sendo alimentados com dieta hiperlipídica, os experimentos foram realizados. Massa magra e massa gordurosa foram medidas através de espectroscopia por ressonância magnética (Bruker BioSpin, Billerica, MA, USA). Sistema de monitoramento metabólico de animais (gaiola metabólica - CLAMS; Columbus Instruments, Columbus, OH, EUA) foi usado para mensurar o consumo de oxigênio (O₂), produção de dióxido de carbono (CO₂), gasto energético, atividade, e consumo calórico e de água.

2.8 Avaliação *in vivo* da sensibilidade à insulina nos camundongos

O clamp normoglicêmico-hiperinsulinêmico foi realizado como descrito anteriormente (Jornayvaz et al., 2011). Para realização, foi implantado um cateter na veia jugular de 6 a 7

dias antes do clamp normoglicêmico-hiperinsulinêmico. Para medida da produção endógena de glicose (EGP) basal, foi realizada a infusão de $[3\text{-}^3\text{H}]$ -glicose (Perkin- Elmer Life Sciences, EUA) em um ritmo de $0.05 \mu\text{Ci}/\text{min}$ por 120 minutos. Após essa infusão basal, o clamp normoglicêmico-hiperinsulinêmico foi conduzido em animais acordados por 140 minutos com uma infusão inicial de insulina por 4 minutos ($29 \text{ mU}/\text{kg}$) seguida por uma infusão contínua de $3 \text{ mU}/\text{kg}\cdot\text{min}^{-1}$ de insulina humana (Novolin; Novo Nordisk). Também foi realizada uma infusão contínua de $[3\text{-}^3\text{H}]$ -glicose ($0.1 \mu\text{Ci}/\text{min}$), e uma infusão variável de dextrose (20%) para manutenção da glicemia normal ($100 - 120 \text{ mg}/\text{dL}$). Após 85 minutos do início do clamp foi injetado um bolus de $10 \mu\text{Ci}$ de 2-deoxy-d-[1- ^{14}C]glicose (PerkinElmer) para estimativa da captação tecidual de glicose estimulada por insulina. Amostras de plasma foram coletadas pela cauda nos tempos 0, 30, 50, 65, 80, 90, 100, 110, 120, 130 e 140 minutos. O corte na cauda para coleta de sangue foi feito pelo menos 2 horas antes da primeira coleta para aclimatização dos animais, de acordo com os procedimentos operante padrão (Ayala et al., 2010). Além disso, os animais receberam i.v. uma solução contendo albumina mimetizando o plasma artificialmente durante o período de infusão com insulina, para compensação do volume de sangue retirado para amostras de plasma. Ao final do clamp, os animais foram anestesiados com uma injeção de pentobarbitol sódico ($150 \text{ mg}/\text{kg}$) e todos os tecidos foram retirados e congelados imediatamente em nitrogênio líquido, e então estocados em -80°C para subsequente análise.

2.9 Mensuração do conteúdo tecidual de lipídeos

Após 6 horas de restrição alimentar, os animais foram eutanasiados usando isoflurano e os tecidos retirados para análise do conteúdo de lipídeos. Triglicerídeos (TG) tecidual foram extraídos usando o método de Bligh e Dyer (Bligh et al., 1959) e mensurado usando um reagente para TG (Diagnostic Chemicals). Para extração de diacilglicerol (DAG), os tecidos foram homogeneizados em uma solução tampão (20 mM Tris-HCl, 1 mM EDTA, 0.25 mM EGTA, 250 mM sacarose, 2 mM PMSF) contendo inibidor de protease (Roche), e as amostras foram centrifugadas em 100000 g por 1 hora. Os supernadantes contendo a fração citosólica foram coletados. A concentração de DAG foi mensurada como descrito anteriormente (Yu et al., 2002). O conteúdo total de DAG citosólico é expresso como a soma das espécies individuais. Ceramidas foram mensuradas como descrito anteriormente (Yu et al., 2002).

2.10 Western Blot

Aortas das ratas foram pulverizadas e homogeneizadas em tampão a 4°C (Triton-X-100 1%, Tris 100mM (pH 7,4), pirofosfato de sódio 100 mM, fluoreto de sódio 100 mM, EDTA 10 mM, ortovanadato de sódio 10 mM e PMSF 2 mM) com homogenizador Polytron PTA 20S (Brinkmann Instruments). Os tecidos dos camundongos (fígado e músculo esquelético - gastrocnêmio) também foram homogeneizados com o mesmo tampão.

Os extratos teciduais foram centrifugados a 12000 rpm a 4 °C por 20 minutos para a remoção do material insolúvel. Após a centrifugação, o conteúdo protéico total foi quantificado utilizando-se o método de Bradford (BioRad). Amostras foram tratadas com tampão de Laemmli contendo DTT 200 mM e 50 a 100 µg de proteína total das aortas e 30 µg proteínas totais solublizadas dos fragmentos do fígado e músculo esquelético dos camundongos foram submetidos a eletroforese em gel de poliacrilamida (extrato total). Em cada gel havia um marcador com peso molecular com valores estabelecidos.

A transferência das proteínas das aortas separadas no gel foi realizada eletricamente para uma membrana de PVDF, por meio de um aparelho da BioRad por 12 h a 230 mA, enquanto as proteínas dos tecidos dos camundongos foram transferidas para membrana de PVDF usando um sistema de transferência semidry (Bio-Rad) por 120 minutos. As membranas de PVDF foram incubadas com uma solução bloqueadora (leite desnatado Molico® 5%, Tris 10 mM, NaCl 150 mM e Tween 20 0,02%) a 4°C por 2 horas para reduzir a ligação inespecífica de proteínas nas membranas.

Em seguida, as membranas que continham extratos totais de aorta foram incubadas com os seguintes anticorpos: anti-Cu/Zn SOD, anti-eNOS, anti-TNF- α , IL-1 β , IL-6, anti-peNOS, anti-gp91phox e anti-p22phox. E as membranas contendo as proteínas dos camundongos foram incubadas com os seguintes anticorpos: fosfo-AKT2^{ser473} (Signalway Antibody, USA), AKT2 (Cell Signaling Technology, Inc., Danvers, MA, USA), PKC ϵ (BD Transduction Laboratories, Lexington, KY, USA), PKC θ (BD Transduction Laboratories, Lexington, KY, USA), ou GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Essas incubações foram feitas com solução bloqueadora (3% de BSA ao invés de leite), por 12 h a 4 °C e a concentração de cada anticorpo variou de 1:200 a 1:1000. Em seguida as membranas foram lavadas com a solução bloqueadora sem leite ou BSA por 30 minutos. Estas membranas foram incubadas com o segundo anticorpo, conjugado com peroxidase, por 2 h em temperatura ambiente e, logo após, com a solução para detecção por

quimioluminescência como descrito no protocolo do kit comercial (ECLPlus, Amersham). A emissão de luz foi detectada e visualizada no sistema de captação de imagens MF-Chemibis – Bio-Imaging System (BioAmerica, EUA) para as membranas contendo extrato de aortas, enquanto a quimioluminescência das membranas contendo extratos dos tecidos dos camundongos foi detectada usando filmes radiográficos, já que esse experimento foi realizado na Universidade de Yale. A intensidade das bandas foi quantificada por densitometria óptica através da utilização de programa de análise de intensidade de bandas (Scion Image Software, MD, EUA).

2.11 Análise estatística

Os resultados foram expressos como médias $\pm$ EPM. As análises estatísticas foram realizadas utilizando-se análise de variância de uma ou duas vias (ANOVA) seguido do teste de Bartlett para a homogeneidade das variâncias e teste de múltiplas comparações Tukey-Kramer para ANOVA de uma via e Bonferroni para ANOVA de duas. As análises estatísticas dos resultados dos camundongos foram realizadas utilizando-se o teste *t* para amostras não pareadas. O nível de significância mínima aceitável foi $p < 0,05$.

4 RESULTADOS

4.1 Estudo da função vascular

Ratas OVX apresentaram aumento do peso corporal, da gordura retroperitoneal e do índice de Lee e redução no peso do útero após 11 semanas da cirurgia (Tabela 1). O tratamento com DHEA não corrigiu essas alterações (Tabela 1).

Tabela 1 – Dados zoomórficos das ratas após 11 semanas de cirurgia.

	SHAM	SHAM+DHEA	OVX	OVX+DHEA
Peso Final (g)	234,7 ± 3,4	239,9 ± 4,8	272,0 ± 5,8*	269,9 ± 6,7*
G. Peri-uterina (g)	2,6 ± 0,3	2,7 ± 0,2	2,7 ± 0,5	2,5 ± 0,3
G. Retroperitoneal (g)	1,3 ± 0,1	1,4 ± 0,1	3,0 ± 0,4*	3,1 ± 0,2*
Índice de Lee	300,4 ± 1,5	299,1 ± 1,9	312,9 ± 1,0**	312,6 ± 2,0**
Peso do útero (mg)	613 ± 103	594 ± 50	176 ± 14**	178 ± 24**

* $p < 0,01$ e ** $p < 0,001$ em relação ao SHAM (n = 10).

Fonte: Camporez (2012).

Na Tabela 2 observam-se as concentrações hormonais dos animais. Após 11 semanas da ovariectomia, houve redução na concentração de progesterona, estradiol e testosterona e aumento na concentração sérica de LH e FSH. Os animais tratados com DHEA apresentaram aumento da concentração plasmática de DHEA, demonstrando eficiência do tratamento. Além disso, o tratamento com DHEA levou a um aumento da concentração de estradiol nas ratas OVX.

Tabela 2 – Perfil hormonal de estradiol, progesterona, testosterona, LH, FSH e DHEA ao final de 11 semanas.

	SHAM	SHAM+DHEA	OVX	OVX+DHEA
LH (ng/mL)	9,6 ± 0,5	9,5 ± 0,9	63,2 ± 8,0*	54,5 ± 5,7*
FSH (ng/mL)	4,8 ± 0,2	5,0 ± 0,5	31,0 ± 3,7*	27,1 ± 2,8*
Progesterona (ng/mL)	26,0 ± 5,8	25,0 ± 4,8	9,5 ± 1,6*	9,7 ± 2,4*
Estradiol (pg/mL)	52,8 ± 4,1	60,4 ± 5,6	28,4 ± 3,7*	57,5 ± 6,9
Testosterona (pg/mL)	94,8 ± 16,6	104,0 ± 11,0	29,3 ± 3,4*	27,6 ± 4,5*
DHEA (pg/mL)	31,7 ± 2,1	46,7 ± 1,9*	26,3 ± 3,8	51,5 ± 3,2*

* p < 0,05 em relação ao SHAM (n = 8 - 10).

Fonte: Camporez (2012).

A Tabela 3 descreve as medidas hemodinâmicas. Observamos ao final das 11 semanas após a cirurgia aumento da pressão arterial sistólica e diastólica nas ratas OVX. O tratamento com DHEA nesses animais corrigiu a elevação na pressão arterial. A frequência cardíaca não apresentou qualquer alteração com a ovariectomia ou o tratamento com DHEA.

Tabela 3 – Pressão arterial sistólica (PAS), pressão arterial diastólica (PAD) e frequência cardíaca das ratas ao final de 11 semanas.

	SHAM	SHAM+DHEA	OVX	OVX+DHEA
PAS (mmHg)	129 ± 2	130 ± 2	139 ± 1*	128 ± 2
PAD (mmHg)	91 ± 1	90 ± 2	100 ± 2*	94 ± 1
Frequência Cardíaca (bpm)	356 ± 9	365 ± 14	384 ± 11	354 ± 10

* p < 0,05 em relação ao SHAM (n = 10).

Fonte: Camporez (2012).

A Figura 1 ilustra graficamente as curvas dose-resposta em anéis de aorta para fenilefrina (agonista α -adrenérgico), acetilcolina (ACh, vasodilatador dependente do endotélio)

e nitroprussiato de sódio (NPS, vasodilatador independente do endotélio). Podemos observar que os anéis de aorta das ratas OVX apresentam aumento da resposta à fenilefrina e redução da resposta à ACh, enquanto não houve nenhuma alteração na resposta ao NPS. O tratamento com DHEA corrigiu as respostas, tanto para fenilefrina quanto para ACh.

Figura 1 – Reatividade vascular para fenilefrina, acetilcolina e nitroprussiato de sódio.

Curva dose-resposta para fenilefrina (A), acetilcolina (B) e nitroprussiato de sódio (C) em anéis de aorta com endotélio intacto dos animais SHAM, OVX e tratados com DHEA. OS resultados estão expressos como média $\pm$ epm. ANOVA duas vias: * $p < 0,01$ em comparação com o grupo SHAM ($n = 9 - 13$).

Fonte: Camporez (2012).

Já que as alterações nas respostas vasculares ocorreram para fenilefrina e ACh, e não para NPS, podemos inferir que a aorta das ratas OVX possuem uma redução na biodisponibilidade do NO, e não uma redução na sensibilidade ao NO. Para investigar a modulação pelo endotélio, mais especificamente pelo NO da resposta à fenilefrina, avaliamos essa resposta em anéis de aorta sem endotélio (E-) ou previamente incubados com L-NAME (inibidor da NOS). A retirada do endotélio aumentou a resposta máxima à fenilefrina em todos os grupos estudados (Tabela 4), não havendo diferença entre os grupos. Semelhantemente, a incubação com L-NAME aumentou a resposta máxima da fenilefrina nos

quatro grupos (Tabela 4). Já que, tanto a retirada do endotélio quanto a incubação da aorta com L-NAME aumentou a resposta à fenilefrina em todos os grupos, retirando a diferença observada no grupo OVX, observa-se que o endotélio, especificamente via NO, exerce uma menor modulação sobre a resposta à fenilefrina nos animais OVX.

Aumento da geração do ânion superóxido também contribui com a redução da biodisponibilidade do NO. Dessa forma, foi realizada reatividade vascular nos anéis de aorta previamente incubados com SOD. A incubação com SOD reduziu a contração máxima induzida pela fenilefrina (Tabela 4) e aumentou a vasodilatação dependente do endotélio à ACh (Tabela 5) apenas em aorta de ratas OVX.

Estes resultados sugerem um importante papel das espécies reativas de oxigênio (EROs) na disfunção vascular observada em ratas OVX. Uma das principais fontes celulares de EROs, em especial o ânion superóxido, é o complexo enzimático NADPH-oxidase. Sendo assim, para investigar o papel da NADPH-oxidase na resposta vascular de ratas OVX, foi realizada reatividade vascular nos anéis de aorta previamente incubados com apocinina (inibidor da NADPH-oxidase). Assim como a SOD, a apocinina reduziu a resposta máxima à fenilefrina (Tabela 4) e aumentou a resposta máxima à ACh (Tabela 5) apenas em aorta de ratas OVX.

Tabela 4 – Efeito da remoção do endotélio (E-), L-NAME, SOD, Apo e salicilato na resposta máxima (Rmax) à fenilefrina.

	SHAM	SHAM+DHEA	OVX	OVX+DHEA
E+	0,84 ± 0,03	0,86 ± 0,06	1,27 ± 0,07*	0,90 ± 0,04
E-	2,69 ± 0,17 [#]	2,71 ± 0,25 [#]	2,54 ± 0,10 [#]	2,66 ± 0,16 [#]
L-NAME	2,53 ± 0,13 [#]	2,37 ± 0,07 [#]	2,33 ± 0,11 [#]	2,42 ± 0,10 [#]
SOD	0,84 ± 0,05	0,80 ± 0,02	0,90 ± 0,02 [#]	0,84 ± 0,04
Apo	0,85 ± 0,09	0,74 ± 0,09	0,88 ± 0,11 [#]	0,71 ± 0,08
Salicilato	0,78 ± 0,08	0,80 ± 0,09	0,87 ± 0,11 [#]	0,83 ± 0,09

Os valores são expressos como média ± EPM (grama de tensão por miligrama de tecido). * p < 0,05 em relação ao SHAM (n = 8 - 10), # p < 0,05 em relação aos anéis E+.

Fonte: Camporez (2012).

Tabela 5 – Efeito da incubação com SOD, Apo e salicilato na resposta máxima (Rmax) à ACh.

	SHAM	SHAM+DHEA	OVX	OVX+DHEA
E+	91,6 ± 2,7	95,7 ± 3,7	74,1 ± 2,7*	90,3 ± 2,4
SOD	93,4 ± 2,5	95,7 ± 1,6	96,5 ± 2,2 [#]	94,9 ± 1,6
Apo	92,5 ± 3,9	94,4 ± 1,6	93,4 ± 1,7 [#]	90,5 ± 2,3
Salicilato	90,0 ± 2,9	90,1 ± 1,7	94,9 ± 2,0 [#]	94,3 ± 2,4

Os valores são expressos como média ± EPM (% de relaxamento). * p < 0,05 em relação ao SHAM (n = 8 - 10),
[#] p < 0,05 em relação aos anéis E+.

Fonte: Camporez (2012).

O estudo da reatividade vascular indica maior biodisponibilidade de EROs em aorta de ratas OVX. A Figura 2 mostra maior marcação para a diidroetidina (DHE) nos cortes de aorta de ratas OVX, indicando maior biodisponibilidade de EROs. Tratamento com DHEA reduziu essa marcação em aorta de ratas OVX, porém não alterou a marcação em aorta de ratas SHAM (Figura 2).

O estresse oxidativo também pode levar a uma ativação de vias inflamatórias por ativação do fator NFκB. Assim, experimentos de reatividade vascular foram conduzidos com a incubação prévia dos anéis de aorta com salicilato. De maneira semelhante à SOD e à apocinina, incubação com salicilato reduziu a resposta contrátil à fenilefrina (Tabela 4) e aumentou a resposta máxima à ACh (Tabela 5) apenas em aorta de ratas OVX.

Figura 2 – Espécies reativas de oxigênio em aortas.

Fotomicrografias representativas de fluorescência de sessões microscópicas de aorta torácica (A) dos animais SHAM, OVX e tratados com DHEA. Os vasos foram marcados com a oxidação da diidroetidina, que produz fluorescência vermelha quando oxidada para brometo de etídio pelo ânio superóxido. Análise quantitativa das fotomicrografias representativas de fluorescência de sessões microscópicas de aorta torácica (B) dos animais SHAM, OVX e tratados com DHEA. Os resultados estão expressos como média $\pm$ epm. ANOVA uma via: * $p < 0,01$ em comparação com o grupo SHAM ($n = 7$).

Fonte: Camporez (2012).

A Figura 3A demonstra que aorta de ratas OVX apresenta redução da expressão total da Cu/Zn-SOD, sem nenhuma alteração da expressão da Mn-SOD (Figura 3B). O tratamento com DHEA das ratas OVX restaurou a expressão desta enzima (Figura 3A). Nessa mesma figura observamos um aumento da gp91phox e p22phox (subunidades da NADPH-oxidase) na aorta das ratas OVX, sendo que o tratamento com DHEA foi capaz de corrigir esse aumento (Figura 3C-D).

Na Figura 4 observa-se que a expressão total da eNOS não foi alterada pela ovariectomia ou tratamento com DHEA. Entretanto, foi encontrado uma redução significativa no grau de fosforilação da eNOS na aorta das ratas OVX, enquanto o tratamento com DHEA corrigiu esta redução (Figura 4).

Figura 3 – Expressão protéica da Cu/Zn-SOD, Mn-SOD e subunidades da NADPH oxidase.

Imagens e análise quantitativa da expressão da Cu/Zn-SOD (A), Mn-SOD (B) e as subunidades da NADPH oxidase gp91phox (C) e p22phox (D) em aorta torácica dos animais SHAM, OVX e tratados com DHEA. Os resultados estão expressos como média ± epm. ANOVA uma via: * $p < 0,01$ em comparação com o grupo SHAM ($n = 8$).

Fonte: Camporez (2012).

Figura 4 – Fosforilação da enzima eNOS em aortas.

Imagens e análise quantitativa da fosforilação da enzima eNOS em aorta torácica dos animais SHAM, OVX e tratados com DHEA. Os resultados estão expressos como média $\pm$ epm. ANOVA uma via: * $p < 0,01$ em comparação com o grupo SHAM ($n = 8$).

Fonte: Camporez (2012).

Observa-se na Figura 5 que a expressão de TNF- α e IL-1 β na aorta das ratas OVX está elevada, enquanto não houve nenhuma alteração na expressão de IL-6. Observa-se também que nas ratas OVX, o tratamento com DHEA reduziu a expressão de TNF- α e IL-1 β (Figura 5).

Uma vez que foi observado um aumento na concentração plasmática de estradiol nas ratas OVX tratadas com DHEA (Tabela 2), a resposta vascular foi avaliada também em um grupo de ratas OVX tratadas com DHEA e com um inibidor da enzima aromatase, para determinar se os efeitos vasculares observados após o tratamento com DHEA seriam dependentes de sua conversão a estradiol. A Figura 6 demonstra que a aorta das ratas OVX tratadas com DHEA possuem uma menor resposta contrátil a fenilefrina e uma maior resposta vasodilatadora à acetilcolina, independentemente do tratamento com o inibidor de aromatase. Estes resultados sugerem que os efeitos do DHEA nas ratas OVX são independentes da conversão a estradiol.

Figura 5 – Expressão de citocinas pro-inflamatórias em aorta.

Imagens e análise quantitativa da expressão de TNF- α (A), IL-1 β (B) e IL-6 em em aorta torácica dos animais SHAM, OVX e tratados com DHEA. Os resultados estão expressos como média $\pm$ epm. ANOVA uma via: * $p < 0,01$ em comparação com o grupo SHAM ($n = 8$).

