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Tese de Doutorado

Modificações no controle da função vascular na prole de ratos diabéticos tipo-1: Contribuição da inervação perivascular e os efeitos do tratamento com losartan.

DIEGO BARBOSA DE QUEIROZ

Orientador: Prof.Dr. Fabiano Elias Xavier

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Tese submetida ao Programa de Bioquímica e Fisiologia da Universidade Federal de Pernambuco, para obtenção do grau de Doutor em Bioquímica e Fisiologia.

ORIENTADOR

Prof. Dr. Fabiano Elias Xavier

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“Modificações no controle da função vascular na prole de ratos diabéticos tipo-1: Contribuição da inervação perivascular e os efeitos do tratamento com losartan”

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“A ciência permanecerá sempre a satisfação do desejo mais alto da nossa natureza, a curiosidade; fornecerá sempre ao homem o único meio que ele possui de melhorar a sua própria sorte”.

(Ernest Renan)

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RESUMO

O conceito da “programação fetal” sugere que um individuo pode ser “programado” durante as fases intra-uterina e perinatal para desenvolver doenças na vida adulta. A literatura mostra que o diabetes materno produz importantes alterações metabólicas na prole adulta, predispondo-os ao surgimento de doenças cardiovasculares. Este estudo analisou se a hipertensão arterial e disfunção vascular observada em ratos adultos submetidos ao diabetes materno estariam mediadas pelas ações da angiotensina II via ativação do receptor AT1 e com as alterações na inervação perivascular em preparações de artéria mesentérica. O diabetes materno foi induzido por estreptozotocina em ratas Wistar. Alterações na homeostasia da glicose, como intolerância a glicose e resistência à insulina foram observados nos ratos adultos com 12 meses de idade provenientes de mães diabéticas (O-DR) e revertidos quando tratados com losartan. Através da medida direta da PA, a PAM dos ratos (O-DR Losartan) apresentaram níveis normotensos quando comparado aos ratos (O-DR). No grupo (O-DR losartan) foi observado um aumento no relaxamento dependente do endotélio e redução na contração à fenilefrina quando comparado aos ratos (O-DR). Para avaliar o envolvimento dos metabolitos derivados do acido araquidônico, foram utilizados inibidores da COX-1 e 2 (indometacina) ou da COX-2 (NS-398), onde ambos não alteraram o relaxamento e contratilidade, significativamente no grupo O-DR tratado com losartan. Ao analisar a inervação perivascular em artéria mesentérica superior, os ratos O-DR de 6 meses de idade apresentaram aumento da inervação adrenérgica com participação da NA e ATP e elevação da inervação nitrergica com aumento da liberação de NO neuronal. Esses resultados sugerem que o Diabetes mellitus durante a fase intrauterina e perinatal causa modificações metabólicas, cardiovasculares e na inervação perivascular em ratos adultos e que estas alterações podem ser explicadas pela participação da ANGII e maior ativação da inervação adrenérgica e nitrérgica nestes distúrbios.

Palavras-chave: Diabetes Gestacional, disfunção endotelial, Angiotensina II e inervação perivascular.

ABSTRACT

The concept of "fetal programming" suggests that an individual can be "programmed" during intrauterine and perinatal stages to develop diseases in adulthood. The literature shows that maternal diabetes cause important metabolic changes in adult offspring, predisposing them to the emergence of cardiovascular diseases. This study examined whether hypertension and vascular dysfunction observed in adult rats subjected to maternal diabetes would be mediated by the actions of angiotensin II via AT1 receptor activation and changes in perivascular innervation in mesenteric artery preparations. Maternal diabetes induced by streptozotocin in Wistar rats. Changes in glucose homeostasis, such as glucose intolerance and insulin resistance was observed in offspring diabetic rats (O-DR) and reversed when treated with losartan. Through direct measurement of BP, MAP of rats (O-DR Losartan) showed normotensive levels compared to offspring diabetic rats (O-DR). In group (O-DR losartan) we observed an increase in the endothelium-dependent relaxation and a reduction in the contraction to phenylephrine compared to the rats (O-DR). To assess the involvement of arachidonic acid derived metabolites, COX-1 and 2 (indomethacin) or COX-2 (NS-398), both did not alter the relaxation and contractility significantly in the group O-DR Losartan. By analyzing the perivascular innervation in the superior mesenteric artery, O-DR-6 months of age showed increased adrenergic innervation with participation of NA and ATP and increased nitrergic innervation with increased release of neuronal NO. These results suggest that diabetes mellitus during intrauterine and perinatal phase causes metabolic, cardiovascular and perivascular innervations changes in offspring diabetic rats and that these alterations can be explained by the participation of ANG II and increased activation of adrenergic and nitrergic innervation in these disorders.

Keywords: Gestational diabetes, endothelial dysfunction, angiotensin II and perivascular innervation.

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INTRODUÇÃO

1. INTRODUÇÃO

O diabetes mellitus gestacional ou materno (DMG) é definido como uma intolerância à glicose que resulta em níveis hiperglicêmicos variáveis, podendo ter inicio ou primeiro diagnosticado durante a gestação. Semelhante a outras formas de hiperglicemia, o DMG é caracterizado por uma insuficiência na produção de insulina endógena pelas células β-pancreáticas, provavelmente resultantes de um mesmo espectro de causas que incluem doenças autoimunes, anormalidades genéticas ou resistência à insulina (Buchanan *et al.*, 2007).

A prevalência do diabetes gestacional dependendo do estudo da população e do diagnostico utilizado, pode alcançar de 1 a 15% de todas as gestações (ADA, 2004). Recentemente, foi proposto um novo critério que diagnosticou em torno de 20% a porcentagem de mulheres que já apresentaram DMG (Metzger *et al.*, 2010) o que torna um potencial problema de saúde pública. A origem dessa epidemia, geralmente, reside tanto no envelhecimento populacional, quanto em fatores ambientais como uma nutrição inadequada e o sedentarismo, nos quais muitos países com economia em rápido crescimento, como é o caso da população brasileira, está particularmente exposta (ADA, 2004).

O DMG durante a gravidez é frequentemente associado com consequências a curto e longo prazo no feto, aumentando os riscos de prematuridade, aborto e de má-formação congênita (Yang *et al.*, 2006; Michael, 2009). A exposição contínua do embrião aos níveis elevados de glicose pode resultar em embriopatia diabética (Chugh *et al.*, 2003), cujo processo é caracterizado por vários tipos de má formação, os quias resultam em problemas no fechamento do tubo neural, anormalidades urogenitais, além de alterações no sistema esquelético, endócrino e cardiovascular (Lucas *et al.*, 1997; Lynch & Wright, 1997; Nold & Georgieff, 2004).

Em relação aos efeitos em longo prazo do diabetes materno sobre o feto, há um grande número de evidências epidemiológicas e experimentais

demonstrando que a exposição intrauterina e/ou perinatal ao diabetes materno está associada com risco elevado de desenvolvimento de obesidade (Lawlor *et al.*, 2011), diabetes mellitus tipo-2 (Clausen *et al.*, 2008) e distúrbios cardiovasculares durante a vida adulta (Bunt *et al.*, 2005;Yang *et al.*, 2006).

2. Fundamentação teórica

No final da década de 80, o grupo do Dr. David Barker identificou o envolvimento dos distúrbios ocorridos durante a fase intrauterina no desenvolvimento de doenças crônicas na vida adulta (Barker & Bagby, 2005; Barker *et al.*, 1989; Barker, 1998). Especificamente, Barker e seus colaboradores evidenciaram que a distribuição geográfica da taxa mortalidade neonatal na Inglaterra e no País de Gales no início do século XIX era próxima da distribuição da taxa de mortalidade por doenças cardiovasculares setenta anos mais tarde. Devido ao fato de que a mortalidade neonatal no início do século XIX era atribuída ao baixo peso após o nascimento, Barker sugeriu que fatores iniciados durante a vida fetal, e que retardam o crescimento, poderiam "programar" ou definitivamente alterar a estrutura e/ ou a fisiologia de sistemas ligados ao desenvolvimento de doenças cardiovasculares na idade adulta (Barker, 1998). Ademais, com base na associação entre peso corporal após o nascimento e a pressão arterial na vida adulta, publicada em 1985 por Wadsworth *et al*., Barker propôs que influências do ambiente fetal poderiam também alterar a pressão arterial na vida adulta (Barker *et al.*, 1989).

Esses dados conduziram à *Hipótese de Barker* ou *Teoria da origem fetal das doenças do adulto* segundo a qual a nutrição deficiente durante a vida intrauterina e infância precoce origina uma adaptação metabólica e/ ou estrutural permanente que aumenta o risco de desenvolvimento de doença coronariana e outras doenças associadas, como a hipertensão arterial, o diabetes e o acidente vascular cerebral, na vida adulta – *Programação Fetal* (Barker, 1998). Essa teoria se baseia no conceito de plasticidade do desenvolvimento, que é a capacidade de um genótipo poder originar diferentes estados morfológicos ou fisiológicos em resposta a exposições diferentes durante o desenvolvimento (Lawlor *et al.*, 2011; Pettitt *et al.*, 1993). Essas adaptações se dariam durante um período de desenvolvimento conhecido como "período crítico", que, para a maioria dos órgãos, ocorre durante a vida intrauterina.