Fonte: Camporez (2012).

Figura 6 – Reatividade vascular para fenilefrina e acetilcolina em animais tratados com inibidor de aromatase (formestane).

A

B

Curva dose-resposta para fenilefrina (A) e acetilcolina (B) em anéis de aorta com endotélio intacto dos animais OVX tratados ou não com DHEA e formestane (inibidor da enzima aromatase). OS resultados estão expressos como média $\pm$ epm. ANOVA duas vias: * $p < 0,01$ em comparação com o grupo SHAM ($n = 8$).
Fonte: Camporez (2012).

4.2 – Estudo da resistência periférica à insulina

Para estudo dos efeitos do DHEA sobre a resistência periférica à insulina em um modelo experimental de menopausa, foram utilizadas camundongos fêmeas ovariectomizadas alimententadas com dieta rica em gorduras.

Na Tabela 6 observa-se que não há diferença no peso corporal final entre os animais OVX e os tratados com DHEA. Entretanto, observa-se uma redução do percentual de gordura após o tratamento com DHEA, sem nenhuma alteração da massa muscular. Também é possível observar que não há nenhuma alteração na concentração plasmática de triglicerídeos (TG), ácidos graxos livres e estradiol. O tratamento com DHEA foi eficiente em elevar a concentração de DHEA nos animais OVX.

Tabela 6 – Dados zoomórficos e perfil lipídico e hormonal dos animais após 4 semanas da cirurgia.

	OVX	OVX+DHEA
Peso Final (g)	27,1 ± 0,6	26,7 ± 0,5
Gordura corporal (%)	23,5 ± 1,1	18,9 ± 0,7*
Massa muscular (%)	64,8 ± 1,5	67,3 ± 1,0
TG plasmático (mg/dL)	41,9 ± 2,2	43,9 ± 2,1
Ácidos Graxos Livres (mmol/L)	0,77 ± 0,07	0,83 ± 0,10
Estradiol plasmático (pg/mL)	9,0 ± 0,6	8,5 ± 0,6
DHEA plasmático (ng/mL)	2,2 ± 0,4	20,9 ± 0,5**

* p < 0,01 e ** p < 0,001 em relação ao grupo OVX .

Fonte: Camporez (2012).

O monitoramento dos animais em gaiola metabólica automatizada permitiu a avaliação do metabolismo corporal total. É possível observar que não há diferença entre os grupos no consumo de O₂, produção de CO₂ e gasto energético (Figura 7A - C). Entretanto, há uma leve

redução do quociente respiratório (RER) nos animais tratados com DHEA, sugerindo uma maior utilização lipídios como substrato energético (Figura 7D). Além disso, observa-se que o tratamento com DHEA levou a uma redução da ingestão calórica (Figura 8A). Não há diferença na ingestão hídrica e atividade (Figura 8B - C).

Figura 7 – Caracterização metabólica dos camundongos fêmeas OVX.

Consumo de oxigênio (A), produção de dióxido de carbono (B), gasto energético (C) e quociente respiratório (D) dos animais OVX e OVX+DHEA após 72 horas em gaiola metabólica. Os resultados estão expressos como média $\pm$ epm. Teste *t* de *student* (n = 8). * p < 0,05 em comparação com o grupo OVX.

Fonte: Camporez (2012).

Figura 8 – Comportamento alimentar e físico dos camundongos fêmeas OVX.

Ingestão calórica (A), ingestão hídrica (B) e atividade (C) dos animais OVX e OVX+DHEA após 72 horas em gaiola metabólica. Os resultados estão expressos como média $\pm$ epm. Teste *t* de *student* ($n = 8$). * $p < 0,05$ em comparação com o grupo OVX.

Fonte: Camporez (2012).

Para avaliação da resistência periférica à insulina *in vivo*, foi realizado o *clamp* normoglicêmico-hiperinsulinêmico nos animais acordados. Observa-se que não há diferenças na glicemia basal e durante o *clamp* (Figura 9A), e também não há diferença entre os grupos na concentração plasmática de insulina basal e ao final do *clamp* (figura 9B). Contudo, na Figura 9C-D pode-se observar que o tratamento com DHEA leva a um aumento da sensibilidade à insulina, já que o ritmo de infusão glicose (GIR) durante o *clamp* para manutenção da normoglicemia foi maior nos animais tratados com DHEA.

Figura 9 – Caracterização *in vivo* da sensibilidade à insulina dos camundongos fêmeas OVX.

Valores de glicemia, insulina e infusão de glicose durante o clamp normoglicêmica-hiperinsulinêmico. Valores da glicemia durante o clamp (A), concentração da insulina basal e após o clamp (B), curva dos valores de glicose infundida durante o clamp (C) e média dos valores de glicose infundida durante os últimos 40 minutos de clamp (D) dos animais OVX e OVX+DHEA. Os resultados estão expressos como média $\pm$ epm. Teste *t* de *student* ($n = 8$). * $p < 0,05$ em comparação com o grupo OVX.

Fonte: Camporez (2012).

Como parte desse aumento da sensibilidade à insulina dos animais OVX+DHEA é devido ao aumento da captação total de glicose estimulada por insulina durante o *clamp* (Figura 10A), foi avaliado a captação de 2-DG nos tecidos. Observamos que o tratamento com DHEA levou a um aumento da captação de 2-DG estimulada por insulina no músculo esquelético (Figura 10B) e no tecido adiposo branco (Figura 10C). Entretanto, não houve nenhuma diferença entre os grupos no tecido adiposo marrom (Figura 10D).

Figura 10 – Captação de glicose e 2-deoxy-glicose (2-DG) estimulada por insulina.

Valores de captação de glicose do corpo inteiro estimulada por insulina durante o clamp (A), captação de 2-DG em músculo esquelético (B), captação de 2-DG em tecido adiposo branco (C) e captação de 2-DG em tecido adiposo marrom (D) do animais OVX e OVX+DHEA após o clamp nomoglicêmico-hiperinsulinêmico. Os resultados estão expressos como média ± epm. Teste *t* de *student* (n = 8). * $p < 0,05$ em comparação com o grupo OVX.

Fonte: Camporez (2012).

Embora não há diferença entre os grupos referente a produção endógena de glicose (EGP) basal (Figura 11A), a supressão da EGP mediada por insulina durante o *clamp* foi maior no grupo tratado com DHEA (Figura 11B-C). Como a maior parte da EGP é proveniente do fígado, podemos presumir que os animais OVX+DHEA possuem uma maior sensibilidade à insulina hepática.

Corroborando com os resultados observados na captação de 2-DG, em que os animais tratados com DHEA apresentaram maior captação no tecido adiposo branco, sugerindo uma maior sensibilidade à insulina nesse tecido, podemos observar que os animais OVX+DHEA apresentaram uma maior supressão da concentração de AGL durante o clamp (figura 12A-C).

Figura 11 – Valores de produção endógena de glicose.

Produção endógena de glicose na condição basal durante duas horas que precederam o clamp (A), produção endógena de glicose durante o clamp (B) e percentual de supressão estimula por insulina (diferença entre antes e depois do clamp) da produção endógena de glicose (C) após o clamp normoglicêmico-hiperinsulinêmico dos animais OVX e OVX+DHEA. Os resultados estão expressos como média $\pm$ epm. Teste *t* de *student* ($n = 8$). * $p < 0,05$ em comparação com o grupo OVX.

Fonte: Camporez (2012).

Figura 12 – Valores de ácidos graxos livres dos camundongos fêmeas OVX.

Concentração plasmática basal de ácidos graxos livres (A), concentração plasmática de ácidos graxos livres após o clamp normoglicêmico-hiperinsulinêmico (B) e percentual de supressão da concentração de ácidos graxos livres estimulada por insulina (C) após o clamp nos animais OVX e OVX+DHEA. Os resultados estão expressos como média $\pm$ epm. Teste *t* de *student* ($n = 8$). * $p < 0,05$ em comparação com o grupo OVX.

Fonte: Camporez (2012).

Existem diversas hipóteses dos mecanismos que levam a resistência à insulina, fato observado nos animais OVX (Anexo 3), e que o tratamento com DHEA foi capaz de reverter. Entre essas hipóteses, o acúmulo ectópico de lipídeos é uma das mais importantes. Dessa forma, foi avaliado o conteúdo de TG e dois de seus intermediários (DAG e ceramidas) no fígado e músculo esquelético. Podemos observar que os animais OVX+DHEA apresentam uma forte tendência de reduzir o conteúdo de TG no fígado ($p = 0,05$; Figura 13A). No músculo esquelético o tratamento com DHEA reduziu o conteúdo de TG. Além disso, é possível observar na Figura 14A-B que o tratamento com DHEA reduziu o conteúdo citoplasmático de DAG, tanto no fígado quanto no músculo esquelético. Também houve uma pequena redução do conteúdo de ceramidas no fígado dos animais OVX+DHEA (Figura 15A), enquanto não houve nenhuma diferença entre os grupos no músculo esquelético (Figura 15B).

Figura 13 – Perfil do conteúdo tecidual lipídico – triglicerídeos (TG).

Conteúdo total de TG no fígado (A) e músculo esquelético (B) na condição basal (6 horas de restrição alimentar) dos animais OVX e OVX+DHEA. Os resultados estão expressos como média $\pm$ epm. Teste *t* de *student* ($n = 8$). * $p < 0,05$ em comparação com o grupo OVX.
Fonte: Camporez (2012).

Figura 14 – Perfil do conteúdo tecidual lipídico – diacilglicerol (DAG).

Conteúdo citosólico de DAG no fígado (A) e músculo esquelético (B) na condição basal (6 horas de restrição alimentar) dos animais OVX e OVX+DHEA. Os resultados estão expressos como média $\pm$ epm. Teste *t* de *student* ($n = 8$). * $p < 0,05$ em comparação com o grupo OVX.
Fonte: Camporez (2012).

Figura 15 – Perfil do conteúdo tecidual lipídico – ceramidas.

Conteúdo total de ceramidas no fígado (A) e músculo esquelético (B) na condição basal (6 horas de restrição alimentar) dos animais OVX e OVX+DHEA. Os resultados estão expressos como média $\pm$ epm. Teste *t* de *student* ($n = 8$). * $p < 0,05$ em comparação com o grupo OVX.
Fonte: Camporez (2012).

Corroborando com a redução do conteúdo citoplasmático de DAG, pode-se observar uma menor ativação da PKC ϵ no fígado (Figura 16A) e PKC θ nos músculo esquelético (Figura 16B) nos animais tratados com DHEA. Sabe-se que a ativação dessas isoformas de PKC tanto no fígado quanto no músculo levam a inibição da sinalização da insulina. Para confirmar essa hipótese foi analisada a fosforilação de AKT2 em ambos os tecidos após o *clamp*. É possível observar um aumento da fosforilação de AKT2 tanto no fígado (Figura 17A) quanto no músculo (Figura 17B).

Figura 16 – Ativação das novas PKCs no fígado e músculo.

Imagens representativas da expressão da PKC ϵ na membrana e citosol no fígado e análise quantitativa da relação membrana/citosol (A) e imagens representativas da expressão da PKC θ na membrana e citosol no músculo esquelético e análise quantitativa da relação membrana/citosol (B) dos animais OVX e OVX+DHEA na condição basal (6 horas de restrição alimentar). Os resultados estão expressos como média $\pm$ epm. Teste *t* de student ($n = 6$). * $p < 0,05$ em comparação com o grupo OVX.

Fonte: Camporez (2012).

Figura 17 – Fosforilação da enzima AKT2.

Imagens representativas e análise quantitativa da fosforilação de AKT2 no fígado (A) e músculo esquelético (B) dos animais OVX e OVX+DHEA na condição estimulada por insulina (pós clamp normoglicêmico-hiperinsulinêmico). Os resultados estão expressos como média $\pm$ epm. Teste *t* de *student* ($n = 8$). * $p < 0,05$ em comparação com o grupo OVX.

Fonte: Camporez (2012).

5 DISCUSSÃO

5.1 Estudo da função vascular

A prevalência de doenças cardiovasculares é menor em mulheres jovens do que em homens da mesma idade ou mulheres após a menopausa (Bur et al., 1995). Isso ocorre, em parte, devido aos efeitos protetores dos estrogênios sobre o sistema cardiovascular (White, 2002) observados em estudos epidemiológicos, clínicos e experimentais. Dessa forma, a redução drástica das concentrações plasmáticas dos estrogênios, que ocorre na menopausa, pode induzir profundos efeitos sobre o sistema cardiovascular, como um aumento da pressão arterial e disfunção endotelial, tanto em mulheres (Mercuro et al., 2004; Virdis et al., 2000) quanto em modelos experimentais de menopausa (Ito et al., 2006; Mendonça et al., 2007; Wassmann et al., 2001). Corroborando com essas informações, nosso estudo demonstra um aumento na pressão arterial, aumento na contração vascular à fenilefrina e redução do relaxamento vascular à ACh após ovariectomia em ratas Wistar.

Interessantemente, os estrogênios parecem que são importantes não somente para fêmeas, já que, camundongos machos *knockout* para aromatase também apresentaram redução na resposta vascular à ACh (Kimura et al., 2003). Em mulheres, terapia de reposição de estrogênios corrigiu a redução do incremento do fluxo sanguíneo no antebraço pela ACh (Virdis et al., 2000) e a elevação da pressão arterial observadas após 1 mês de ovariectomia (Mercuro et al., 2004). No entanto, os efeitos da reposição hormonal sobre o sistema cardiovascular ainda são controversos. Apesar dos efeitos biológicos favoráveis dos estrogênios sobre o sistema cardiovascular serem reconhecidos, inúmeros ensaios clínicos randomizados falharam em observar benefícios da terapia de reposição hormonal na incidência de eventos cardiovasculares em mulheres na menopausa (Huley et al., 1998; Prentice et al., 2009; Rossouw et al., 2002). No presente estudo, demonstramos que o tratamento com DHEA, no lugar de terapia de reposição com estrogênio, reduz a pressão arterial e protege ratas ovariectomizadas contra a disfunção endotelial. Esses resultados foram, em parte, devido à proteção do DHEA contra o estresse oxidativo e redução da expressão de fatores inflamatórios.

A concentração plasmática de DHEA reduz durante o processo de envelhecimento e isso tem sido relacionado à obesidade e à resistência à insulina, além do aumento da mortalidade por doenças cardiovasculares (Barrett-Connor et al., 1986; Nestler et al., 1988;

Schriock et al., 1988;). Tanto homens quanto mulheres em idade avançada submetidos ao tratamento com DHEA por 6 meses apresentaram redução da gordura visceral e subcutânea, além do aumento da sensibilidade à insulina (Villareal, Holloszy, 2004). Ademais, em um estudo duplo-cego, mulheres na menopausa tratadas com DHEA por 12 semanas demonstraram melhora na função endotelial (Williams et al., 2004).

Em estudos experimentais com animais, o tratamento com DHEA apresentou diversos efeitos benéficos, como redução da fibrose no miocárdio em ratos diabéticos (Aragno et al., 2008), retardo na formação de placas ateroscleróticas em coelhos (Hayashi et al., 2000), redução do ganho de peso corporal e depósito de gordura (Han et al., 1998), aumento da sensibilidade à insulina (Campbell et al., 2004) e secreção de insulina em ilhotas pancreáticas isoladas de ratos velhos (Medina et al., 2006) e redução da pressão arterial em ratas ovariectomizadas (Bhuiyan e Fukunaga, 2010). Além disso, em estudos *in vitro*, DHEA aumenta a proliferação endotelial (Williams et al., 2004) e protege células endoteliais contra apoptose (Liu et al., 2007), sendo que tais efeitos são independentes da ativação do receptor para estrogênio. Dessa forma, DHEA parece possuir diversas ações sobre o sistema cardiovascular, as quais poderiam representar uma ação direta ou indireta, pela redução de fatores de risco cardiovasculares, como o ganho de peso corporal e resistência à insulina. Entretanto, nossos resultados sugerem que os efeitos do DHEA sobre as alterações vasculares e pressão arterial não foram relacionados com a redução de fatores de risco cardiovasculares, já que o ganho de peso corporal e depósitos de gordura não foram influenciados pelo tratamento com DHEA.

A correção da resposta vascular alterada para fenilefrina e ACh observada nas aortas das ratas ovariectomizadas pelo tratamento com DHEA está relacionada ao aumento da produção de NO, baseado no fato que o tratamento com DHEA corrigiu a redução da fosforilação da eNOS, já que a fosforilação da eNOS em serina-1179 aumenta a atividade enzimática e a produção de NO (Dimmeler et al., 1999). Ainda, a correção da fosforilação da eNOS pelo tratamento com DHEA também explicaria o efeito no aumento da modulação da resposta contrátil à fenilefrina pelo NO, observado nas ratas ovariectomizadas. Recentemente, Bhuiyan e Fukunaga (2010) demonstraram que o tratamento com DHEA aumenta a fosforilação da eNOS também em rins de ratas OVX, levando a uma redução da pressão arterial. Além disso, estudos *in vitro* demonstraram que o DHEA estimula diretamente a fosforilação da eNOS em células endoteliais (Formoso et al., 2006; Liu e Dillon, 2004). Ainda, o efeito do tratamento com DHEA na resposta vascular para fenilefrina e ACh nas ratas ovariectomizadas parece não estar relacionado ao aumento da capacidade do músculo

liso vascular de relaxar em resposta ao NO exógeno, já que a resposta vascular ao nitroprussiato de sódio foi semelhante em todos os grupos.

Além de uma redução da função da eNOS, outro fator importante relacionado à disfunção endotelial é o estresse oxidativo. O aumento da disponibilidade de ânion superóxido em células vasculares provoca um declínio na biodisponibilidade do NO, levando a disfunção endotelial (Cai e Harrison, 2000). Nossos resultados indicam que além da redução da fosforilação da eNOS, o estresse oxidativo também pode estar envolvido na reduzida biodisponibilidade do NO em aorta de ratas ovariectomizadas. De fato, a resposta vascular para fenilefrina e ACh em anéis de aorta foram corrigidas pela enzima SOD. Além disso, aortas das ratas ovariectomizadas apresentaram aumento no conteúdo de EROs. Portanto, a disfunção endotelial observada nas ratas castradas parece ser causada tanto pela reduzida fosforilação da eNOS, quanto pelo estresse oxidativo. Essa disfunção endotelial causada pelo estresse oxidativo após a retirada dos estrogênios já tem sido mostrada em alguns trabalhos. Viridis et al. (2000) observaram que a disfunção endotelial resultante da privação aguda de estrogênios foi causada pelo estresse oxidativo. Em ratas SHR ovariectomizadas foi demonstrado redução da resposta vasodilatadora ao carbacol em anéis de aorta associada ao aumento na produção de ânion superóxido (Wassmann et al., 2001). Além disso, os estrogênios protegem camundongos fêmeas ovariectomizadas contra o aumento da produção de EROs em aortas, bem como a indução por angiotensina II da produção celular de EROs em músculo liso vascular (Strehlow et al., 2003).

Alguns estudos mostraram um potencial efeito antioxidante do DHEA. No presente estudo também foi demonstrado um papel do DHEA na redução do conteúdo de ânion superóxido na aorta de ratas ovariectomizadas. Esse efeito pode representar um segundo mecanismo pelo qual o tratamento com DHEA corrigiu a resposta vascular para fenilefrina e ACh na aorta das ratas ovariectomizadas. Yorek et al. (2002) observaram que o DHEA reduziu a produção de ânion superóxido em arteríolas de ratos diabéticos. Ainda, Aragno et al. (2006) também demonstraram redução no conteúdo de EROs no coração de ratos diabéticos tratados com DHEA. Além disso, parece que o DHEA possui efeitos antioxidantes não apenas sobre o sistema cardiovascular, já que em ratos diabéticos o DHEA reduziu o dano no músculo esquelético induzido por estresse oxidativo (Aragno et al., 2004). Parâmetros de estresse oxidativo no plasma e em células periféricas mononucleares do sangue em indivíduos diabéticos também foram reduzidos pelo tratamento com DHEA (Brignardello et al., 2007; Yorek et al., 2002).

Uma razão para o estresse oxidativo observado na menopausa e ovariectomia poderia ser a falta da modulação negativa pelos estrógenos da atividade e/ou expressão de enzimas antioxidantes ou produtoras de EROs, como NADPH-oxidase, que é a principal fonte de ânion superóxido associado à disfunção endotelial (Cai e Harrison, 2000). Tsuda et al. (2005) demonstraram que a aorta de camundongos fêmeas possui menor atividade e expressão da NADPH-oxidase do que aorta de camundongos machos. Entretanto, nesse mesmo trabalho foi observado que a ovariectomia promove um aumento na atividade e expressão da NADPH-oxidase em fêmeas. Além disso, sistemas antioxidantes também são reduzidos pela retirada dos estrogênios, o que contribui para o estresse oxidativo induzido pela ovariectomia. Deficiência de estrogênio reduz a expressão gênica de SOD em aorta de camundongos fêmeas ovariectomizadas, o que foi relacionado ao aumento da disponibilidade do ânion superóxido; e a terapia de reposição de estrogênio preveniu esse efeito (Strehlow et al., 2003).

No presente estudo demonstramos que as alterações vasculares induzidas pelo estresse oxidativo envolve maior ativação da NADPH-oxidase, uma vez que a inibição dessa enzima com apocinina, semelhantemente à SOD, foi capaz de corrigir tais alterações. Corroborando com essa hipótese, observamos também aumento da expressão protéica das subunidades da NADPH-oxidase. O fato de que aortas de ratas ovariectomizadas apresentarem redução da expressão da enzima Cu/Zn-SOD, nos nossos resultados, indica que além de maior geração de ânion superóxido, a ovariectomia promoveu também redução do sequestro desse ânion. Estes resultados explicariam a disfunção endotelial causada pelo estresse oxidativo nas ratas castradas. Além disso, foi observada após o tratamento com DHEA uma correção na expressão protéica tanto da Cu/Zn-SOD quanto da NADPH-oxidase, o qual poderia ser o principal resultado do tratamento com DHEA. Pois, este efeito levou a uma redução do conteúdo total de EROs e consequentemente melhora na função vascular das ratas ovariectomizadas.

A ação do DHEA sobre a enzima SOD tem sido demonstrada também em outras condições. Tratamento com DHEA aumenta a atividade da SOD em aorta de ratos envelhecidos (Wu et al., 2007), previne o dano oxidativo em icterícia obstrutiva em ratos aumentando a atividade da SOD no fígado (Celebi et al., 2004) e também eleva a atividade da SOD no fígado de ratos diabéticos (Aragno et al., 1999).

A indução de citocinas pró-inflamatórias pelas EROs é bem estabelecida. O aumento do conteúdo celular de EROs leva a uma maior degradação do fator I κ B α , consequentemente, a uma maior translocação do NF κ B para o núcleo da célula e maior atividade transcricional. O

resultado final desse mecanismo pode ser o aumento da expressão de citocinas pró-inflamatórias e moléculas de adesão (Droge, 2002).

Além disso, o excesso de tecido adiposo observado na obesidade, que também está presente nas ratas ovariectomizadas, também é responsável pela produção de citocinas pró-inflamatórias, as quais estão relacionadas com a disfunção endotelial associada com a obesidade, além do estresse oxidativo e resistência à insulina (Berg e Scherer, 2005; Kobayasi et al., 2010). No presente trabalho, observamos um aumento na expressão protéica local de TNF- α e IL-1 β na aorta das ratas ovariectomizadas. Esse aumento das citocinas pode estar associado com a disfunção endotelial observada nas ratas castradas. Isso ocorre, em parte, pois o TNF- α pode reduzir a biodisponibilidade do NO pelo aumento da produção de EROs, o que também foi observado em nosso estudo, através do aumento da atividade da NADPH-oxidase (Li et al., 2002). Essa associação entre citocinas pró-inflamatórias, em particular o TNF- α , e disfunção endotelial foi demonstrado em outros estudos. Kobayasi et al. (2010) observaram que camundongos fêmeas submetidas a dieta hiperlipídica por 16 semanas apresentam redução do relaxamento vascular dependente do endotélio. Esse resultado foi associado a elevação protéica de TNF- α na aorta. Além disso, em artérias coronárias, tanto de ratos obesos Zucker e camundongos diabéticos, foi observado aumento da expressão local de TNF- α , além do aumento da atividade da NADPH-oxidase e produção de EROs (Gao et al., 2007, 2010). Isso foi associado, em ambos os modelos animais, a redução do relaxamento dependente do endotélio.

Esse aumento das citocinas pró-inflamatórias observado nas ratas ovariectomizadas parece ser um importante fator que contribui para disfunção endotelial presente nesses mesmos animais. Já que, foi observado em ratas castradas que após a incubação dos anéis de aorta com salicilato (inibidor do NF κ B) houve uma correção na resposta vascular à ACh e PHE. Isso demonstra o papel da via inflamatória do NF κ B na resposta vascular alterada nas ratas com ausência dos hormônios sexuais. Assim como, observado por Kobayasi et al. (2010), a inibição aguda do NF κ B em aorta de camundongos fêmeas alimentadas com dieta hiperlipídica leva a uma melhora da resposta à ACh, melhorando o relaxamento dependente do endotélio.

Além da ação antioxidante do tratamento com DHEA, reduzindo o conteúdo total de EROs nas ratas ovariectomizadas, foi observado no presente estudo uma redução da expressão protéica das citocinas pró-inflamatórias na aorta das ratas castradas após o tratamento com DHEA. Isso pode ser outro importante fator pelo qual o tratamento com DHEA restaurou a função endotelial nas ratas castradas, já que foi demonstrado que o TNF- α reduz o

relaxamento vascular dependente do endotélio, em parte, devido à redução da biodisponibilidade do NO (Wimalasundera et al., 2003). Essa ação anti-inflamatória do DHEA, reduzindo a expressão de citocinas, também foi demonstrado em outros estudos. Recentemente, Aragno et al. (2009) observaram que coelhos alimentados com dieta hiperlipídica apresentam aumento da expressão protéica do NF κ B e expressão gênica do TNF- α , levando a uma elevação do conteúdo de EROs. Nesse mesmo estudo foi observado que o tratamento com DHEA protegeu os animais contra esses efeitos. Ainda em coração, ratos Wistar diabéticos ou Zucker apresentam aumento do conteúdo de EROs e TNF- α , o que foi revertido após o tratamento com DHEA (Aragno et al., 2006). Dessa forma, assim como observado tanto em nosso estudo como em outros (Aragno et al., 2006, 2009), o tratamento com DHEA apresenta efeitos antioxidantes e anti-inflamatórios, protegendo contra o estresse oxidativo e reduzindo a expressão de citocinas.

A ovariectomia em animais, como modelo experimental de menopausa, induz diversos efeitos deletérios sobre o sistema cardiovascular. Como observado no presente estudo, as ratas apresentam disfunção endotelial, com redução do relaxamento dependente do endotélio e aumento da contração estimulado por fenilefrina. Esses resultados foram associados ao estresse oxidativo e aumento do conteúdo de fatores inflamatórios na aorta. Outros trabalhos também demonstraram que a retirada dos estrogênios induz disfunção endotelial, associado com o aumento da produção de EROs (Squadrito et al., 2000; Wassmann et al., 2001; Widder et al., 2003). Além disso, a importância dos hormônios sexuais, em particular o estrogênio, é também observado em diversos estudos, já que a reposição hormonal com estradiol em ratas ovariectomizadas reverte a disfunção endotelial e estresse oxidativo (Squadrito et al., 2000; Wassmann et al., 2001; Widder et al., 2003). No presente estudo, podemos observar que o tratamento com DHEA exerceu efeitos similares ao estradiol nas ratas castradas. Além disso, também foi observado que após o tratamento com DHEA a concentração plasmática de estradiol nas ratas ovariectomizadas estavam elevadas, similares as ratas SHAM, sugerindo que o DHEA poderia exercer suas ações através de sua conversão à estradiol. Dessa forma, foi realizado o experimento de reatividade vascular nas ratas castradas após o tratamento com formestane (inibidor da aromatase). Observamos que mesmo após a inibição da enzima aromatase, responsável pela conversão de andrógenos à estrógenos, o tratamento com DHEA foi capaz de corrigir a resposta vascular para fenilefrina e ACh. Esses resultados sugerem que apesar do DHEA ser um precursor de estrógenos, esse hormônio exerce seus principais efeitos diretamente, reduzindo a produção de EROs e fatores inflamatórios, independente de sua conversão para estradiol.

5.2 Estudo da resistência periférica à insulina

Em nosso estudo demonstramos que a ovariectomia promoveu aumento do peso corporal e conteúdo de gorduras (Anexo A), confirmando o importante papel dos hormônios sexuais no controle do peso corporal.

O papel dos hormônios sexuais na composição corporal foi demonstrado em outros estudos. Em um estudo longitudinal com mulheres de 43 anos ou mais acompanhadas por 4 anos, foi observado um aumento do peso corporal e peso de gordura visceral apenas nas mulheres que passaram para menopausa (Lovejoy et al., 2008). Ainda, ratas Sprague-Dawley também apresentaram aumento do peso corporal após ovariectomia (Chen e Heiman, 2001). Rogers et al. (2009) também demonstraram que a ovariectomia induz uma redução no gasto energético, associado ao aumento do peso corporal. Os estrogênios parecem ter uma participação crítica no controle do peso corporal. Heine et al. (2000) observaram que camundongos *knockout* para o receptor de estrogênio α (ER α), de ambos os sexos, apresentaram aumento do peso corporal e do conteúdo de gordura. O aumento do tecido adiposo abdominal, após menopausa/ovariectomia, pode ser devido a alterações no metabolismo do tecido adiposo. Alguns estudos têm demonstrado que estrogênios influenciam a atividade da lipase hormônio sensível no tecido adiposo e a lipólise. Foi demonstrado que mulheres na pré-menopausa possuem uma maior atividade da lipase hormônio sensível e maior atividade lipolítica visceral do que em depósitos de gordura femural. As diferenças regionais nos depósitos de gordura não foram observadas em mulheres na menopausa (Rebuffe-Scrive et al., 1986). Essa redução na atividade lipolítica no tecido adiposo visceral pode contribuir para o aumento do peso corporal e obesidade após a menopausa.

A relação entre a obesidade e resistência à insulina é bem estabelecida. Cefalu et al. (1995) demonstraram que tanto em homens quanto em mulheres, entre 23 e 83 anos de idade, a gordura intra-abdominal correlacionou-se negativamente com a sensibilidade à insulina e tolerância à glicose. Além disso, a obesidade induzida por dieta, hiperlipídica ou de cafeteria, também induz resistência à insulina em modelos experimentais (Caricilli et al., 2008; Prada et al., 2006).

A insulina exerce sua ação através da ativação de uma cascata de sinalização intracelular, que se inicia após sua ligação a um receptor de membrana (Saltiel e Kahn, 2001). A resistência à insulina detectada em modelos experimentais de obesidade está associada à redução da sinalização da insulina em tecidos alvo como fígado, tecido adiposo, hipotálamo e músculo esquelético. Camundongos ou ratos alimentados com dieta rica em gorduras ou dieta

de cafeteria apresentam redução da fosforilação do IR, IRS-1/2 e Akt, além da associação dos IRS-1/2 com a PI3-K em fígado, músculo esquelético e hipotálamo (Caricilli et al., 2008; Carvalho-Filho et al., 2009; De Souza et al., 2005; Lalli et al., 2008; Prada et al., 2006).

Riant et al. (2009) observaram resistência à insulina em camundongos fêmeas castradas e alimentadas com dieta hiperlipídica, enquanto os animais SHAM alimentados com essa dieta e ovariectomizadas alimentadas com dieta padrão não apresentaram resistência à insulina. Nesse mesmo estudo também foi demonstrado uma redução da fosforilação de Akt estimulada por insulina em ratas ovariectomizadas alimentadas com a dieta hiperlipídica.

Os estrogênios possuem efeitos reconhecidos sobre o metabolismo da glicose. Mulheres em período fértil possuem uma maior sensibilidade à insulina do que homens da mesma idade, enquanto as mulheres na menopausa apresentam uma redução da sensibilidade à insulina comparada com as mulheres férteis, e se igualam a homens da mesma idade (Manco et al., 2006). Margolis et al. (2004) demonstraram em um ensaio clínico duplo-cego e randomizado que mulheres na menopausa que fizeram reposição hormonal com estrogênio+progestogênio reduziram a incidência de DM2 e melhoraram a sensibilidade à insulina. O principal hormônio que exerce papel favorável sobre o metabolismo da glicose e sensibilidade à insulina parece ser o estradiol, pois Puah e Bailey (1985) demonstraram em camundongos fêmeas, que a resistência à insulina no músculo esquelético induzida pela ovariectomia foi prevenida com o tratamento com estradiol, enquanto o tratamento com progesterona não exerceu nenhum efeito. Corroborando com esses dados, também foi observado, em ratas Sprague-Dawley ovariectomizadas, aumento da resistência à insulina, o que foi prevenido pelo tratamento com estradiol ou exercício físico (Saengsirisuwan et al., 2009).

Os efeitos protetores do estradiol parecem ser mediados pela isoforma ER α , já que camundongos fêmeas *knockout* para ER α castradas e alimentadas com dieta hiperlipídica não respondem ao tratamento com estradiol, permanecendo resistentes à insulina (Riant et al., 2009). Além disso, foi observado intolerância à glicose em camundongos, de ambos os sexos, *knockout* para ER α (Heine et al., 2000), além de apresentarem redução da expressão de GLUT-4 no músculo esquelético (Barros et al., 2009). Por outro lado, camundongos *knockout* para ER β apresentam hipoglicemia, melhora da tolerância à glicose e aumento da expressão de GLUT-4 (Barros et al., 2009).

No presente trabalho demonstramos que o tratamento com DHEA melhorou a sensibilidade à insulina em camundongos fêmeas ovariectomizadas e alimentadas com dieta hiperlipídica. Embora não foi possível observar nenhuma alteração no peso corporal,

observamos uma redução do percentual corporal de gordura após o tratamento com esse hormônio.

A redução da concentração circulante de DHEA tem sido relacionada ao aumento da gordura visceral, da concentração de insulina plasmática e redução da ação insulínica, tanto em humanos quanto em modelos experimentais. Em estudos experimentais foram descritos efeitos anti-diabetogênicos em camundongos ob/ob (Coleman et al., 1982), melhora da resistência periférica à insulina em ratos Wistar (Campbell et al., 2004 ; Mukasa et al., 1998) e redução do tecido adiposo em ratos fa/fa (Cleary e Zisk, 1986; Cleary et al., 1988; Gansler et al., 1985) com tratamento com DHEA. Em nosso estudo observamos aumento da sensibilidade corporal à insulina, o que pode ser explicado pelo aumento da sensibilidade hepática e muscular à insulina. Observamos que os animais tratados com DHEA possuem uma maior supressão da produção endógena de glicose pela insulina durante o clamp. Sendo o fígado responsável pela maior parte da produção endógena de glicose, podemos presumir que essa maior supressão é devido ao aumento da sensibilidade hepática à insulina. Quanto ao músculo esquelético, observamos que os animais tratados com DHEA apresentam uma maior captação de 2-DG estimulada por insulina. Corroborando com os dados obtidos no clamp, foi observado aumento da fosforilação de AKT2 no fígado e no músculo esquelético dos animais OVX+DHEA.

Existem diversas hipóteses que buscam explicar o desenvolvimento da resistência à insulina associado a obesidade, situação observada na falta de estrogênios. Aumento das citocinas plasmáticas e teciduais devido a inflamação e aumento dos adipócitos pode levar a resistência à insulina, já que algumas citocinas como o fator de necrose tumoral alfa (TNF α) e a resistina são potentes inibidores da sinalização da insulina (Camporez et al., 2011; Jazet et al., 2003). Outras hipóteses que tentam explicar a resistência à insulina e o desenvolvimento do DM2 são o aumento da produção de espécies reativas de oxigênio (EROS) e estresse de retículo endoplasmático, ambos presentes na obesidade (Ando e Fujita, 2009; Hotamisligil, 2008). Outra hipótese é o aumento da concentração plasmática de triglicerídeos (TG) e ácidos graxos livres (AGL), o que pode levar ao desequilíbrio entre a captação tecidual de lipídios, a oxidação e o armazenamento, levando ao aumento do conteúdo intracelular de intermediários como o diacilglicerol (DAG) e ativação das isoformas das novas proteínas kinase C (nPKC), as quais inibem a ação da insulina no fígado e músculo (Samuel et al., 2010). Estudos experimentais com camundongos transgênicos confirmam essa última hipótese. É possível observar que camundongos *knockout* para acetil CoA carboxilase-2 (ACC2), os quais tiveram reduzida concentração de DAG, portanto reduzida ativação de PKC θ , são protegidos contra a

resistência à insulina induzida por dieta hiperlipídica (Choi et al., 2007a). Além disso, confirmando a hipótese DAG/PKC, camundongos *knockout* para PKC θ demonstraram serem protegidos contra resistência à insulina induzida por dieta hiperlipídica (Kim et al., 2004). Ainda, camundongos envelhecidos com aumento da expressão da enzima catalase na mitocôndria em músculo esquelético possuem aumento da atividade mitocondrial, e portanto redução do conteúdo de DAG e aumento da sensibilidade à insulina (Lee et al., 2010).

No fígado também é possível observar esse fenômeno. Ratos alimentados com dieta hiperlipídica apresentaram aumento do conteúdo de DAG no fígado, o que foi associado com a ativação da PKC ϵ e, por sua vez, reduzida fosforilação de IRS-2 e resistência hepática à insulina (Samuel et al., 2004). Isso também foi observado in animais alimentados com frutose, os quais apresentaram aumento de DAG no fígado, aumento da ativação da PKC ϵ , e, portanto reduzida sensibilidade hepática à insulina (Nagai et al., 2009). Camundongos geneticamente modificados também demonstraram o papel do DAG e PKC ϵ na resistência à insulina no fígado. Reduzida expressão da PKC ϵ no fígado mediada por tratamento com oligonucleotídeo *antisense* protegeu os animais contra resistência hepática à insulina induzida por gordura (Samuel et al., 2007). Igualmente, camundongos *knockout* para a enzima mitocondrial glicerol-3-fosfato aciltransferase ou camundongos com reduzida expressão de diacilglicerol O-aciltransferase-2, os quais apresentam conteúdo reduzido de DAG no fígado, estão protegidos contra resistência hepática à insulina induzida por gordura, associado a uma reduzida ativação da PKC ϵ (Choi et al., 2007b; Neschen et al., 2005). Um outro modelo experimental interessante que corrobora com a hipótese do DAG são os camundongos transgênicos ob/ob que possuem maior adiponectina expressa. Esses camundongos são deficientes em leptina (ob/ob), portanto obesos, embora possuam altas concentrações de adiponectina, uma citocina produzida no tecido adiposo e que melhora a sensibilidade à insulina. Apesar da maior obesidade presente nesses animais do que nos camundongos ob/ob regulares, eles não apresentam resistência hepática à insulina. Isso foi associado com um baixo conteúdo de DAG no fígado, e reduzida ativação da PKC ϵ (Kim et al., 2007). Além disso, reduzida oxidação de lipídeos está relacionada ao aumento de DAG no fígado, e então resistência hepática à insulina (Zhang et al., 2007). Ainda, é possível observar o papel direto do DAG na resistência hepática à insulina em camundongos com aumento da expressão da enzima diacilglicerol aciltransferase 2 (DGAT2). Esses camundongos apresentaram severa resistência à insulina no fígado associada com aumento do conteúdo intracelular de DAG (Jornayvaz et al., 2011).

De fato, no nosso estudo a melhora da sensibilidade à insulina nos animais tratados com DHEA foi associada a uma redução do acúmulo ectópico de lipídios, em particular o DAG, tanto no fígado quanto no músculo. Isso foi associado a uma reduzida ativação da PKC ϵ no fígado e PKC θ no músculo, o que levou a um aumento da sensibilidade à insulina. Esse reduzido acúmulo ectópico de lipídios pode ser resultado da redução do percentual de gordura observado após o tratamento com DHEA. Isso, por sua vez, pode ter sido causado por duas maneiras. A primeira delas é a redução da ingestão calórica observada nos animais OVX+DHEA. Outros trabalhos com roedores também observaram tal efeito. Catalina et al. (2001) demonstraram que o DHEA possui efeitos sobre a ingestão calórica em camundongos, reduzindo a ingestão calórica desses animais. Outro estudo observou efeitos similares em ratos Zucker, tanto no tratamento com DHEA usando injeções intraperitoneal ou intracérebro-ventricular (Wright et al., 1995). Outro motivo para os efeitos do DHEA sobre a composição corporal é o aumento da oxidação de lipídios. Observamos que o tratamento com DHEA reduziu o quociente respiratório dos animais, sugerindo uma maior utilização de lipídios como substrato energético, embora essa redução tenha sido pequena, ficando difícil a determinação do impacto fisiológico desse efeito.

Contudo, em humanos existem resultados menos concordantes quanto aos efeitos do DHEA. Enquanto alguns estudos demonstram que a suplementação com DHEA induz uma melhora da ação insulínica com redução da adiposidade (Kawano et al., 2003; Lasco et al., 2001; Villareal e Holloszy, 2004), outros apresentam que o DHEA não exerce efeito algum na sensibilidade à insulina e obesidade (Basu et al., 2007; Nair et al., 2006), embora, recentemente, outro estudo em humanos demonstrou que o tratamento com DHEA é benéfico (Weiss et al., 2011), reduzindo a resistência à insulina e marcadores inflamatórios. Esse estudo ainda discute que a condição do indivíduo ao iniciar o tratamento com DHEA pode ser de fundamental importância para a observação dos efeitos, sendo assim, indivíduos em condições deletérias maiores, como obesidade, envelhecimento e diabetes, poderiam ser os maiores beneficiados. Além disso, outro ponto a favor é que a suplementação com DHEA parece possuir uma aparente segurança, não apresentando efeitos colaterais (Panjari et al., 2009), enquanto a reposição hormonal com estradiol em mulheres na menopausa apresenta diversos riscos, como aumento da incidência de câncer de mama, tromboembolismo venoso e acidente vascular cerebral (Prentice et al., 2009).

6 CONSIDERAÇÕES FINAIS

Em resumo, esse estudo confirma, primeiramente, o papel central do estresse oxidativo e das citocinas pró-inflamatórias na indução da disfunção endotelial em ratas com privação de estrogênio. Além disso, indica um potencial papel da terapia com DHEA, ao invés da reposição hormonal com estrogênio, como antioxidante e antiinflamatório, melhorando a resposta vascular à PHE e ACh, reduzindo a pressão arterial sistólica e diastólica, e modulando a expressão protéica da Cu/Zn-SOD e NADPH-oxidase e grau de fosforilação da eNOS na aorta de ratas ovariectomizadas.