Em países subdesenvolvidos e em desenvolvimento a desnutrição intrauterina e prematuridade são duas das influências que podem induzir tais efeitos. Entretanto, a hipótese do fenótipo poupador propõe respostas metabólicas e fisiológicas protetoras à desnutrição no início do desenvolvimento do feto, permitindo a formação dos órgãos vitais como o cérebro e assim possibilitando a sobrevivência do organismo (Huxley *et al.*, 2000; Plagemann *et al.*, 1998; Law & Shiell, 1996). Embora os países desenvolvidos e em desenvolvimento sejam acometidos pelo mesmo problema, o principal fator determinante para o baixo peso ao nascer não parece ter a mesma causa. Nesses países, o aumento do aporte calórico adicionado à obesidade materna converge para o risco de diabetes gestacional e macrossomia fetal, podendo levar ao desenvolvimento de doenças cardiovasculares na vida adulta. Estes estudos que relacionam peso ao nascer com doenças cardiovasculares seguem uma trajetória em forma de "U", com maior peso ao nascer também levando a um aumento do risco de doenças cardiovasculares na vida adulta (Huxley *et al.*, 2000; Law & Shiell, 1996).

Inicialmente, a maioria dos estudos sobre "programação fetal" enfatizaram os efeitos da desnutrição materna e sua relação com algumas doenças na vida adulta. Na última década já surgiram alguns estudos demonstrando que a exposição fetal à hiperglicemia materna tem uma contribuição importante para o aparecimento de doenças desde a vida intrauterina até a vida adulta (Weiss *et al.*, 2000; Dabelea *et al.*, 2000). Dörner & Plagemann (1987), estiveram entre os primeiros a fornecer evidencias de que um ambiente hiperglicêmico na fase intrauterina predispõe ao aparecimento de obesidade e diabetes na idade adulta. Eles propuseram que a hiperinsulinemia na fase fetal e/ou neonatal durante um período critico da organogênese cerebral conduz a uma permanente má formação dos centros regulatórios hipotalâmicos para o metabolismo, e até mesmo dos sistemas regulatórios energéticos. Tais dados suportaram um grande número de estudos epidemiológicos mostrando a importância do DMG no desenvolvimento da obesidade, intolerância a glicose, diabetes tipo-2, síndrome metabólica entre

outras doenças crônicas (Yu *et al.*, 2011; Monasta *et al.*, 2010; Harder *et al.*, 2001; Alcolado *et al.*, 2002).

Os primeiros estudos realizados com essa finalidade foram com os índios *Pima* dos Estados Unidos (Knowler *et al.*, 1978; Lillioja *et al.*, 1993), uma população com uma elevada prevalência de diabetes tipo-2. Estes estudos mostraram que a exposição intrauterina ao diabetes, adicionado à predisposição genética, é um fator de risco independente que leva ao desenvolvimento de várias alterações (Lindsay *et al.*, 2000; Dabelea *et al.*, 2000). Nessa população, foi observado um aumento de seis vezes na incidência de diabetes tipo-2 em filhos de mães diabéticas e pré-diabéticas quando comparado às mães que não apresentavam diabetes (Franks *et al.*, 2006). Além disso, esses indivíduos apresentaram altos níveis de pressão arterial sistólica e aumento no risco de desenvolver obesidade na infância (Pettitt *et al.*, 1993; Pettitt *et al.*, 1983; Dabelea *et al.*, 2000). Outro estudo prospectivo importante chamado de *The Framinghan Offspring Study* mostrou um alto risco de intolerância à glicose ou diabetes tipo-2 em filhos de mães jovens que apresentavam diabetes, consistindo com um dos efeitos da exposição a um ambiente intrauterino com altas concentrações de glicose (Meigs *et al.*, 2000). Catalano & Hauguel (2011) sugeriram que a hiperglicemia materna causa um aumento na transferência de glicose para o feto, levando a um aumento na produção de insulina fetal. Esse aumento da insulina promove um aumento do crescimento fetal e macrossomia que pode estar vinculado ao aparecimento de desordens metabólicas na vida adulta.