Ainda, nosso estudo demonstra que o tratamento alternativo com DHEA em camundongos fêmeas OVX, ao invés do estradiol, aumenta a sensibilidade à insulina, provavelmente devido a redução da ingestão calórica que levou a uma redução do percentual de gordura, que por sua vez reduziu o acúmulo ectópico de lipídios.

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Anexos

Anexo A – Composição corporal dos animais dos camundongos fêmeas SHAM e OVX.

Peso corporal inicial (A), peso corporal final (B) e percentual de gordura corporal (C) dos animais SHAM e OVX. Os resultados estão expressos como média $\pm$ epm. Teste *t* de *student* ($n = 8$). * $p < 0,05$ em comparação com o grupo SHAM.

Fonte: Camporez (2012)

Anexo B – Concentração plasmática de E2 e DHEA.

Concentração plasmática de estradiol (A) e DHEA (B) dos animais SHAM e OVX. Os resultados estão expressos como média $\pm$ epm. Teste *t* de *student* ($n = 8$). * $p < 0,05$ em comparação com o grupo SHAM.

Fonte: Camporez (2012).

Anexo C – Medida *in vivo* da sensibilidade à insulina.

Glicemia dos animais durante o clamp normoglicêmica-hiperinsulinêmico (A), valores da infusão de glicose durante o clamp normoglicêmica-hiperinsulinêmico (B) e média dos valores da infusão de glicose nos últimos 40 minutos do clamp normoglicêmica-hiperinsulinêmico (C) nos animais SHAM e OVX. Os resultados estão expressos como média $\pm$ epm. Teste *t* de *student* (n = 8). * p < 0,05 em comparação com o grupo SHAM.

UNIVERSIDADE DE SÃO PAULO

CERTIFICADO

Certificamos, nos termos do artigo 74, parágrafo único, inciso 5, alínea "b", do Estatuto da Universidade de São Paulo, que **João Paulo Gabriel Camporez** portador(a) da identidade: **1.529.478-ES - SESPES** concluiu o curso de Extensão Universitária na modalidade de *Difusão*: *Curso de Proteção Radiológica* sob a responsabilidade: **Instituto de Ciências Biomédicas**.

São Paulo, 08 de fevereiro de 2008

Prof. Dr. Luiz Roberto Giorgetti de Britto
Diretor

Prof. Dr. Jose Maria Alvarez Mosig
Presidente da Comissão de Cultura e Extensão Universitária

PRÓ-REITORIA DE
CULTURA E EXTENSÃO
UNIVERSITÁRIA

Yale University

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February 21st, 2011

IACUC #: 2008-07277

(Please refer to this number when
inquiring about this project)

PRINCIPAL INVESTIGATOR: Gerald Shulman

TITLE OF PROTOCOL: In Vivo NMR Studies of Carbohydrate Metabolism

CURRENT APPROVAL PERIOD: 06-30-10 TO 06-30-11

CHANGES APPROVED: New procedure: Ovariectomy; New agent: Estrogen, DHEA

The Institutional Animal Care and Use Committee (IACUC) granted approval the above entitled protocol effective 02-13-11. A copy of your approved modification is attached to this letter.

Yale University has an approved Animal Welfare Assurance (#A3230-01) on file with the NIH Office for Protection from Research Risks. The Assurance was approved May 16, 2007.

Approval of the Yale University IACUC does not, in and of itself, constitute approval for implementation of this project. Other levels of review and approval may be required (e.g. Environmental Health and Safety, Radiation Safety), and the project should not begin until the proper approvals have been obtained.

If you have any questions, or require any additional assistance, please do not hesitate to phone the IACUC Office at 785-5992.

Elizabeth Eynon
IACUC Vice Chair
On behalf of the IACUC

Record of Completion

This is to confirm that

Joao Paulo Camporez

Has successfully completed the following Course on 11/12/10

01. Radiation Safety Basics Part I-Web Training

Environmental Health & Safety
Course Owner

04/19/2011
Date

Dehydroepiandrosterone protects against oxidative stress-induced endothelial dysfunction in ovariectomized rats

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Non-technical summary It is well known that cardiovascular disease is more frequent in postmenopausal than in premenopausal women. Moreover, it has been shown that dehydroepiandrosterone (DHEA), a steroid hormone secreted by adrenal glands, reduces during ageing. Its reduced plasma level has been related to increased prevalence of obesity, insulin resistance and cardiovascular disease. We show that DHEA treatment in ovariectomized rats, an experimental model of menopause, reduces blood pressure and improves vascular function. Furthermore, DHEA reduced reactive oxygen species (ROS), correcting the reduced protein expression of Cu/Zn-SOD, an antioxidant protein, and increased protein expression of NADPH oxidase, a pro-oxidant protein. This work shows the potential effect of DHEA upon correction of endothelial dysfunction observed on oestrogen deprivation.

Abstract Cardiovascular disease is less frequent in premenopausal women than in age-matched men or postmenopausal women. Moreover, the marked age-related decline in serum dehydroepiandrosterone (DHEA) level has been associated to cardiovascular disease. The aim of this study was to evaluate the effects of DHEA treatment on vascular function in ovariectomized rats. At 8 weeks of age, female Wistar rats were ovariectomized (OVX) or sham (SHAM) operated and 8 weeks after surgery both groups were treated with vehicle or DHEA (10 mg kg⁻¹ week⁻¹) for 3 weeks. Aortic rings were used to evaluate the vasoconstrictor response to phenylephrine (PHE) and the relaxation responses to acetylcholine (ACh) and sodium nitroprusside (SNP). Tissue reactive oxygen species (ROS) production and SOD, NADPH oxidase and eNOS protein expression were analysed. PHE-induced contraction was increased in aortic rings from OVX compared to SHAM, associated with a reduction in NO bioavailability. Furthermore, the relaxation induced by ACh was reduced in arteries from OVX, while SNP relaxation did not change. The incubation of aortic rings with SOD or apocynin restored the enhanced PHE-contraction and the impaired ACh-relaxation only in OVX. DHEA treatment corrected the increased PHE contraction and the impaired ACh-induced relaxation observed in OVX by an increment in NO bioavailability and decrease in ROS production. Besides, DHEA treatment restores the reduced Cu/Zn-SOD protein expression and eNOS phosphorylation and the increased NADPH oxidase protein expression in the aorta of OVX rats. The present results suggest an important action of DHEA, improving endothelial function in OVX rats by acting as an anti-oxidant and enhancing the NO bioavailability.

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Abbreviations ACh, acetylcholine; DHEA, dehydroepiandrosterone; NO, nitric oxide; ORT, oestrogen replacement therapy; OVX, ovariectomized rats; PHE, phenylephrine; ROS, reactive oxygen species; SNP, sodium nitroprusside.

Introduction

It is known that oestrogen has profound effects on the cardiovascular system (White, 2002). Indeed, cardiovascular disease is much less frequent in premenopausal women than in age-matched men or postmenopausal women (Burt *et al.* 1995).

Endothelial dysfunction is a common factor in the development of many cardiovascular diseases. In line with this, endothelial function was shown to be impaired in ovariectomized women (Virdis *et al.* 2000) and hypertensive female rats (Wassmann *et al.* 2001; Widder *et al.* 2003). Indeed, it has been observed that ovariectomy reduces vascular response to acetylcholine in Sprague–Dawley rats (Squadrito *et al.* 2000; Lam *et al.* 2006), female hypertensive rats (Wassmann *et al.* 2001; Widder *et al.* 2003), and Wistar rats (Paredes-Cabejal *et al.* 1995). Ovariectomy also increases vascular response to phenylephrine in Sprague–Dawley rats (Lam *et al.* 2006), and Wistar rats (Paredes-Cabejal *et al.* 1995). This impairment in endothelial function was associated with an increment in superoxide anion production (Wassmann *et al.* 2001; Lam *et al.* 2006) as well as a reduction in eNOS protein expression (Widder *et al.* 2003). Moreover, ovariectomy promoted an increment in blood pressure of normotensive women (Mercuro *et al.* 2004), as well as in female Wistar rats (Mendonça *et al.* 2007), and in hypertensive rats (Ito *et al.* 2006).

Oestrogen replacement therapy (ORT) in ovariectomized women and female rats prevents the deleterious effect of ovariectomy by reducing blood pressure and increasing antioxidant defence and eNOS protein expression. However, large controlled clinical trials have not shown benefits of ORT, or have even shown deleterious effects, such as an increment of venous thromboembolic disease, stroke and breast cancer incidence (Prentice *et al.* 2009). Therefore, new therapeutic strategies to prevent oestrogen deprivation-induced deleterious effects on the cardiovascular system will be valuable.

Dehydroepiandrosterone (DHEA) has been considered a potential alternative hormone for ORT, due to its apparent safe, antioxidant and metabolic effects (Labrie *et al.* 2005). DHEA and its sulfated form (DHEAS) are the most abundant cholesterol-derived hormones in humans and their plasma concentrations progressively decline with age. An inverse relation between plasma DHEA concentration and obesity, diabetes and incidence of cardiovascular disease has been observed (Barrett-Connor *et al.* 1986; Nestler *et al.* 1988; Schriock *et al.* 1988). Moreover, DHEA treatment protects heart of diabetic rats against oxidative stress (Aragno *et al.* 2008); it furthermore protects endothelial cells against apoptosis (Liu *et al.* 2007), and stimulates ser1177 eNOS phosphorylation (Formoso *et al.* 2006).

It is well known that ovariectomized female rats present endothelial dysfunction and high blood pressure levels (Wassmann *et al.* 2001; Widder *et al.* 2003). Therefore, DHEA evokes beneficial mechanisms that could reduce endothelial dysfunction and reduce the cardiovascular risk present after surgical menopause in rats. Thus, the present work hypothesizes that DHEA could be an alternative hormonal therapy, and provide benefits to the vascular system of postmenopausal women. To evaluate this hypothesis, the aim of the present study was to assess the effects of DHEA treatment on endothelium-dependent relaxation, focusing on the mechanisms involved, mainly related to NO and oxidative stress, in an experimental menopause models in rats.

Methods

Animal care and protocol

All experimental procedures were approved by and conducted in accordance with the guidelines of the Animal Care and Use Committee of the Biomedical Institute of Sao Paulo University, according to the guidelines of the Brazilian Societies of Experimental Biology. Seven-week-old female Wistar rats were obtained from colonies maintained at the Institute of Biomedical Sciences of the University of São Paulo. At 8 weeks of age, the rats were anaesthetized with a ketamine–xylazine–acepromazine mixture (64.9, 3.2 and 0.78 mg kg⁻¹, respectively, i.p.) and were ovariectomized (OVX) or sham-operated (SHAM). After 8 weeks of surgery, OVX and SHAM rats were treated with vehicle (soybean oil) or DHEA (10 mg kg⁻¹ week⁻¹, s.c.) for 3 weeks. The DHEA administration protocol was based on studies published by us and others (Campbell *et al.* 2004; Medina *et al.* 2006; Jacob *et al.* 2009). During all the procedures rats were housed in a constant room temperature environment, with 12:12 h light–dark cycle, with free access to standard rat chow and tap water. Rats were randomly divided into four groups: (1) SHAM, (2) SHAM+DHEA, (3) OVX, and (4) OVX+DHEA.

Biometrics characteristics

At the end of the treatment period, the body weight gain, retroperitoneal fat pad and uterus weight were determined.

Arterial blood pressure measurement

Rats were anaesthetized with a ketamine–xylazine–acepromazine mixture (64.9, 3.2 and 0.78 mg kg⁻¹, respectively, i.p.) and allowed to breathe room air spontaneously. The right carotid artery was cannulated

with a polyethylene catheter (PE-50 with heparinized saline) that was exteriorized in the mid-scapular region. After 24 h, arterial pressure and heart rate were measured in conscious animals by a pressure transducer (model TRA 021, PanLab, Barcelona, Spain) and recorded by using an interface and software for computer data acquisition (Power Lab 4/25; ADInstruments, Sydney, Australia). Heart rate was determined from the interbeat intervals.

Vascular reactivity study

Vascular function was evaluated in aortic rings from all groups studied. We used the aortic rings as a model to study vascular function because in this artery the main endothelium-dependent vasodilator factor is NO. Thus, since the aim of the present study is to assess the role of NO and reactive oxygen species on the DHEA treatment in OVX rats, the aortic rings are the best model to answer our questions without misinterpretation. Segments of the thoracic aorta (4 mm in length), free of connective tissue, were mounted in an isolated tissue chamber containing Krebs–Henseleit solution (containing (in mM): 118 NaCl, 4.7 KCl, 25 NaHCO₃, 2.5 CaCl₂·2H₂O, 1.2 KH₂PO₄, 1.2 MgSO₄·7H₂O, 11 glucose, and 0.01 EDTA), gassed with 95% O₂–5% CO₂, and maintained resting tension of 1 g at 37°C at pH 7.4 as previously published by our group (Davel *et al.* 2008). In order to analyse the influence of the endothelium on vascular responses, the endothelial layer was mechanically removed in certain experiments by rubbing the lumen with a needle. Isometric tension was recorded by using an isometric force transducer (Letica TRI 210, Barcelona, Spain) connected to an acquisition system (MP100, Biopac Systems, Goleta, CA, USA).

After 30 min equilibration, concentration–response curves to the α_1 -adrenoceptor agonist phenylephrine (PHE, 10^{−10}–10^{−4} mol l^{−1}), to the endothelium-dependent vasodilator acetylcholine (ACh, 10^{−11}–10^{−5} mol l^{−1}) and to the nitric oxide donor sodium nitroprusside (SNP, 10^{−11}–10^{−5} mol l^{−1}) were obtained. At the end of the experiment, the presence or absence of functional endothelium was verified in all aortic rings by observing whether relaxation occurred on exposure to ACh (10^{−5} mol l^{−1}) or not. The endothelium was considered intact if the aortic ring relaxed more than 80% to ACh, while endothelial denudation was confirmed by less than 10% relaxation. At the end of experiments, aortic rings were allowed to dry for at least 24 h and then were weighed. Vasoconstrictor responses to PHE are expressed as gram (g) of tension per milligram (mg) of tissue. Vasodilator concentration–response curves were obtained in the aortic rings pre-contracted with PHE (~10^{−6} mol l^{−1}), which is approximately 80% of maximal contraction. Relaxation induced by ACh and SNP was expressed as the percentage of the tonus obtained with PHE.

The role of NO and superoxide anion in the response to PHE and ACh was evaluated by incubating some aortic rings with the non-selective NOS inhibitor L-N^G-nitro-L-arginine methyl ester (L-NAME; 100 μ mol l^{−1}), the superoxide anion scavenger superoxide dismutase (SOD; 150 U ml^{−1}) or the inhibitor of NADPH oxidase, apocynin (100 μ mol l^{−1}), added 30 min before the construction of the concentration–response curves to PHE or ACh.

For each concentration–response curve to PHE and ACh, the maximal response ($R_{\max}$) and the agonist concentration log resulting in 50% of the $R_{\max}$ (log EC₅₀) were calculated using non-linear regression analysis (GraphPad Prism software, USA).

Reactive oxygen species measurement in aorta

Thoracic aortas were carefully dissected out and cleaned of connective tissue. Aortas were then divided into cylindrical segments 4 mm in length and were first immersed in an embedding medium (tissue freezing medium) and then frozen and kept at −80°C until superoxide anion was measured. The oxidative fluorescent dye hydroethidine was used to evaluate the *in situ* production of reactive oxygen species (ROS) as previously described (Hernanz *et al.* 2004). Transverse aortic sections (10 μ m) were obtained in a cryostat from previously frozen aorta and collected on glass slides and left to reach equilibrium for 30 min at 37°C in phosphate-buffered saline (PBS). Fresh buffer containing hydroethidine (5 μ mol l^{−1}) was topically applied to each tissue section and coverslipped. Slides were incubated in a light-protected, humidified chamber at 37°C for 30 min. Control sections received the same volume of PBS. Images were obtained with a Nikon E1000 microscope equipped for epifluorescence (excitation at 488 nm; emission at 610 nm). Fluorescence was detected with a 585 nm long-pass filter. The fluorescence intensity was quantified using ImageJ software (NIH) and was expressed as arbitrary units.

Western blot analysis

For analysis of eNOS, ser1177 phosphorylated eNOS, SOD and NADPH oxidase protein expression, aortas were dissected out, cleaned of connective tissue, frozen in liquid nitrogen and kept at −70°C until the day of analysis. They were homogenized in RIPA lysis buffer containing protease inhibitor cocktail (1:5000 dilution), sodium fluoride (100 mM), sodium pyrophosphate (10 mM), sodium orthovanadate (100 mM), and PMSF (10 mM). Proteins from homogenized aorta (75 μ g of protein extracts) were electrophoretically separated by 7.5% or 12% SDS-PAGE and then transferred to polyvinylidene

Table 1. Body weight gain, retroperitoneal fat pad and uterus weight from ovariectomized (OVX) or sham-operated (SHAM) rats treated with vehicle or DHEA for 3 weeks

	SHAM	SHAM + DHEA	OVX	OVX + DHEA
Body weight gain (g)	47.8 ± 3.0	49.2 ± 2.6	83.3 ± 4.3*	79.3 ± 4.5*
Retroperitoneal fat pad (mg (100 g BW) ⁻¹)	0.5 ± 0.04	0.6 ± 0.06	1.1 ± 0.09*	1.1 ± 0.10*
Uterus weight (mg)	613 ± 103	594 ± 50	176 ± 14*	178 ± 24*

The values are expressed as means ± SEM. Two-way ANOVA: *P < 0.01 in comparison to SHAM and SHAM+DHEA.

Table 2. Changes in systolic (SBP) and diastolic blood pressure (DBP) and in heart rate from ovariectomized (OVX) or sham-operated (SHAM) rats treated with vehicle or DHEA for 3 weeks

	SHAM	SHAM + DHEA	OVX	OVX + DHEA
SBP (mmHg)	129.2 ± 2.5	130.5 ± 2.6	139.6 ± 1.8*	128.4 ± 2.7‡
DBP (mmHg)	91.5 ± 1.0	90.2 ± 2.6	100.2 ± 2.3*	94.0 ± 1.6‡
Heart rate (bpm)	356.8 ± 9.1	365.2 ± 14.6	384.0 ± 11.9	354.5 ± 10.0

The values are expressed as mean ± SEM. Two-way ANOVA: *P < 0.05 in comparison to SHAM and ‡*P* < 0.05 in comparison to OVX.

difluoride membranes overnight at 4°C using a Mini Trans-Blot Cell system (Bio-Rad) containing 25 mM Tris, 190 mM glycine, 20% methanol and 0.05% SDS.

After blockade of non-specific sites with 5% non-fat dry milk, membranes were incubated overnight at 4°C with the following primary antibodies: anti-eNOS (1:1000, BD Transduction Laboratories, Lexington, KY, USA), anti-phospho eNOS^{ser1177} (1:1000, Cell Signaling Technology, Inc., Danvers, MA, USA), anti-Cu/Zn SOD (1:2000, Sigma-Aldrich, Germany), anti-Mn SOD (1:2000, Sigma-Aldrich), anti-gp91phox (1:1000, Upstate Biotechnology, Inc., Lake Placid, NY, USA) and anti-p22phox (1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After washing (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20), membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:1500, Bio-Rad Laboratories, Hercules, CA, USA) for gp91phox, p22phox, Mn-SOD and phospho eNOS, and with anti-mouse IgG (1:1500, Bio-Rad) for Cu/Zn-SOD and eNOS. The same membranes were used to determine α -actin protein expression using a mouse monoclonal antibody (1:1500, Sigma-Aldrich). Membranes were thoroughly washed, and immune complexes were detected using an enhanced luminol chemiluminescence system (ECLPlus, Amersham GE Healthcare, UK) and subjected to MF-Chemibis-Bio-Imaging System (BioAmerica Inc., Miami, FL, USA). Signals on the immunoblot were quantified with Scion Image software.

Total expression of the proteins is given as the ratio between the optical density for the specific protein and the signal for α -actin protein expression.

Drugs

Dehydroepiandrosterone, phenylephrine hydrochloride, acetylcholine hydrochloride, sodium nitroprusside, SOD (bovine erythrocyte), L-NAME and apocynin were purchased from Sigma-Aldrich (USA); stock solutions (10 mmol l⁻¹) were prepared in distilled water, except for DHEA, which was suspended in soybean oil (10 mg ml⁻¹).

Statistical analysis

Results were expressed as means ± SEM, and *n* represents the numbers of animals used in each experiment. All results were analysed by two-way ANOVA followed by the Bonferroni *post hoc* test. A value of *P* < 0.05 was considered significant.

Results

Biometrics and haemodynamic parameters

OVX rats displayed elevated body weight gain (SHAM: 47.8 ± 3.0 vs. OVX: 83.3 ± 4.3 g; *n* = 10, *P* < 0.01) and retroperitoneal fat pad (SHAM: 0.5 ± 0.04 vs. OVX: 1.1 ± 0.09 mg (100 g BW)⁻¹; *n* = 10, *P* < 0.01) (Table 1). However, they presented reduced uterus weight (SHAM: 613 ± 103 vs. OVX: 176 ± 14 mg; *n* = 10, *P* < 0.01) as compared to SHAM rats (Table 1). DHEA treatment did not modify these parameters; body weight gain (SHAM+DHEA: 49.2 ± 2.6 and OVX+DHEA: 79.3 ± 4.5 g; *n* = 10), retroperitoneal fat pad (SHAM+DHEA: 0.6 ± 0.06 and OVX+DHEA: 1.1 ± 0.10 mg (100 g BW)⁻¹; *n* = 10) and uterus

weight (SHAM+DHEA: 594 ± 50 and OVX+DHEA: 178 ± 24 mg; $n = 10$), compared to respective groups treated with vehicle (Table 1).

Table 2 shows that OVX rats presented higher systolic and diastolic blood pressure as compared to SHAM rats, while heart rate was not different in different groups. DHEA treatment corrected the systolic and diastolic blood pressure in OVX rats (Table 2). However, it did not change the blood pressure levels in SHAM rats as well as heart rate in both groups (Table 2).

Vascular reactivity

The contraction induced by PHE was increased in aorta from OVX rats compared with SHAM (Fig. 1A) ($R_{\max}$: SHAM 0.84 ± 0.03 vs. OVX 1.27 ± 0.07 g of tension per mg of tissue; $n = 11$, $P < 0.01$). On the other hand, the endothelium-dependent relaxation induced by ACh was reduced in aorta from OVX rats (Fig. 1B) ($R_{\max}$: SHAM 91.6 ± 2.7 vs. OVX $74.1 \pm 2.7\%$ of relaxation; $n = 9$, $P < 0.01$), while there was no change in the relaxation response to the NO donor SNP (Fig. 1C). DHEA treatment in OVX rats corrected both the increased PHE-induced contraction (Fig. 1A) ($R_{\max}$: OVX+DHEA 0.90 ± 0.04 g of tension per mg of tissue; $n = 11$) and the impaired ACh-induced relaxation (Fig. 1B) ($R_{\max}$: OVX+DHEA $90.3 \pm 2.4\%$ of relaxation; $n = 9$); while DHEA treatment in SHAM rats did not change either PHE-induced contraction (Fig. 1A) ($R_{\max}$: SHAM+DHEA 0.86 ± 0.06 g of tension per mg of tissue; $n = 11$) or ACh-induced relaxation (Fig. 1B) ($R_{\max}$: SHAM+DHEA $95.7 \pm 3.7\%$ of relaxation; $n = 9$). There were no changes in EC_{50} values among groups for each drug studied (data not shown).

Effects of DHEA treatment on the endothelial and NO modulation of phenylephrine-induced contraction in OVX rats

Endothelium and NO modulation of PHE-induced contraction were evaluated by endothelium denudation or L-NAME incubation, respectively. An increase in $R_{\max}$ to PHE was observed in all groups of animals after endothelium removal (Table 3) or L-NAME incubation (Table 3). It is important to note that in the absence of endothelial cells or in the presence of nitric oxide inhibitor (L-NAME) there were no differences among groups. EC_{50} values were similar in all conditions (data not shown).

Effects of DHEA treatment on the role of superoxide anion in the phenylephrine-induced contraction in OVX rats

To evaluate the possible role of superoxide anion modulation, derived from NADPH oxidase, on PHE-induced contraction, SOD and apocynin were

incubated in some aortic rings 30 min before the concentration–response curves to PHE. Both SOD and apocynin significantly reduced the $R_{\max}$ to PHE in aorta from OVX rats, whereas they did not have any effects in

Figure 1. Vascular reactivity to phenylephrine, acetylcholine and sodium nitroprusside

Concentration–response curve to phenylephrine (A), acetylcholine (B) and sodium nitroprusside (C) in endothelium-intact aortic rings from SHAM, OVX and DHEA-treated rats. Results are expressed as means $\pm$ SEM. Two-way ANOVA: * $P < 0.01$ in comparison to SHAM ($n = 9$ –13).

Table 3. Effect of endothelium denudation (E⁻), N^G-nitro-L-arginine methyl ester (L-NAME), SOD and apocynin (Apo) on the maximal response (R_{max}) to phenylephrine

	SHAM	SHAM + DHEA	OVX	OVX + DHEA
E ⁺	0.84 ± 0.03	0.86 ± 0.06	1.27 ± 0.07*	0.90 ± 0.04‡
E ⁻	2.69 ± 0.17‡	2.71 ± 0.25‡	2.54 ± 0.10‡	2.66 ± 0.16‡
L-NAME	2.53 ± 0.13‡	2.37 ± 0.07‡	2.33 ± 0.11‡	2.42 ± 0.10‡
SOD	0.84 ± 0.05	0.80 ± 0.02	0.90 ± 0.02‡	0.84 ± 0.04
Apo	0.85 ± 0.09	0.74 ± 0.09	0.88 ± 0.11‡	0.82 ± 0.07

The values are expressed as mean ± SEM (g of tension per mg of tissue). Two-way ANOVA: *P < 0.01 in comparison to SHAM, †*P* < 0.01 in comparison to OVX, ‡*P* < 0.01 in comparison to E⁺ rings (endothelium intact).

Table 4. Effect of SOD and apocynin (Apo) on the maximal response (R_{max}) to acetylcholine

	SHAM	SHAM + DHEA	OVX	OVX + DHEA
E ⁺	91.6 ± 2.7	95.7 ± 3.7	74.1 ± 2.7*	90.3 ± 2.4†
SOD	93.4 ± 2.5	95.7 ± 1.6	96.5 ± 2.2‡	94.9 ± 1.6
Apo	92.5 ± 3.9	94.4 ± 1.6	93.4 ± 1.7‡	90.5 ± 2.3

The values are expressed as mean ± SEM (% of relaxation). Two-way ANOVA: *P < 0.01 in comparison to SHAM, †*P* < 0.01 in comparison to OVX, ‡*P* < 0.01 in comparison to E⁺ rings (endothelium intact).

aorta from SHAM and DHEA-treated rats (Table 3). There were no differences among groups in EC₅₀ in any situation (data not shown).

Effects of DHEA treatment on the role of superoxide anion in the acetylcholine-induced relaxation in OVX rats

The involvement of superoxide anion in ACh-induced relaxation was also evaluated. Both SOD and apocynin significantly enhanced the R_{max} to ACh in aortic rings from OVX rats, whereas they did not have any effect in aorta from SHAM and DHEA-treated rats (Table 4). In addition, there were no differences among groups in EC₅₀ to ACh in the presence of SOD or apocynin (data not shown).

Reactive oxygen species production

In order to evaluate tissue reactive oxygen species (ROS) production, dihydroethidium (DHE) staining was performed in aorta. As observed in Fig. 2 dihydroethidium-emitted fluorescence intensity was higher in aorta from OVX rats than in SHAM, indicating increased ROS production (Fig. 2). Treatment with DHEA for 3 weeks did not change ROS production in aorta

from SHAM rats, although it corrected the enhanced ROS production in aorta from OVX rats (Fig. 2).

Western blot analysis of endothelial nitric oxide synthase, superoxide dismutase and subunits of NADPH oxidase

Ovariectomy or DHEA treatment did not change endothelial nitric oxide synthase (eNOS) protein expression in aorta (Fig. 3A). However, in aorta from OVX rats there was observed a reduction on eNOS phosphorylation compared to SHAM (Fig. 3B). Furthermore, DHEA treatment in OVX rats corrected this reduction (Fig. 3B).

In addition, Cu/Zn-SOD protein expression was reduced in aorta from OVX rats and corrected by DHEA treatment (Fig. 4A), while there was no change in Mn-SOD protein expression among groups (Fig. 4B). Besides reduction of Cu/Zn-SOD protein expression, the aorta from OVX rats displayed an increased protein expression of gp91phox and p22phox (Fig. 5) NADPH oxidase subunits. Moreover, DHEA treatment of OVX rats corrected the increased of both NADPH oxidase subunits in aorta (Fig. 5).

Discussion

It has been shown that oestrogens have protective effects on the vascular system (White, 2002), based on epidemiological, clinical and molecular findings. In line with this affirmation, the loss of oestrogens may have profound effects on the cardiovascular system, with increase in blood pressure and endothelial dysfunction, both in women (Virdis *et al.* 2000; Mercuro *et al.* 2004) and in experimental models of menopause (Wassmann *et al.* 2001; Ito *et al.* 2006). In the present study we demonstrated that DHEA treatment reduces blood pressure and protects against oxidative stress-induced endothelial dysfunction in ovariectomized rats.

The favourable biological effects of oestrogen in the cardiovascular system have been recognized, in spite of the fact that several randomized clinical trials have failed to observe a benefit of hormone replacement therapy on the incidence of cardiovascular events in postmenopausal women (Hulley *et al.* 1998; Rossouw *et al.* 2002; Prentice *et al.* 2009). On the other hand, oestrogen replacement therapy corrected, in ovariectomized women, endothelial dysfunction (Virdis *et al.* 2000) and increased blood pressure (Mercurio *et al.* 2004). It is well known that ovariectomized rats display endothelial dysfunction, showing reduction in vascular response to ACh and increase in vascular response to PHE (Wassmann *et al.* 2001; Wong *et al.* 2006). Furthermore, Mendonça *et al.* (2007) found increased blood pressure after 11 weeks of ovariectomy in female Wistar rats. Interestingly, oestrogen seems to be important not only for females, since aromatase knockout male mice displayed reduced vascular response to ACh (Kimura *et al.* 2003). In line with those observations, our study shows an enhancement in blood pressure, increased vascular contraction to PHE and reduced endothelium-dependent relaxation to ACh following ovariectomy in rats.

Supporting the hypothesis of the present study, 3 weeks of DHEA treatment reduced the systolic and diastolic blood pressure, and corrected vascular responses to PHE and ACh in ovariectomized rats. The plasma DHEA level reduces during the ageing process and this has been related to the prevalence of body weight gain, obesity and insulin resistance, and mortality by cardiovascular disease (Barrett-Connor *et al.* 1986; Nestler *et al.* 1988;

Schriock *et al.* 1988). Both aged men and aged women submitted to DHEA treatment for 6 months displayed visceral and subcutaneous fat reduction, besides increased insulin sensitivity (Villareal & Holloszy, 2004). Moreover, in a double-blind study, DHEA-treated postmenopausal women for 12 weeks showed an enhancement of endothelial function (Williams *et al.* 2004). Considering that the adrenal steroid DHEA is the unique sexual steroid hormone source in postmenopausal women (Labrie, 2010) and the risks associated with ORT (Prentice *et al.* 2009), the data presented here concerning the mechanism of DHEA action suggest that DHEA could be used as a successful alternative hormone replacement therapy for postmenopausal women.

Indeed, some studies using animal models demonstrated that DHEA treatment induces several beneficial effects. It may reduce cardiac fibrosis in diabetic rats (Aragno *et al.* 2008), delay atherosclerotic plaque formation in rabbits (Hayashi *et al.* 2000), reduce weight gain and fat deposition in rats (Han *et al.* 1998), increase insulin sensitivity (Campbell *et al.* 2004) and insulin secretion in rats (Medina *et al.* 2006), and reduce blood pressure in ovariectomized rats (Bhuiyan & Fukunaga, 2010). In addition, DHEA increases *in vitro* endothelial proliferation (Williams *et al.* 2004), and protects endothelial cells against apoptosis (Liu *et al.* 2007), both effects independently of oestrogen receptors. Besides, the present study has for the first time shown that DHEA treatment *in vivo* corrects both contractile and relaxation responses to PHE and ACh in OVX rats. Therefore, DHEA has several effects on the cardiovascular system, which

Figure 2. Reactive oxygen species in aorta

Representative fluorescence photomicrographs of microscopic sections of thoracic aorta from SHAM, OVX and DHEA-treated rats. Vessels were labelled with the oxidative dye hydroethidine, which produces a red fluorescence when oxidized to ethidium bromide by superoxide anion. Quantitative analysis for fluorescence photomicrographs of microscopic sections of thoracic aorta from SHAM, OVX and DHEA-treated rats. Results are expressed as means $\pm$ SEM. Two-way ANOVA: * $P < 0.01$ in comparison to SHAM and # $P < 0.01$ in comparison to OVX ($n = 7$).

could be direct or indirect, which reduce cardiovascular risk factors such as body weight gain or insulin resistance.

The present results suggest that the correction of increase in PHE-induced contraction and decrease in ACh-induced relaxation observed in aortas from ovariectomized rats by DHEA treatment in part are related

to an increment in eNOS phosphorylation, improving NO production, since the eNOS phosphorylation on ser-1177 enhances the NO production by eNOS (Dimmeler *et al.* 1999). In line with the present results obtained in aorta, Bhuiyan & Fukunaga (2010) showed a decrease in eNOS phosphorylation in kidney from ovariectomized Wistar rats. Moreover, Widder *et al.* (2003) demonstrated

Figure 3. eNOS protein expression and phosphorylation in aorta

Representative Western blots (top) and quantitative analysis (bottom) for total eNOS protein expression (A) and ser1177 eNOS phosphorylation (B) in thoracic aorta from SHAM, OVX and DHEA-treated rats. Results are expressed as means ± SEM. Two-way ANOVA: * $P < 0.01$ in comparison to SHAM and # $P < 0.01$ in comparison to OVX ($n = 9$).

Figure 4. Cu/Zn-SOD and Mn-SOD protein expression in aorta

Representative Western blots (top) and quantitative analysis (bottom) for Cu/Zn-SOD (A) and Mn-SOD (B) protein expression in thoracic aorta from SHAM, OVX and DHEA-treated rats. Results are expressed as means ± SEM. Two-way ANOVA: * $P < 0.01$ in comparison to SHAM and # $P < 0.01$ in comparison to OVX ($n = 8$).

that ovariectomy is related to a reduction of eNOS protein expression in aorta from SHR rats, which was not observed in our study using aorta from Wistar rats. Recently, consistent with our results, Bhuiyan & Fukunaga (2010) also showed that DHEA enhances eNOS phosphorylation in kidney from ovariectomized rats, leading to blood pressure reduction. In addition, there are some *in vitro* studies which have demonstrated that DHEA directly stimulates eNOS phosphorylation in endothelial cells (Liu & Dillon, 2004; Formoso *et al.* 2006). Furthermore, our results exclude a possible role of DHEA treatment on impaired ability of vascular smooth muscle to relax in response to exogenous NO donor, since the endothelium-independent relaxation induced by SNP was similar in all groups and was not changed by DHEA treatment.

Besides the reduction of NO production by eNOS, the increased superoxide anion in vascular cells provokes a decline in NO bioavailability, leading also to endothelial dysfunction (Cai & Harrison, 2000). The main source of superoxide anion in the vascular system is the enzyme NADPH oxidase (Cai & Harrison, 2000). Similar to other studies (Wassmann *et al.* 2001; Tsuda *et al.* 2005), here we observed that beyond decrease in eNOS phosphorylation, oxidative stress is also involved in the reduced NO bioavailability in aorta from OVX rats. Therefore, endothelial dysfunction in OVX rats is due to both reduced eNOS phosphorylation and oxidative stress. In fact, PHE and ACh response of aortic rings from OVX rats were also corrected by scavenging superoxide anion and inhibiting NADPH oxidase. Reinforcing our results, aorta from OVX rats displayed an increase in ROS production. Oxidative stress-induced endothelial dysfunction by oestrogen withdrawal has already been shown by several studies. Viridis *et al.* (2000) observed that endothelial dysfunction resulting from acute oestrogen deprivation in women was caused by oxidative stress. In SHR OVX rats reduction in relaxation response to carbachol in aortic rings associated with increased superoxide anion production was demonstrated (Wassmann *et al.* 2001). Moreover, oestrogen protects female OVX mice against increase in ROS production in aorta, and angiotensin II-induced intracellular ROS production in vascular smooth muscle (Strehlow *et al.* 2003).

Several studies have shown a potential antioxidant effect of DHEA. The present study also demonstrates a role of DHEA in reducing superoxide anion content in aorta from OVX rats. This effect may represent the mechanism by which DHEA treatment corrects the vascular response to PHE and ACh in aorta from OVX rats. Yorek *et al.* (2002) observed that DHEA reduced plasma thiobarbituric acid-reactive substances (TBARS) and superoxide anion production in arterioles from diabetic rats, and Aragno *et al.* (2006) also demonstrated reduction in ROS content in hearts from diabetic rats

treated with DHEA. It seems that DHEA has an antioxidant effect that is not only in the cardiovascular system, since it reduced oxidative stress-induced skeletal muscle damage in diabetic rats (Aragno *et al.* 2004). Furthermore, oxidative stress parameters in plasma and in peripheral blood mononuclear cells in diabetic subjects were significantly decreased by DHEA treatment (Brignardello *et al.* 2007). Besides, *in vitro* studies have shown direct

Figure 5. NADPH oxidase subunits protein expression in aorta Representative Western blots (top) and quantitative analysis (bottom) for gp91phox (A) and p22phox (B) protein expression in thoracic aorta from SHAM, OVX and DHEA-treated rats. Results are expressed as means $\pm$ SEM. Two-way ANOVA: * $P < 0.01$ in comparison to SHAM and # $P < 0.01$ in comparison to OVX ($n = 8$).

effects of DHEA on oxidative stress parameters. DHEA reduced ROS production and NF- κ B activation in endothelial cells treated with TNF- α (Gutiérrez *et al.* 2007), and protected endothelial cells against apoptosis (Liu *et al.* 2007) and stimulated their proliferation (Williams *et al.* 2004).

As discussed above and demonstrated in the present study, menopause and ovariectomy induce oxidative stress, mainly in the cardiovascular system. One reason for this outcome could be the modulation of the activity or expression of antioxidant enzymes. Tsuda *et al.* (2005) demonstrated that aorta from female mice displayed less NADPH oxidase activity and expression than aorta from male mice, while ovariectomy showed an increase in NADPH oxidase activity and expression in females. This outcome has an important role in endothelial dysfunction observed in OVX rats, since NADPH oxidase is the main source of superoxide anion associated with endothelial dysfunction (Cai & Harrison, 2000). In addition, antioxidant systems are also depressed by oestrogen withdrawal, which contributes to oxidative stress-induced endothelial dysfunction in OVX rats. Oestrogen deficiency decreased SOD mRNA expression in aorta from OVX mice, which was related to an increase in the presence of the superoxide anion, and oestrogen replacement therapy prevented this downregulation (Strehlow *et al.* 2003).

In our study we also observed a modulation of SOD and NADPH oxidase protein expression following ovariectomy, displaying reduced Cu/Zn-SOD protein expression and increased gp91phox and p22phox (subunits of NADPH oxidase) protein expression, which would explain the oxidative stress-induced endothelial dysfunction. Besides, we observed a correction of Cu/Zn-SOD downregulation and gp91phox and p22phox increment by DHEA treatment, which could be the main result of DHEA treatment, leading to reduced ROS production and consequently enhanced vascular function in OVX rats. Besides the vascular antioxidant effect by modulating expression of enzymes involved in oxidant/antioxidant activity, the present work shows that DHEA treatment also enhances SOD activity in aorta from aged rats (Wu *et al.* 2007), prevents oxidative injury in obstructive jaundice in rats by increasing SOD activity in liver (Celebi *et al.* 2004), and also raises SOD activity in liver from diabetic rats (Aragno *et al.* 1999). However, the present study is the first to demonstrate that DHEA treatment can modulate Cu/Zn-SOD, gp91phox and p22phox (NADPH oxidase) protein expression.

In summary, this study confirms the pivotal role of oxidative stress-induced endothelial dysfunction in OVX rats. Furthermore, it indicates the potential role of DHEA therapy, instead of oestrogen replacement therapy, as an antioxidant, improving endothelial function,

reducing systolic and diastolic blood pressure, and modulating eNOS phosphorylation, and Cu/Zn-SOD, gp91phox and p22phox protein expression in aorta from OVX rats.

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Author contributions

J.P.G.C., E.H.A. and C.R.O.C. contributed to the conception and design of the experiments. J.P.G.C. performed and analysed most of the experiments in this study, with technical assistance from E.H.A., A.P.D., C.R.F. and L.V.R. All authors analysed and interpreted data. J.P.G.C., L.V.R. and C.R.O.C. wrote the article. Each of the authors approved the final version of the manuscript. There were no conflicts of interest.

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Influence of the Hepatic Eukaryotic Initiation Factor 2 α (eIF2 α) Endoplasmic Reticulum (ER) Stress Response Pathway on Insulin-mediated ER Stress and Hepatic and Peripheral Glucose Metabolism^{*[5]}

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Recent studies have implicated endoplasmic reticulum (ER) stress in insulin resistance associated with caloric excess. In mice placed on a 3-day high fat diet, we find augmented eIF2 α signaling, together with hepatic lipid accumulation and insulin resistance. To clarify the role of the liver ER stress-dependent phospho-eIF2 α (eIF2 α -P) pathway in response to acute caloric excess on liver and muscle glucose and lipid metabolism, we studied transgenic mice in which the hepatic ER stress-dependent eIF2 α -P pathway was inhibited by overexpressing a constitutively active C-terminal fragment of GADD34/PPP1R15a, a regulatory subunit of phosphatase that terminates ER stress signaling by phospho-eIF2 α . Inhibition of the eIF2 α -P signaling in liver led to a decrease in hepatic glucose production in the basal and clamped state, which could be attributed to reduced gluconeogenic gene expression, resulting in reduced basal plasma glucose concentrations. Surprisingly, hepatic eIF2 α inhibition also impaired insulin-stimulated muscle and adipose tissue insulin sensitivity. This latter effect could be attributed at least in part by an increase in circulating IGFBP-3 levels in the transgenic animals. In addition, infusion of insulin during a hyperinsulinemic-euglycemic clamp induced conspicuous ER stress in the 3-day high fat diet-fed mice, which was aggravated through continuous dephosphorylation of eIF2 α . Together, these data imply that the hepatic ER stress eIF2 α signaling pathway affects hepatic glucose production without altering hepatic insulin sensitivity. Moreover, hepatic ER stress-dependent eIF2 α -P signal-

ing is implicated in an unanticipated cross-talk between the liver and peripheral organs to influence insulin sensitivity, probably via IGFBP-3. Finally, eIF2 α is crucial for proper resolution of insulin-induced ER stress.

Recent studies suggest that endoplasmic reticulum (ER)³ stress links hepatic lipid accumulation, as seen in conditions such as obesity, to hepatic insulin resistance (1–3). The ER folds unfolded polypeptide chains into proteins with distinct functions. Accumulation of unfolded proteins in the ER activates at least three transmembrane signal transducers, namely inositol requiring protein-1, activating transcription factor-6, and protein kinase RNA-like ER kinase (PERK) (4–6). In concert, ER effector signaling constitutes the unfolded protein response (UPR). Disequilibrium between the unfolded protein load entering the ER and the actual ER capacity to properly fold these proteins causes ER stress, which ultimately leads to a cell suicide signal mediated by the pro-apoptotic factor C/EBP homologous protein. Prior to this event, a negative feedback loop consisting of eIF2 α and activating transcription factor-4, inducing C/EBP homologous protein, is initiated. C/EBP homologous protein increases transcription of the phosphatase GADD34, which dephosphorylates and inactivates eIF2 α , which partially blocks PERK-mediated signaling (3–5).

Activation of ER stress markers, and by inference ER stress levels, is increased in livers and adipose tissue, but not skeletal muscle, of obese humans and mice (7, 8). Levels of ER stress markers are further modulated by feeding, fatty acids, glucose, cytokines, and adipokines (3, 5, 9). Moreover, the PI3K complex has recently been shown to mediate the translocation of X-box binding protein 1 (XBP-1) to the nucleus (9).

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³ The abbreviations used are: ER, endoplasmic reticulum; PERK, protein kinase RNA-like ER kinase; eIF2 α -P, phospho-eIF2 α ; HFD, high fat diet; UPR, unfolded protein response; GC, C-terminal fragment of Gadd34; Alb, albumin promoter; Mops, 4-morpholinepropanesulfonic acid; GRP78/Bip, glucose responsive protein 78; XBPs, spliced XBP-1.

Genetic and pharmacological manipulations affecting the level of ER stress and the strength of the response to it affect diverse aspects of intermediary metabolism, e.g. ectopic fat accumulation and glucose homeostasis (5–9, 11). The liver maintains one of the highest protein synthesis rates in the body, with the majority of these peptides being processed by the ER (10), and it would therefore be expected to be prone to the metabolic changes induced by ER stress. However, the details of the relationship between ER stress and hepatic glucose and lipid metabolism and the causal links between them remain poorly understood. Therefore, to gain further understanding of the problem defined above, we took a reductionist approach and sought to clarify the role of the metabolic consequences of selective enfeeblement of the eIF2 α strand of the UPR in the liver.

Previous work had shown that it is possible to selectively promote eIF2 α dephosphorylation in the liver by an active C-terminal fragment of Gadd34 (GC), which is under the control of the albumin promoter (Alb:GC). This transgene continuously suppresses eIF2 α -mediated signaling by dephosphorylation at the serine 51 residue (11). This focused physiological perturbation led to protection from HFD-mediated liver steatosis and impaired glycogen accumulation in the fed state. These alterations correlated with changes in expression of genes encoding key enzymes in lipid and glucose metabolism in the liver and revealed the importance of the eIF2 α -P strand of the UPR to hepatic intermediary metabolism. However, the broader physiological impact of this perturbation was not assessed.

Here we undertook such an assessment and subjected Alb:GC Tg mice and control mice to a short term high fat diet, which has previously been shown to exclusively induce hepatic lipid accumulation and hepatic insulin resistance, without resulting in differences in body weight in both rodents (12) and humans (13).

EXPERIMENTAL PROCEDURES

Generation of Mice—The mice were generated as described (11) on a FvB/n background, and only Tg mice or WT littermates were used for these studies. All of the breeding was carried out at the New York University Animal Facilities and the Yale University Animal Facilities according to institutional regulations and the National Institutes of Health Guide for the Use and Care of Laboratory Animals.

Basal Study—The mice were housed at the Yale University Animal Facility under controlled temperature ($22 \pm 2^\circ\text{C}$) and lighting (12 h of light, 7 a.m. to 7 p.m.; 12 h of dark, 7 p.m. to 7 a.m.) with free access to water and food. Alb:GC Tg mice and littermate control mice were fed a regular chow diet (TD2018; Harlan Teklad, Madison, WI). Twelve-week-old mice were fed a high fat diet (HFD, 55% fat by calories; TD 93075; Harlan Teklad), which was withheld 14 h prior to the experiments. Fat and lean body masses were assessed by ^1H magnetic resonance spectroscopy (Bruker BioSpin, Billerica, MA). Comprehensive animal metabolic monitoring system (CLAMS; Columbus Instruments, Columbus, OH) was used as described (14, 15) to evaluate activity, food consumption, and energy expenditure. Energy expenditure and respiratory quotient were calculated

from the gas exchange data [energy expenditure = $(3.815 + 1.232 \times \text{respiratory quotient}) \times \text{VO}_2$], with respiratory quotient determined as the ratio of VCO_2 to VO_2 . Activity was measured in both horizontal and vertical directions using infrared beams to count the beam breaks during a specified period. Feeding was measured by recording the difference in the scale measurement of the center feeder from one time point to another. Drinking was assessed by a computed system counting consumed water droplets.

Hyperinsulinemic-Euglycemic Clamp Studies—Hyperinsulinemic-euglycemic clamp studies were conducted as described (16, 17). In brief, at 7 a.m. after an overnight fast, the mice were continuously infused with $0.05 \mu\text{Ci}/\text{min}$ [$3\text{-}^3\text{H}$]glucose over 2 h to assess basal glucose turnover. Hyperinsulinemic-euglycemic clamp studies were then conducted for 140 min with a primed continuous infusion of human insulin (21 milliunits/kg prime over 3 min, 3 milliunits/kg/min of infusion; Novo Nordisk) and a variable infusion of 20% dextrose to maintain euglycemia (120 mg/dl). $0.1 \mu\text{Ci}/\text{min}$ [$3\text{-}^3\text{H}$]glucose was continuously infused to determine insulin-stimulated glucose uptake and endogenous glucose production after the basal period. $10\text{-}\mu\text{Ci}$ bolus of 2-deoxy-d-[$1\text{-}^{14}\text{C}$]glucose (PerkinElmer Life Sciences) was injected after 85 min to estimate the insulin-stimulated tissue glucose uptake. Samples were taken at 0 and 135 min for plasma fatty acid and insulin concentrations. At study completion, the mice were anesthetized, and tissues were harvested within 3 min with liquid N_2 -cooled aluminum tongs and stored at -80°C for subsequent analysis.

Biochemical Analysis and Calculations—Plasma glucose was analyzed by the glucose oxidase method on a Beckman analyzer (Beckman Coulter, Inc., CA). Plasma insulin was measured by RIA using kits from Linco (Millipore). Plasma fatty acid concentrations were determined using an acyl-CoA oxidase-based colorimetric kit (Wako Chemicals USA, Inc.). Serum IGFBP-3 concentrations were measured in duplicates using ELISAs (Mediagnost GmbH, Reutlingen, Germany). The intra- and interassay coefficients of variation were 2.0–5.9% and 2.4–8.4%, respectively. Plasma [^3H]glucose was measured by scintillation counting of $\text{ZnSO}_4/\text{Ba}(\text{OH})_2$ deproteinized serum, dried to remove $^3\text{H}_2\text{O}$. Basal and insulin-stimulated whole body glucose turnover rates = ($[\text{3-}^3\text{H}]\text{glucose infusion (in dpm)}/(\text{plasma glucose specific activity (dpm per mg)})$) at the end of the basal period and during the final 30 min of the clamp, respectively. Hepatic glucose production = (rate of total glucose appearance) – (glucose infusion rate). Plasma concentration of $^3\text{H}_2\text{O}$ = ^3H counts [wet] – [dry]. Whole body glycolysis was estimated by the rate of increase in plasma $^3\text{H}_2\text{O}$ concentration by linear regression of measurements at 90, 100, 110, 120, 130, and 140 min. Tissue [^{14}C]2-deoxyglucose-6-phosphate content was measured following sample homogenizing, and the supernatant's [^{14}C]2-deoxyglucose-6-phosphate was separated from 2-deoxyglucose by ion exchange column as previously described (16, 17).

Tissue Lipid Measurements—Tissue triglyceride was extracted using the method of Bligh and Dyer (18) and measured using a DCL triglyceride reagent (Diagnostic Chemicals Ltd).

AKT2 Activity—AKT2 activity was assessed in protein extracts from muscle harvested after hyperinsulinemic-euglycemic clamp study using methods previously described (12, 19).

mRNA Quantification by Real Time PCR—Liver total RNA was isolated using the RNeasy kit per manufacturer's instructions (Invitrogen). cDNA was synthesized from 2 μ g of total RNA and random hexamers StrataScript reverse transcriptase (Stratagene). The abundance of transcripts was assessed by real time PCR on an Opticon 2 (Bio-Rad) with a SYBR Green detection system (Stratagene Corporation). For each run, samples were run in duplicate for both the gene of interest and actin and/or GAPDH. The expression data for each gene of interest and actin and GAPDH were normalized for the efficiency of amplification, as determined by a standard curve included on each run. Primer sequences are displayed in the [supplemental Table S2](#).

Immunoblots—Liver was ground with a mortar and pestle and mixed with 1 ml of lysis buffer (50 mM Tris-HCl buffer, pH 7.5, at 4 °C), 50 mM NaF, 5 mM NaPPi, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM benzamide, 1 mM PMSF, glycerol (10% v/v), Triton X-100 (1% v/v), 1 μ M TSA, and 50 mM nicotinamide) and homogenized for 30 s. Homogenates were spun at $20,800 \times g$ for 10 min at 4 °C, and protein concentrations were determined. SDS gel electrophoresis was performed by using precast Bis-Tris 4–12% gradient polyacrylamide gels in the Mops buffer system (Invitrogen). After transfer to nitrocellulose membranes, membranes were incubated in blocking buffer (5% milk) for 1 h and immunoblotted with GRP78 (Cell Signaling Technology) or β -actin or GAPDH (Cell Signaling Technology, MA) as indicated. After the incubation with the primary antibody, the membranes were washed three times for 15 min with TBS (10 mM Tris-HCl, pH 7.4, 0.5 M NaCl) plus Tween 20 (0.2% v/v) (TBST). The membranes were immersed in blocking buffer and a corresponding IgG-conjugated secondary antibody and were incubated for 2 h. The membranes were then washed three times for 5 min using TBST. Proteins were then detected with enhanced chemiluminescence (Thermo Fisher Scientific Inc.), and autoradiographs were quantified by using densitometry (Image J) (20).

Cell Culture of Primary Mouse Hepatocytes—Primary hepatocytes were isolated by the Yale Liver Center by collagenase digestion. The cells were washed three times with recovery medium, and equal amount of cells (10^6 cells) were incubated at 37 °C in GlutaMAX^{TM} medium supplemented with insulin, FBS, dexamethasone, and penicillin and streptomycin. After 4 h, the medium was changed to DMEM low glucose medium, and the cells were incubated overnight. Then the cells were washed twice with Hanks' balanced salt solution at 37 °C. Half of the wells were pretreated with 100 nM wortmannin. After 30 min, the cells were incubated with or without 100 nM insulin for 4 h. Then cells were washed and harvested for further mRNA extraction.

Cytokine Array—Mouse cytokine array (RayBio) was conducted as described by the manufacturer. Briefly, the membranes were incubated with blocking buffer at room temperature, and all of the bubbles were removed. After 30 min, blocking buffer was decanted, and 1 ml of diluted sample (with blocking buffer) was applied to membranes and incubated

FIGURE 1. Effect of a 3-day HFD on hepatic triglyceride content, hepatic insulin responsiveness, and markers of hepatic ER stress in WT mice. A, liver triglyceride content was increased by 20% in 3-day HFD-fed mice (3d HFD) compared with regular chow (RC)-fed mice. B, 3-day HFD-fed mice had hepatic insulin resistance compared with regular chow-fed mice, as reflected by increased hepatic glucose production during the hyperinsulinemic-euglycemic clamp studies ($n = 3$). C, phosphorylation of eIF2 α was increased with 3 days of HFD, indicating increased PERK signaling ($n = 5$). D, mRNA expression of ER stress markers: GRP78, XBP1s, and CHOP were not induced in 3-day HFD mice compared with regular chow-fed mice despite increased hepatic triglyceride content and hepatic insulin resistance. $n = 3$ –5.

overnight at 4 °C. Then samples were washed three times with 2 ml of wash buffer at room temperature with gentle shaking for at least 5 min/wash. 1 ml of diluted biotin conjugated antibody was added to each membrane, and membranes were then incubated overnight at 4 °C. After washing, 2 ml of 1,000-fold diluted HRP-conjugated streptavidin was added to each membrane, and the membranes were incubated overnight at 4 °C and then washed. For detection, 250 μ l of each detection buffer was added to the membrane and mixed. The membranes were then positioned protein side up on a clean plastic sheet, and mixed detection buffer was pipetted onto the membrane and incubated at room temp for 2 min. All of the air bubbles were removed. After 2 min, the membranes were exposed to x-ray film.

Statistical Analysis—The data are expressed as means $\pm$ S.E. The results were assessed using two-tailed unpaired Student's t test or one-way analysis of variance. A p value less than 0.05 was considered significant.

RESULTS

Three Days of High Fat Feeding Induces Hepatic Lipid Accumulation, Insulin Resistance, and eIF2 α Signaling—To better characterize the influence of ER stress on the development of lipid induced hepatic insulin resistance, we used a dietary model of 3 days of high fat feeding (12). Body weights, weight gain, and body composition after 3 days of high fat feeding or regular chow diet did not differ ([supplemental Fig. S1, A and B](#)) between the groups. Hepatic triglyceride concentrations were increased by $\sim$ 20% in the 3-day HFD group (Fig. 1A). Basal

TABLE 1

Body composition, metabolic cage, and biochemistry

Parameter	WT	Alb:GC Tg	P value
Body weight (gram)	31 ± 0.4	30 ± 0.7	0.6
Weight gain (gram)	0.3 ± 0.1	1.3 ± 0.4	0.4
Body fat (%)	18.4 ± 0.8	20.2 ± 1	0.2
Lean mass (%)	68.8 ± 1.2	67.8 ± 1.1	0.5
V _{O2} [ml/(kg·h)]	3280 ± 90	3190 ± 110	0.5
V _{CO2} [ml/(kg·h)]	2920 ± 60	2810 ± 100	0.4
Respiratory quotient (V _{CO2} /V _{O2})	0.89 ± 0.01	0.88 ± 0.01	0.6
Energy expenditure [KCal/(kg·h)]	16 ± 0.4	15.5 ± 0.5	0.4
Feeding [KCal/(kg·h)]	19.0 ± 0.2	19.2 ± 0.5	0.9
Drinking [ml/(kg·h)]	0.5 ± 0.03	0.6 ± 0.04	0.4
Activity (counts/h)	40 ± 4	32 ± 2	0.1
Fasted plasma fatty acids (mEq/ml)	1.1 ± 0.1	1.1 ± 0.1	0.6
Clamp plasma fatty acids (mEq/ml)	0.6 ± 0.1	0.7 ± 0.1	0.8
Plasma triglycerides (mg/dl)	140 ± 10	130 ± 10	0.6
Muscle triglycerides (mg/g)	8.4 ± 2	6.2 ± 1	0.2

insulin concentrations were 15 ± 2 and 45 ± 7 microunits/ml in the regular chow and the 3-day HFD group, respectively ($p < 0.01$). During the hyperinsulinemic-euglycemic clamp, insulin concentrations were similar between groups, and hepatic glucose production was nearly 2-fold higher in the high fat-fed mice (Fig. 1B) compared with the regular chow fed mice. Concomitantly, 3 days of HFD led to a 140% induction of eIF2 α signaling as evidenced by enhanced phosphorylation of eIF2 α (Fig. 1C). mRNA expression of key hepatic ER stress makers spliced XBP-1, GRP78, and CHOP (Fig. 1D) did not change measurably. These data identify eIF2 α activation as one of the early events in lipid-induced hepatic ER stress. Therefore, we next examined the contribution of eIF2 α in the early development of hepatic insulin resistance.

Blocking of the Hepatic eIF2 α -mediated ER Stress Pathway in Vivo Decreases Hepatic Glucose Production—We first examined the influence of the hepatic eIF2 α pathway on energy metabolism *in vivo* using a model with silencing of eIF2 α signaling specifically in liver (Alb:GC Tg mice). Alb:GC Tg and littermate control mice were fed a high fat diet for 3 days. Body weight and weight gain were similar between groups (Table 1 and Fig. 2A). The proportions of whole body fat and whole body lean mass compared with body weight were also similar between groups (Table 1). Oxygen consumption (V_{O2}), carbon dioxide production (V_{CO2}), energy expenditure, respiratory quotient, caloric and water intake, and locomotor activity did not differ between groups (Table 1). Thus, blocking of the hepatic eIF2 α -mediated pathway on a moderate level did not interfere with body weight regulation, whole body energy expenditure, or caloric intake.

To examine whether the attenuation of hepatic eIF2 α signaling would protect Alb:GC Tg mice from lipid-induced hepatic insulin resistance, we performed hyperinsulinemic-euglycemic clamp studies. Basal plasma glucose concentrations were reduced by 16% in the Alb:GC Tg mice (Fig. 2B) compared with wild type littermate control mice. Basal and clamped plasma insulin concentrations did not differ between groups (Fig. 2C). Basal and insulin suppressed endogenous glucose production were reduced by 23 and 16%, respectively, in the Alb:GC Tg mice (Fig. 2, D and E, and supplemental Table S1), indicating that eIF2 α signaling mainly affects hepatic glucose production without additional effect on hepatic insulin sensitivity. In line with this finding, expression of gluconeogenic gene transcrip-

FIGURE 2. Fasting- and insulin-stimulated hepatic and peripheral glucose metabolism during the hyperinsulinemic-euglycemic clamp in Alb:GC Tg mice and WT littermate control mice (also see supplemental Table S1). A, body weights of WT and the Alb:GC Tg mice after 3 days of HFD ($n = 8$ /group). B, fasting plasma glucose concentrations were reduced in the Alb:GC transgenic mice compared with the WT mice ($n = 5$ –6/group). C, basal and clamp insulin concentrations in Alb:GC Tg mice and WT mice ($n = 6$ –8/group). D, basal rates of hepatic glucose production were lower in the Alb:GC Tg mice compared with the WT mice ($n = 6$ –8/group). E, during the hyperinsulinemic-euglycemic clamp, endogenous glucose production was not further reduced by insulin in the Alb:GC Tg mice, indicating no measurable effect of eIF2 α on hepatic insulin sensitivity. F, C/EBP transcription factors and PEPCK mRNA expressions were reduced in the Alb:GC Tg mice compared with the WT mice ($n = 6$ –8/group). *, $p < 0.05$.

tion factors CCAAT enhancer-binding protein (C/EBP) transcription factors C/EBP α and C/EBP β (Fig. 2F) and cytosolic PEPCK (Fig. 2F) were reduced in the Alb:GC Tg mice. These data demonstrate that reducing signaling through the hepatic eIF2 α -mediated pathway reduces hepatic glucose production through suppression of key gluconeogenic genes without affecting hepatic insulin sensitivity during the clamp.

Blocking the Hepatic eIF2 α -mediated ER Stress Signaling Pathway Induces Peripheral Insulin Resistance—Surprisingly, Alb:GC Tg mice required an $\sim 40\%$ lower glucose infusion rate to maintain euglycemia during the hyperinsulinemic-euglycemic clamp (Fig. 3, A and B, and supplemental Table S1), indicating reduced whole body insulin sensitivity despite lower hepatic glucose production rates (Fig. 2, D and E). Insulin-stimulated peripheral glucose uptake was reduced by 22% in the Alb:GC Tg mice, indicating increased peripheral insulin resistance (Fig. 3C). These data demonstrate differential regulation of hepatic and peripheral insulin sensitivity in mice following liver-specific attenuation of eIF2 α -P signaling and provide a first indication that modulation of hepatic ER stress signaling pathways leads to a cross-talk between the liver and periphery.

Blocking of the Hepatic eIF2 α -mediated ER Stress Pathway Induces Insulin Resistance in Skeletal Muscle and White Adi-

FIGURE 3. Hepatic eIF2 α signaling affects peripheral insulin sensitivity. A, plasma glucose concentrations in Alb:GC Tg mice and WT mice during the hyperinsulinemic-euglycemic clamp ($n = 6-8$ /group). B, glucose infusion rates (GINF) tended to be lower in the Alb:GC Tg mice compared with the WT mice during the hyperinsulinemic-euglycemic clamp ($n = 6-8$ /group). C, insulin-stimulated peripheral glucose uptake was decreased in the Alb:GC Tg mice compared with the WT mice ($n = 6-8$ /group).

pose Tissue—We next addressed the question of which peripheral insulin-sensitive organs are responsible for the insulin resistance in the Alb:GC Tg mice. Glucose uptake into the mixed fiber type gastrocnemius muscle was reduced by 37% in the Alb:GC Tg mice (Fig. 4A), and into white adipose tissue, glucose uptake was reduced by 25% (Fig. 4B). Accordingly, insulin-stimulated AKT2 activity in gastrocnemius muscle of Alb:GC Tg mice was decreased by 37% (Fig. 4C).

Because the GADD34 active fragment encoding transgene is expressed selectively in the liver, we considered that the transgene's effect on peripheral insulin sensitivity and glucose metabolism might be mediated by factors secreted from the liver. Insulin-like growth factors and their binding proteins are synthesized and secreted by the liver, can cause peripheral insulin resistance (21), and have previously been shown to be regulated by the PERK/eIF2 α pathway (22). Hepatic mRNA expression of IGFBP-3, the prevailing IGF-binding protein, was increased by 25% ($p < 0.05$). Accordingly, plasma IGFBP-3, which has been shown to mediate peripheral insulin resistance independently of IGF-1 (23), was also increased by 30% in the Alb:GC Tg mice (Fig. 4D). IGFBP-3 has been shown to facilitate

FIGURE 4. Insulin-stimulated glucose uptake in muscle and white adipose tissue, insulin-stimulated AKT2 activity in muscle, and plasma IGFBP-3 levels in Alb:GC Tg and WT littermate control mice. A, insulin-stimulated 2-deoxyglucose (2-DOG) uptake by the gastrocnemius muscle was decreased in Alb:GC Tg mice compared with the WT mice at the end of the hyperinsulinemic-euglycemic clamp ($n = 6-8$ /group). B, insulin-stimulated 2-deoxyglucose uptake by white adipose tissue (WAT) was reduced in Alb:GC Tg mice compared with the WT mice at the end of the hyperinsulinemic-euglycemic clamp ($n = 6$ /group). C, insulin-stimulated Akt2 activity in gastrocnemius muscle was reduced in Alb:GC Tg mice compared with WT mice at the end of the hyperinsulinemic-euglycemic clamp ($n = 5$ /group). D, plasma IGFBP-3 concentrations were increased in the Alb:GC Tg mice ($n = 6$ /group). E, Smad-2 phosphorylation was increased in skeletal muscle of Alb:GC Tg mice ($n = 5$).

the phosphorylation of the TGF- β receptor regulated Smad-2 (24, 25). Consistent with the potential role of increased circulating levels of IGFBP-3 promoting peripheral insulin resistance, we found that Smad-2 phosphorylation was induced 2-fold in skeletal muscle of the Alb:GC Tg mice (Fig. 4E). We also measured other factors, which might cause peripheral insulin resistance, such as plasma fatty acids, triglycerides, and muscle triglyceride concentrations, which did not differ between wild type and Alb:GC Tg mice (Table 1). Cytokine arrays also did not show genotype-specific differences in levels of plasma cytokines, (except for platelet factor 4, which is not known to have a metabolic function (supplemental Fig. S2)). Taken together, these data demonstrate that modulation of the eIF2 α arm of the UPR pathway in the liver can promote peripheral insulin resistance, which at least in part, may be due to

FIGURE 5. Effect of blocking the hepatic eIF2 α signaling pathway on markers of hepatic ER stress before and at the end of the hyperinsulinemic-euglycemic (HE) clamp. A, hepatic expression of XBP1 mRNA was increased following the hyperinsulinemic-euglycemic clamp in Alb:GC Tg mice compared with WT littermate mice. B, hepatic expression of CHOP mRNA was increased in the Alb:GC Tg mice compared with WT littermate mice following the hyperinsulinemic-euglycemic clamp in Alb:GC Tg mice compared with WT littermate mice. C, hepatic protein expression of GRP78 was increased following the hyperinsulinemic-euglycemic clamp in Alb:GC Tg mice (Tg) compared with WT littermate mice (WT) ($n = 6$ /group).

increased expression, secretion, and signaling of IGFBP-3 in muscle.

Blocking the Hepatic eIF2 α -mediated ER Stress Signaling Pathway Renders Mice Hypersensitive to Insulin-induced ER Stress *in Vivo*—It has recently been suggested that the eIF2 α arm of the UPR attenuates aspects of XBP1 signaling in response to sustained insulin action (9). To determine the role of eIF2 α signaling in this aspect directly *in vivo*, we compared hyperinsulinemic-euglycemic clamped mice with attenuated eIF2 α signaling to clamped control wild type mice. Basal mRNA expression levels of ER stress markers GRP78, XBP1, and CHOP were similar in livers from Alb:GC Tg mice and wild type littermate control mice after 3 days of HFD (Fig. 5, A and B; GRP78/BiP not shown). During insulin stimulation, mRNA expression of ER stress markers XBP1 (Fig. 5A), CHOP (Fig. 5B), and protein expression of GRP78 (Fig. 5C) in Alb:GC Tg mice livers were markedly increased compared with wild type littermate control mice.

Insulin Signaling Elevates ER Stress Markers through PI3K *In Vitro* and *In Vivo*—To determine whether insulin *per se* could activate these pathways *in vivo*, we examined these pathways in mice under basal and hyperinsulinemic-euglycemic clamped conditions. Hyperinsulinemia resulted in a 2–3-fold increase in the mRNA expression of the ER stress markers XBP1 and CHOP (Fig. 6A) and a 2-fold increase in the protein expression of GRP78 (Fig. 6B) compared with unclamped control mice. To further examine how insulin induces ER stress, primary mouse hepatocytes were incubated with insulin. Hepatocytes re-

FIGURE 6. Insulin induces hepatic ER stress *in vitro* and *in vivo*. Primary hepatocytes were incubated with control medium or medium supplemented with 100 nM insulin for 4 h with or without pretreatment with the PI3K inhibitor wortmannin. A, insulin infusion during hyperinsulinemic-euglycemic clamp induces hepatic mRNA expression of XBP1 and CHOP *in vivo* ($n = 8$ /group). B, insulin infusion during the hyperinsulinemic-euglycemic clamp induces hepatic GRP78 protein expression ($n = 8$ /group). Lanes c, clamp; lanes b, basal. C, insulin induces mRNA expression of CHOP in primary hepatocytes. The PI3K inhibitor wortmannin (Wortm.) inhibits the insulin-mediated induction of CHOP ($n = 3$ /group). D, insulin induces mRNA expression of XBP1 in primary hepatocytes. The PI3K inhibitor wortmannin inhibits the insulin-mediated induction of XBP1 ($n = 3$ /group).

sponded with a 2-fold increase expression in CHOP (Fig. 6C) and an ~30% increase in expression of XBP1 (Fig. 6D) compared with untreated primary hepatocytes. Blocking of the insulin-signaling cascade with the PI3K inhibitor wortmannin completely abrogated insulin-induced expression of CHOP and XBP1 (Fig. 6, C and D). Thus, insulin induces ER stress through a PI3K-dependent mechanism. Taken together with our observation that the hepatic steatosis and hepatic insulin resistance occur without these transcriptional changes after 3 days of high fat feeding, these data suggest that hepatic ER stress induced by caloric excess is at least in part mediated by hyperinsulinemia through PI3K and that reduced eIF2 α signaling, as observed in obesity (9), further aggravates insulin-induced hepatic ER stress.

DISCUSSION

The development of insulin resistance in obesity is characterized by both ectopic lipid accumulation and activation of markers of hepatic ER stress. These observations have led to the hypothesis that ER stress may play a causal role in hepatic insulin resistance.

We show that ectopic lipid accumulation in the liver, induced by short term high fat feeding, causes selective hepatic insulin resistance with activation of the ER stress pathway involving eIF2 α -P. The major novel finding in our study is that selective inhibition of the eIF2 α -P signaling in the liver does not improve hepatic insulin sensitivity but leads to reduced fasting

plasma glucose concentrations caused by reductions in hepatic glucose production by gluconeogenic gene expression and that this liver specific transgene induces a cross-talk between liver and peripheral glucose metabolism.

The unanticipated peripheral insulin resistance in skeletal muscle and white adipose tissue is indicative of the cross-talk between the liver and the periphery mediated by hepatic ER stress signaling pathways. This finding shows for the first time that hepatic ER stress pathways can control peripheral insulin-stimulated glucose metabolism. This observation adds a novel concept to the assembly of mechanisms proposed to link hepatic ER stress to metabolic regulation. In accordance with reduced peripheral insulin sensitivity, we found decreased insulin signaling, as highlighted by reduced insulin-stimulated AKT2 activity, in gastrocnemius muscle. The fact that insulin-stimulated glucose uptake was reduced in both skeletal muscle and white adipose tissue to a similar degree suggests that a liver-derived circulating factor generated in the Alb:GC Tg mice might be responsible for these effects. Plasma cytokine and lipid levels, as well as intramuscular lipid content, did not show corresponding changes, but hepatic IGFBP-3 mRNA expression and circulating levels of IGFBP-3 were increased in the Alb:GC Tg mice. IGFBP-3 has been shown to cause decreased insulin-stimulated muscle and white adipose tissue glucose uptake (23), and IGFBP-3 has been reported to induce Smad-2 signaling (24, 25), which was also present in Alb:GC Tg skeletal muscle. It is therefore possible that increased hepatic production of IGFBP-3 results in increased plasma concentrations of IGFBP-3, which induces peripheral insulin resistance in conditions of hepatic silencing of eIF2 α -P. More studies are needed to decipher the mechanism by which the ER stress response is connected to IGFBP-3.

Previous studies revealed reduced fasting glucose concentrations and improved glucose tolerance, as shown with an intraperitoneal glucose tolerance test in mice with abrogate hepatic eIF2 α signaling on a long term HFD (11). Therefore, our study was undertaken to examine how specific organs contribute to changes in glucose and lipid metabolism in these mice. For this reason, we assessed tissue-specific rates of insulin-stimulated glucose metabolism using the hyperinsulinemic-euglycemic clamp combined with radiolabeled glucose tracers. Our finding of reduced overall insulin sensitivity in the Alb:GC Tg mice differs from the previous findings in long-term HFD fed Alb:GC Tg mice, which were shown to have improved glucose tolerance compared with WT controls, as assessed by an IPGTT (11). These disparate observations might be explained by the fact that short term high fat feeding exclusively affects hepatic insulin sensitivity (12), whereas long term high fat feeding also leads to skeletal muscle insulin resistance caused by ectopic lipid accumulation in skeletal muscle. The chronic ectopic lipid effect in skeletal muscle might be a stronger inducer of insulin resistance than the protection from insulin resistance through reduced IGFBP-3 concentration, as observed in our 3-day HFD-fed WT mice.

To determine the initial events in the insulin resistance and ectopic lipid accumulation that occur with caloric excess, we used a model of short term high fat diet (12, 13). Dietary fat has been involved in the induction of the eIF2 α pathway at least in

part by the activation of double stranded RNA-dependent protein kinase (26). Our data indicate that a 3-day fat-based caloric excess is sufficient to induce eIF2 α , along with hepatic lipid accumulation and insulin resistance. However, our data mainly demonstrate a role of eIF2 α in hepatic glucose production through induction of gluconeogenic gene expression without a direct effect on insulin sensitivity during insulin administration. In contrast, we found that insulin concentrations in the pathophysiological range induce markers of unresolved ER stress (CHOP) *in vivo* and *in vitro* in a PI3K-dependent fashion, which is aggravated with reduced eIF2 α signaling. Insulin signaling with the regulatory subunits of PI3K, p85 α and p85 β , is involved in the nuclear translocation of XPB and, thus, the proper resolution of induced ER stress (9, 27). In insulin-resistant states, insulin-mediated translocation of XPB appears to be blunted, ER stress cannot be properly resolved, and the eIF2 α pathway gets progressively activated (9). In our mice, eIF2 α signaling was partly abrogated, and therefore, the induction of ER stress through insulin led to an exaggerated, compensatory response through the remaining ER stress arms. Together, these data suggest that once insulin resistance develops, high insulin concentrations might further aggravate ER stress and contribute to the metabolic deterioration in this setting (28).

Continuous activation of eIF2 α in conditions with increased insulin conditions, such as insulin resistance, might lead to activation of C/EBP β , as shown in our and other mice studies (11), which increases on the one hand hepatic glucose production and on the other hand hepatic *de novo* lipogenesis.

The findings also have potential clinical implications for patients with Wolcott-Rallison syndrome, which is caused by mutations in the *EIF2AK3* gene. This gene encodes PERK, and mutations in the *EIF2AK3* gene reduce its ability to transduce ER stress signaling via eIF2 α , similar to what occurs in the Alb:GC Tg mice. Patients with Wolcott-Rallison syndrome have a rare form of neonatal diabetes, with impaired β -cell function (29, 30). Given our findings of increased peripheral insulin resistance in Alb:GC Tg mice, it is possible that increased peripheral insulin resistance may also be a contributing factor to the development of neonatal diabetes in these patients.

In summary, we show that reducing the hepatic eIF2 α signaling pathway leads to reduced hepatic glucose production through reduced hepatic gluconeogenic gene expression. Moreover, we show that modulating hepatic ER stress pathways induces a cross-talk between the liver and the periphery to modulate peripheral glucose homeostasis, mediated at least in part by increased hepatic production of IGFBP-3. This finding adds a novel concept to the assembly of mechanisms proposed to link hepatic ER stress to metabolic regulation.

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Supplementary Figure 1

S1: A) Body weight after 3 days of HFD and B) Weight gain after 3 days of either regular mouse chow or 3 days of HFD

Supplementary Figure 2

S2: Plasma cytokine array from WT and Alb:GC Tg mice. Platelet factor 4 (PF4) is reduced in Alb:GC Tg mice. PF4 is not known to have metabolic functions. Red boxes also show IL6 and TNF α , two cytokines, which have been implied in metabolic regulation

Cellular Mechanism by which Estradiol Protects Mice From

Diet-Induced Hepatic and Muscle Insulin Resistance

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Abstract

Metabolic diseases are less frequent in premenopausal women than in age-matched men or postmenopausal women. Moreover, estrogen replacement therapy reduces incidence of type 2 diabetes in postmenopausal women. The aim of this study was to evaluate the metabolic effects of estrogen replacement therapy in an experimental model of menopause. At 8 weeks of age, female mice were ovariectomized (OVX) or sham (SHAM) operated. OVX mice were treated with vehicle or estradiol. All mice were fed with high fat diet. OVX displayed reduced whole-body energy expenditure. Also, OVX mice had impaired glucose tolerance and whole-body insulin resistance. E2 treated mice displayed increased hepatic and muscular insulin sensitivity, while SHAM mice displayed only increased muscular insulin sensitivity compared to OVX mice. Diacylglycerol (DAG) was higher in skeletal muscle from OVX mice, while in liver E2 treatment reduced the DAG accumulation. The insulin resistance and DAG content were associated with increased PKC ϵ activation in liver of SHAM and OVX mice, and PKC θ activation in skeletal muscle of OVX mice. The present results suggest that E2 plays a pivotal role on whole-body energy homeostasis, increasing oxygen consumption and energy expenditure, leading to increased whole-body insulin sensitivity in OVX mice.

Key words: insulin resistance, estrogen, obesity, diacylglycerol, and PKC.

Introduction

The estrogen loss during the menopause in women is associated with increased visceral adipose tissue and, in turn, related metabolic diseases, such as insulin resistance, diabetes, and cardiovascular disease. The beneficial effects of estrogens on insulin action and glucose homeostasis are supported by studies showing that insulin sensitivity is higher in premenopausal women compared with age-matched men, and metabolic-related cardiovascular diseases and type 2 diabetes are less frequent in those women (Nuutila et al. 1995; Donahue et al. 1997). Also, estrogen deficiency leads to increased fat mass and body weight in postmenopausal women, associated with increased intra-abdominal fat (Lovejoy et al. 2008). This increased fat mass observed in menopausal women could be explained by energy expenditure, since menopausal transition leads to decreased energy expenditure in women. Interestingly, menopausal women also displayed a reduced fat oxidation and spontaneous physical activity (Lovejoy et al. 2008). Finally, it is possible to observe that the estrogen replacement therapy in postmenopausal women reduces diabetes incidence in these women (Margolis et al. 2004).

Experimental studies have also shown that estrogen plays an important role on metabolic homeostasis. Increased body weight associated with increased fat mass is observed in both ovariectomized mice (Rogers et al. 2009) and rats (Camporez et al. 2011). Also, Aromatase knockout (ArKO) mice, both female and male, display increased body weight, fat mass, and adipocyte hypertrophy compared with wild type mice, showing the importance of estrogen lack on fat accumulation (Jones et al. 2000).

The physiological effects of estrogen are mediated by two classical receptors, estrogen receptor alpha (ER α) and beta (ER β). Estrogen binds to its receptor, inducing

conformational changes, which allow recruitment of coactivator or corepressor molecules, and binding to estrogen response element (ERE) on DNA to regulate target genes. Besides, it has been described that some estrogen effects are membrane dependent and occur through a G protein-coupled membrane receptor, which, in turn, cannot be explained by nuclear mechanism mentioned above. Estrogen binds to G protein-coupled receptor 30 (GPR30), a G protein-coupled membrane receptor presented on membranes of different cells, which mediates the rapid non-genomic estrogen effects (Carmeci et al. 1997).

Some studies suggest that estrogen effects on metabolic homeostasis are mediated by ER α . Since, ER α knockout (ER α KO) mice, both female and male, display increased body weight gain and fat mass associated with impaired glucose tolerance (Heine et al. 2000), and *in vivo* stimulation of ER α in ovariectomized rats increases insulin-stimulated skeletal muscle glucose uptake (Gorres et al. 2011). On the other hand, ER β knockout (ER β KO) mice do not display that metabolic phenotype (Foryst-Ludwig et al. 2008).

Taken together, in both humans and rodents, estrogen seems to play a pivotal role on the control of body weight and fat mass, and, in turn, glucose homeostasis. Thus, the aim of the present work was to evaluate the metabolic effects of estrogen replacement therapy in an experimental model of estrogen deprivation (ovariectomized mice). We observed that estrogen treatment increased energy expenditure, hepatic and muscular insulin sensitivity in those mice. Also, those effects were associated with reduced ectopic lipids accumulation and novel PKC activation in both liver and skeletal muscle.

Methods

Animal care and protocol

All experimental procedures were approved by and conducted in accordance with the Institutional Animal Care and Use Committee guidelines of Yale University School of Medicine. At 8 weeks of age, female mice were anaesthetized with a ketamine-xylazine-acepromazine mixture (64.9, 3.2 and 0.78 mg kg⁻¹, respectively, I.P.) and were ovariectomized (OVX) or sham-operated (SHAM). To study the effect of chronic estradiol (E2) administration, OVX mice were *s.c.* implanted with pellets releasing either placebo or E2 (0.1 mg for 60 days, i.e. 70µg/kg/d; Innovative Research of America, Sarasota, FL). During all procedures mice were in a constant room temperature environment, with 12:12 h light:dark cycle, with free access to water and high fat diet (60% fat, 20% carbohydrate, 20% protein, D12492, Research Diets, NJ, USA). Mice were, then, randomly divided into three groups: sham-operated (SHAM), ovariectomized (OVX), and ovariectomized treated with E2 (OVX+E2).

Basal studies

After 4 weeks of surgery, under high fat diet feeding, the experiments were done. Fat and lean body masses were assessed by ¹H-magnetic resonance spectroscopy (Bruker BioSpin, Billerica, MA). Comprehensive animal metabolic monitoring system (CLAMS; Columbus Instruments, Columbus, OH) was used to evaluate O₂ consumption, CO₂ production, energy expenditure, activity, and food consumption. Drinking was assessed by a computer system counting consumed water droplets.

In vivo glucose homeostasis

Glucose tolerance tests were performed after an overnight fast. Mice were injected intraperitoneally with 1 mg/kg glucose, and blood was collected by tail bleed at set times (0, 15, 30, 45, 60, 90, 120 min) for plasma insulin and glucose measurements. Hyperinsulinemic-euglycemic clamps were performed as previously described (Jornayvaz et al. 2011). To do this, a jugular venous catheter was implanted 6 to 7 days before hyperinsulinemic-euglycemic clamps. To assess basal whole-body glucose turnover, [3-³H]-glucose (HPLC purified; Perkin- Elmer Life Sciences) was infused at a rate of 0.05 μ Ci/min for 120 min into the jugular catheter. Following the basal period, hyperinsulinemic-euglycemic clamps were conducted in conscious mice for 140 min with a 4 min primed (29 mU/kg) followed by a continuous [3 mU/(kg-min)] infusion of human insulin (Novolin; Novo Nordisk), a continuous infusion of [3-³H]-glucose (0.1 μ Ci/min), and a variable infusion of 20% dextrose to maintain euglycemia (100-120 mg/dL). 10 μ Ci bolus of 2-deoxy-d-[1-¹⁴C]glucose (PerkinElmer) was injected after 85 minutes to estimate the insulin-stimulated tissue glucose uptake. Plasma samples were obtained from the tip of the tail at 0, 30, 50, 65, 80, 90, 100, 110, 120, 130, and 140 min. The tail incision was made at least 2 hours before the first blood sample was taken to allow for acclimatization, according to standard operating procedures (Ayala et al. 2010). Also, mice received an i.v. albumin-containing solution mimicking artificial plasma during the insulin-stimulated period of the clamp to compensate for volume loss secondary to blood sampling. At the end of the clamps, mice were anesthetized with pentobarbital sodium injection (150 mg/kg) and all tissues were taken within 4 min, snap-frozen in liquid nitrogen, and stored at -80°C for subsequent analysis.

Liver and skeletal muscle lipids measurements

Tissue triglycerides (TG) were extracted using the method of Bligh and Dyer (Bligh et al. 1959) and measured using a DCL triglyceride reagent (Diagnostic Chemicals). For DAG extraction, livers were homogenized in a buffer solution (20 mM Tris-HCl, 1 mM EDTA, 0.25 mM EGTA, 250 mM sucrose, 2 mM PMSF) containing a protease inhibitor mixture (Roche), and samples were centrifuged at 100,000 g for 1 h. The supernatants containing the cytosolic fraction were collected. Diacylglycerol (DAG) levels were then measured as previously described (Yu et al. 2002). Total cytosolic DAG content is expressed as the sum of individual species. Ceramide was measured as previously described (Yu et al. 2002). All lipid measurements were done in animals under basal conditions following a 6-h fast.

Immuno-blotting analysis

Tissues were homogenized in RIPA lysis buffer. Proteins from homogenized liver, skeletal muscle, or brown adipose tissue (30µg of protein extracts) were electrophoretically separated by 4-12% SDS-PAGE (Invitrogen) and then transferred to polyvinylidene difluoride membranes (Millipore) using a semidry transfer cell (Bio- Rad) for 120 min. After blockade of non-specific sites with 5% non-fat dry milk, membranes were incubated overnight at 4°C with the following primary antibodies: phospho-AKT2^{ser473} (Signalway Antibody, USA), AKT2 (Cell Signaling Technology, Inc., Danvers, MA, USA), PKCε (BD Transduction Laboratories, Lexington, KY, USA), PKCθ (BD Transduction Laboratories, Lexington, KY, USA), or GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After washing (10 mM Tris, 100 mM NaCl,

and 0.1% Tween 20), membranes were incubated peroxidase-conjugated anti-rabbit, or anti-mouse, or anti-goat antibody. Membranes were thoroughly washed, and immune complexes were detected using an enhanced luminol chemiluminescence system (ECL; Thermo Scientific, USA) and subjected to photographic films. Signals on the immunoblot were quantified by optical densitometry (Scion Image Software, MD, USA).

Total RNA preparation, and Real-Time quantitative PCR Analysis

Total RNA was extracted from frozen livers using RNeasy 96-kit (Qiagen), then 1 µg of RNA was reverse transcribed into cDNA with the use of the Quantitect RT kit (Qiagen) as per manufacturer's protocol. The abundance of transcripts was assessed by real-time PCR on a 7500 Real-Time PCR system (Applied Biosystems) with a SYBR Green detection system. Samples were run in duplicate for both the gene of interest and GAPDH and data were normalized for the efficiency of amplification, as determined by a standard curve included on each run. Primers used are shown in Table S1.

Statistical Analysis

Data are expressed as means $\pm$ SEM. Results were assessed using one-way ANOVA (GraphPad; Prism 5) followed by Tukey's multiple comparison test, as appropriate. A *P* value less than 0.05 was considered significant.

Results

Biometrics and basal biochemistry parameters

OVX mice displayed elevated body weight compared to SHAM mice, after 4 weeks of surgery, even though they did not display any differences before the surgery (Table 1). Also, associated with increased body weight, OVX mice had an elevated fat mass (Table 1), showing that estrogen deprivation leads to obesity in rodents. E2 treatment in OVX mice corrected both increased body weight and fat mass (Table 1). There is no difference among the groups of mice in regard to basal glucose, basal insulin, plasma fatty acids, and plasma triglycerides (Table 1).

Metabolic parameters

Figure 1 (A - D) shows that OVX mice displayed reduced oxygen consumption and CO₂ production, in both light and dark hours. Associated with reduced oxygen consumption those mice also displayed reduced energy expenditure, independently of day time (Figure 1 F and H). OVX mice treated with E2 displayed similar phenotype to SHAM mice, correcting the reduced oxygen consumption and energy expenditure observed on OVX mice (Figure 1 A - G). Interesting, during the light hours OVX mice displayed similar activity compared to SHAM mice, while during the dark hours OVX mice are less active than SHAM mice (Figure 1 F and H). Those results suggests that the reduced energy expenditure observed in OVX mice is not, primary, due to reduced activity, since during the light hours the activity between SHAM and OVX is not different. Also interesting is that, although E2 treated mice displayed increased oxygen consumption and energy expenditure compared to OVX mice, they are less activity than OVX mice during the light hours, and there is no difference on activity between

OVX+E2 and OVX mice during the dark hours (Figure 1 F and H), showing that E2 treatment is able to enhance the oxidative metabolism in those mice.

Although OVX mice are obese compared to SHAM mice, they displayed reduced caloric intake corrected by body weight (Figure 1 I) when compared to both SHAM and E2 treated mice. It is also possible to observe that OVX displayed reduced drinking (Figure 1 J). Those results suggest a higher feeding efficiency of OVX mice. There is no difference among the groups when the results, both caloric intake and drinking, are expressed by absolute amount (data not shown).

Glucose tolerance and insulin sensitivity

OVX mice showed impaired glucose tolerance compared to SHAM mice (Figure 2 A), and E2 treatment corrected that. Also, OVX mice had higher plasmatic insulin levels during the IPGTT compared to both SHAM mice and E2 treated mice (Figure 2 B), suggesting that OVX mice are insulin resistance. During the hyperinsulinemic-euglycemic clamp OVX mice displayed decreased glucose infusion rate compared to SHAM and E2 treated mice (Figure 2 C and D), confirming that OVX mice are more insulin resistance. Insulin-stimulated whole body glucose uptake was also reduced in OVX mice (Figure 2 E). Interesting, endogenous E2 was not capable to protect SHAM mice against hepatic insulin resistance. Both SHAM and OVX mice displayed similar insulin-stimulated suppression of endogenous glucose production (Figure 2 F and G). On the other hand, suppression of endogenous glucose production by insulin was around 100% in E2 treated mice (Figure 2 F and G). The insulin-stimulated plasma fatty acid

suppression was lower in OVX mice compared to both SHAM and E2 treated mice (Figure 2 H).

Corroborating with reduced whole body glucose uptake observed in OVX mice, insulin-stimulated 2-deoxy-glucose uptake was lower in OVX mice, in both skeletal muscle and white adipose tissue, compared to SHAM and E2 treated mice (Figure 3 A and B), while there is no difference in brown adipose tissue (Figure 3 C).

Liver and skeletal muscle lipids content

It is possible to observe that E2 treatment in OVX mice reduced TG and DAG content in liver, while there is no in TG difference between SHAM and OVX mice (Figure 4 A). DAG is increased in OVX compared to SHAM (Figure 4 C). OVX mice displayed increased TG and DAG content in skeletal muscle compared to both SHAM and E2 treated mice (Figure 4 B and D). There is no difference in ceramides content in liver and muscle between the groups (Figure E and F).

Novel PKC activation and AKT2 phosphorylation

Associated with insulin resistance observed in clamp experiments and lipids content (DAG in particular), E2 treated mice displayed reduced PKC ϵ activation (translocation to membrane) in liver (Figure 5 A). Also, OVX mice showed increased PKC θ activation (translocation to membrane) in skeletal muscle compared to both SHAM and E2 treated mice (Figure 5 B). In line, insulin-stimulated AKT2 phosphorylation is increased in liver from E2 treated mice (Figure 6 A) and decreased in skeletal muscle from OVX mice (Figure 6 B), fitting the novel PKC data.

mRNA expression in liver and skeletal muscle

Due to increased lipids content in liver from SHAM and OVX mice, and skeletal muscle from OVX mice, it was evaluated mRNA expression of several protein related to lipids synthesis (Figure 7 A and B). E2 treatment decreased expression of several proteins which play a role on lipids synthesis, among them SREBP1c which is an important transcriptional factor that controls the lipids synthesis. Also, E2 treated mice displayed increased UCP-2 expression in liver (Figure 7 A). Interesting, OVX mice showed reduced expression of CEACAM1 in liver, which could explain the high plasmatic insulin concentration observed in those mice after clamp.

OVX mice displayed increased mRNA expression of SREBP1c, DGAT2, and PPAR γ in skeletal muscle compared to both SHAM and E2 treated mice (Figure 7 B).

Discussion

It has been shown that estrogen plays an important role on metabolic homeostasis (Barros and Gustafsson, 2011), based on epidemiological, clinical and molecular findings. In line with this affirmation, the loss of estrogen may have profound effects on glucose homeostasis and body composition in both menopause women (Lovejoy et al. 2008) and rodents (Jone et al. 2000; Rogers et al. 2009). In the presenting study, we showed that endogenous estrogen is important to protect against high fat diet-induced skeletal muscle insulin resistance, while E2 treatment in estrogen-deprived mice increased insulin sensitivity in both liver and skeletal muscle. Also, we showed that

estrogen is important for whole body oxygen consumption and ectopic lipids accumulation.

The favorable metabolic effects of estrogen have been recognized, since it is possible to observe increased body fat mass and visceral fat during menopausal transition in women (Lovejoy et al. 2008). The increased fat mass could be explained by the reduced energy expenditure in postmenopausal women (Lovejoy et al. 2008). Those observation in women fit with the present study, which shows that OVX mice displayed reduced whole-body oxygen consumption and energy expenditure, leading to increased body weight associated with increased body fat mass. The importance of estrogen on body weight and fat mass maintenance is also observed in transgenic mice. Both ER α knockout mice and aromatase knockout mice displayed increased body weight and fat mass in both male and female (Heine et al. 2000; Jones et al. 2000). Also, those results were associated with reduced energy expenditure in transgenic mice (Ribas et al. 2010). As observed in this work, Rogers et al. (2009) also observed reduced energy expenditure in OVX mice associated with increased fat mass. But, for the first time we showed that chronic E2 replacement therapy corrected the reduced energy expenditure and whole-body oxygen consumption. All those observation suggest that the lack of estrogen could play a pivotal role on reduced whole-body energy metabolism, which, in turn, would lead to increased fat mass and obesity. Those effects of E2 treatment on whole-body energy metabolism could be explained, at least partially, by central actions of E2. Recently, Xu et al. (2011) showed that the estrogen effects, through ER α , on metabolic homeostasis might be mediated by central nervous system (CNS), since ER α knockout mice

specifically in CNS displayed increased body weight and fat mass, and decreased energy expenditure.

The obesity observed in OVX mice was also associated with whole-body insulin resistance and impaired glucose tolerance. Riant et al. (2009) also observed impaired glucose tolerance and insulin resistance in OVX mice fed with HFD, and E2 replacement reverted these effects. However, we showed that endogenous estrogen protected female mice from HFD-induced insulin resistance only in skeletal muscle, displaying hepatic insulin resistance as well as OVX mice. The E2 treatment, on the other hand, was able to protect OVX mice from insulin resistance in both liver and skeletal muscle. This paradox could be explained by the plasmatic E2 concentration. Although the E2 concentration in plasma from OVX treated mice might be considered still physiologic (Bryzgalova et al. 2008; Rogers et al. 2009), those mice displayed a higher plasmatic E2 concentration compared to SHAM mice, which could lead to increased E2 action in liver, and then protect those mice from hepatic insulin resistance. Other point to be considered is the progesterone level, since SHAM mice must display high concentration of both E2 and progesterone produced in ovaries, while OVX mice display low concentration of both hormones, and OVX treated mice display high concentration of E2 and low concentration of progesterone, since the hormonal replacement therapy was done only with E2. This is a possible explanation, since Nelson et al. (1994) showed that progesterone administration in rats reduced the insulin-induced suppression of endogenous hepatic glucose production, but does not have any effects on insulin-mediated peripheral glucose utilization. Therefore, it is possible that the higher level of progesterone in SHAM mice prevented the beneficial E2 effects in liver as observed in OVX treated mice.

Moreover, the possible mechanism that led OVX mice to display insulin resistance under HFD is the ectopic lipid accumulation. OVX mice displayed increased TG and DAG content in skeletal muscle compared to SHAM and E2 treated mice. In liver, both SHAM and OVX mice showed higher TG and DAG content compared to E2 treated mice. It is well known that cytosolic DAG accumulation leads to novel PKC activation (PKC ϵ in liver and PKC θ in muscle) (Erion and Shulman, 2010), and it was also observed in the present study. Also, it has been shown that novel PKC activation, in both liver and skeletal muscle, is able to impair proximal insulin signaling (Erion and Shulman, 2010; Jornayvaz et al. 2011; Lee et al. 2010), leading to reduced AKT2 activation as observed in our study, and then insulin resistance. Several studies have been shown that DAG-novel PKCs pathway is directly related to insulin resistance. Jornayvaz et al. (2011) observed that increased DAG accumulation in mice overexpressing DGAT2 in liver led to severe hepatic insulin resistance. Corroborating with those observations, Choi et al. (2007) showed that reduced DGAT2 expression in liver by antisense oligonucleotide (ASO) treatment protected rats from HFD-induced hepatic insulin resistance. Therefore, it seems that cytosolic DAG accumulation-induced novel PKC activation plays a pivotal mechanistically role on insulin resistance in estrogen deprived mice.

Besides the reduced whole-body energy expenditure in OVX mice, which would explain the ectopic lipid accumulation associated with insulin resistance, the expression of enzymes and transcriptional factors related to lipids synthesis could also explain the increased TG and DAG content. Indeed, it has been shown that lipid synthesis proteins expression may be modulated by estrogen. Rogers et al. (2009) observed that OVX mice

displayed increased mRNA expression of PPAR γ , FAS, and ACC1 associated with histological evidence of hepatic steatosis. Also, it was shown that E2 treatment reduced expression of lipogenic enzymes in white adipose tissue from high fat fed mice (Bryzgalova et al., 2008). D'Eon et al. (2005) showed that E2 treatment decreased some lipogenic enzymes in white adipose tissue, liver, and skeletal muscle. We also observed similar effects of estrogen. OVX mice displayed increased expression of lipogenic enzyme in skeletal muscle compared to both SHAM and E2 treated mice, while E2 treatment reduced the expression of those enzymes in liver from OVX mice. Those data fit with TG and DAG content in both liver and muscle. Also, the increased expression of UCP-2 in liver of E2 treated mice could explain the reduced lipid content and higher hepatic insulin sensitivity observed in those mice.

In summary, we found that estrogen plays a pivotal role on whole-body energy homeostasis, increasing oxygen consumption and energy expenditure, leading to increased whole-body insulin sensitivity in OVX mice. Also, this study showed that estrogen is able to reduce the ectopic lipid accumulation, leading to reduced DAG content in both live and muscle, which, in turn, leads to reduced novel PKC activation and increased insulin sensitivity. Therefore, estrogen could be of crucial importance as an approach, in particular for postmenopausal women, for the treatment or prevention of type 2 diabetes and obesity.

Author contributions:

J.P.G.C. contributed to the conception and design of the experiments, performed and analyzed most of the experiments in this study, and wrote the manuscript. F.R.J.

contributed with technical assistance and discussion. S.K. contributed with technical assistance and discussion. H.Y.L. contributed with technical assistance and discussion. B.A.G. contributed with technical assistance. M.K. contributed with technical assistance. V.T.S. contributed to the conception and design of the experiments, discussion and technical expertise. C.R.O.C. contributed to the conception and design of the experiments, discussion and technical expertise. M.J.J. contributed to the conception and design of the experiments, discussion, technical assistance, technical expertise, and reviewed the manuscript. G.I.S. contributed to the conception and design of the experiments, discussion, technical expertise, and wrote the manuscript.

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No potential conflicts of interest relevant to this article were reported.

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Figure Legends

Figure 1 – OVX mice display reduced whole-body energy metabolism. A: Light hours of oxygen consumption. B: Light hours of CO₂ production. C: Dark hours of oxygen consumption. D: Dark hours of CO₂ production. E: Light hours of whole-body energy expenditure. F: Light hours of activity. G: Dark hours of whole-body energy expenditure. H: Dark hours of activity. I: 24 hours of caloric intake. J: 24 hours of drinking. Significance between groups was determined by one-way ANOVA. Data represent the mean $\pm$ SEM.

Figure 2 – OVX mice display impaired glucose and whole-body insulin resistance. A: Plasma glucose following intraperitoneal injection of glucose (1 mg/kg). B: Plasma insulin following intraperitoneal injection of glucose (1 mg/kg). C: Plasma glucose and glucose infusion rate (GIR) during the euglycemic-hyperinsulinemic clamp. D: Average of GIR during the last 40 minutes of euglycemic-hyperinsulinemic clamp. E: Insulin-stimulated whole-body glucose uptake during the euglycemic-hyperinsulinemic clamp. F: Basal endogenous glucose production. G: Insulin-stimulated EGP suppression during the euglycemic-hyperinsulinemic clamp. H: Insulin-stimulated plasma fatty acids suppression during the euglycemic-hyperinsulinemic clamp. Significance between groups was determined by one-way ANOVA. * $p < 0.05$ compared to SHAM mice. Data represent the mean $\pm$ SEM.

Figure 3 – OVX mice display reduced 2-deoxy-glucose uptake. A: Insulin-stimulated 2-deoxy-glucose (2-DG) uptake in skeletal muscle (Gastro). B: Insulin-stimulated 2-DG uptake in white adipose tissue. C: Insulin-stimulated 2-DG uptake in brown adipose

tissue. Significance between groups was determined by one-way ANOVA. Data represent the mean $\pm$ SEM.

Figure 4 – OVX mice display increased ectopic lipid accumulation. A: Triacylglycerol (TG) content in liver. B: TG content in skeletal muscle (Gastro). C: Diacylglycerol (DAG) content in liver. D: DAG content in skeletal muscle (Gastro). E: Ceramides content in liver. F: Ceramides content in skeletal muscle (Gastro). Significance between groups was determined by one-way ANOVA. Data represent the mean $\pm$ SEM.

Figure 5 – OVX mice display increased novel PKC activation in liver and muscle. A: PKC ϵ expression in membrane and cytosol fractions from liver. PKC θ expression in membrane and cytosol fractions from skeletal muscle. Significance between groups was determined by one-way ANOVA. Data represent the mean $\pm$ SEM.

Figure 6 – OVX mice display reduced insulin-stimulated AKT2 phosphorylation. A: Insulin-stimulated AKT2 phosphorylation in liver (post clamp tissue). B: Insulin-stimulated AKT2 phosphorylation in skeletal muscle (post clamp tissue). Significance between groups was determined by one-way ANOVA. Data represent the mean $\pm$ SEM.

Figure 7 – OVX mice display increased mRNA expression of lipogenic enzymes. A: mRNA expression from liver. B: mRNA expression from skeletal muscle. Significance between groups was determined by one-way ANOVA. Data represent the mean $\pm$ SEM.

Table 1 - Biometrics and basal biochemistry parameters

	SHAM	OVX	OVX+E2
Estradiol (pg/mL)	24.1 ± 2.6	8.3 ± 0.3*	39.1 ± 5.2 [#]
Initial Body Weight (g)	17.06 ± 0.1	16.80 ± 0.2	16.83 ± 0.2
Final Body Weight (g)	22.84 ± 0.2	30.22 ± 1.1*	23.34 ± 0.3 [#]
Body Fat (%)	11.13 ± 0.6	28.04 ± 1.8*	5.82 ± 0.2 [#]
Basal Glucose (mg/dL)	107 ± 6.6	132 ± 14	110 ± 7.7
Basal Insulin (μU/mL)	12.8 ± 3.1	12.8 ± 3.0	12.7 ± 2.5
Plasma FA (mmol/L)	0.86 ± 0.08	0.61 ± 0.04	0.91 ± 0.13
Plasma TG (mg/dL)	58.3 ± 8.3	61.5 ± 4.9	60.3 ± 6.0

Figure 1 - Metabolic parameters

Light hours

Dark hours

Figure 2 - Glucose tolerance and Insulin sensitivity

Figure 3 - 2-DG uptake

A

B

C

Figure 4 - Tissue lipids content

A

B

C

D

E

F

Figure 5 – Novel PKC activation

Figure 6 – Insulin-stimulated AKT2 phosphorylation

A

B

Figure 7 – mRNA expression in liver and skeletal muscle

Supplemental
Table S1 - Primers sequence

	Forward	Reverse
SREBP1c	CAGCTCAGAGCCGTGGTGA	TTGATAGAAGACCGGTAGCGC
PPARg	ATGCCAAAAATATCCCTGGTTTC	GGAGGCCAGCATGGTGTAGA
ACC1	GCCATTGGTATTGGGGCTTAC	CCCGACCAAGGACTTTGTTG
ACC2	GACGCCCCGAGGATCTGAAG	GGGACAGGGACGTACTGATC
FAS	GCTGCGGAAACTTCAGGAAAT	AGAGACGTGTCACTCCTGGACT
DGAT2	ACTCTGGAGGTTGGCACCAT	GGGTGTGGCTCAGGAGGAT
UCP2	ATGGTTGGTTTCAAGGCCACA	CGGTATCCAGAGGGAAAGTGAT
CEACAM1	AATCTGCCCCTGGCGCTTGGAGCC	AAATCGCACAGTCGCCTGAGTACG
GAPDH	CTCCACTCACGGCAAATTCA	ATGGGCTTCCCGTTGATGA