Nesse contexto, estudos realizados com modelos animais também demonstraram ser um ótimo instrumento para permitir a observação dos impactos causados pelo diabetes gestacional nos seus descendentes e compreender os mecanismos envolvidos. Uma variedade de modelos murinos tem sido estabelecida administrando estreptozotocina, um agente antibiótico com toxicidade especifica para as células β-pancreáticas, em ratas fêmeas grávidas (Yessoufou *et al.*, 2011; Aerts *et al.*, 1997). Li *et al.* (2012) sugeriram que a exposição intrauterina a níveis elevados de glicose pode interferir no desenvolvimento fetal alterando os mecanismos de regulação homeostática em

longo prazo. Essa exposição durante a gravidez torna o individuo mais susceptivel para o desenvolvimento de complicações vasculares e metabólicas na fase adulta, podendo resultar em DM2, obesidade e hipertensão arterial na vida adulta. Grill *et al*. (1991) e Fujisawa *et al*. (2007) demonstraram o desenvolvimento de intolerância à glicose e resistência à insulina em ratos com 4 e 6 meses de idade provenientes de ratas diabéticas induzidas com estreptozotocina. Manderson *et al*. (2002) demonstraram no plasma da prole desses animais, um aumento na concentração de moléculas de adesão celular, alterações metabólicas e uma maior predisposição a doenças vasculares. Wichi *et al*. (2005) mediram a atividade tecidual da enzima conversora de angiotensina (ECA) no coração, pulmão, rins e fígado de ratos adultos provenientes de ratas diabéticas, e observaram que a atividade desta enzima estava elevada, o que poderia em parte justificar o desenvolvimento de hipertensão arterial nesses ratos.

Como comentado anteriormente, a hiperglicemia materna tem um papel chave no surgimento de alterações na homeostase de seus descendentes, podendo estar envolvida na fisiopatogenia de várias doenças crônicas como diabetes, obesidade e hipertensão na idade adulta. Embora o mecanismo exato relacionado com à origem destas doenças não esteja completamente elucidado, vários são os fatores que se apresentam como possíveis candidatos. Mudanças no sistema renina-angiotensina (SRA) (Wichi *et al*., 2005), no balanço do oxido nítrico (NO) (Cavanal *et al*., 2007) e a participação das espécies reativas de oxigênio (ROS) (Abe & Berk, 1998) no sistema vascular parecem ter envolvimento destacado no que se refere à elevação da pressão arterial e no aparecimento de doenças cardiovasculares em indivíduos submetidos ao diabetes materno.

2.1 Diabetes gestacional e doenças cardiovasculares

O aumento da pressão arterial é um fator de risco importante para o surgimento de doenças do coração, acidentes vasculares cerebrais e doença

renal. Estima-se que uma em cada seis pessoas em todo o mundo são hipertensas e a expectativa é que este número aumente para 1,5 bilhões até o ano de 2025 (Kearney *et al.*, 2005). No Brasil, a prevalência de hipertensão está estimada entre 5 e 30% da população dependendo da área analisada (Picon *et al.*, 2012). Embora muitos dos mecanismos fisiopatológicos da hipertensão tenham sido elucidados, sua etiologia relacionada ao diabetes ainda permanece pouco conhecida. Evidências epidemiológicas e experimentais têm fornecido significativas informações a respeito dos mecanismos ligados às desordens ocorridas durante a fase intrauterina e a etiologia da programação fetal da hipertensão e das alterações vasculares em longo prazo (Gomes & Gil, 2011; Intapad & Alexander, 2013; Racasan *et al.*, 2005).

Em gestações complicadas pelo diabetes materno, o aparecimento de alterações cardiovasculares pode ser observado inicialmente durante o terceiro trimestre da vida uterina fetal (Ojeda *et al.*, 2008). O coração do feto apresenta uma reduzida contratilidade ventricular quando comparado com outros que não foram submetidos ao diabetes materno, sugerindo que um ambiente intrauterino hiperglicêmico induz alterações biomecânicas no sistema cardiovascular (Rasanen & Kirkinen, 1987). Além disso, crianças expostas ao DM durante a vida fetal exibem aumento da pressão sanguínea associada com aumento do peso corporal (Silverman *et al.*, 1991; Bunt *et al.*, 2005).

A Hipertensão arterial sistêmica é caracterizada por resistência vascular periférica elevada que, por sua vez, parece estar relacionada principalmente com alterações intrínsecas da parede vascular de natureza estrutural e/ ou funcional (Shepherd, 1990). Estas alterações incidem especialmente nas células endoteliais, as células musculares lisas e os componentes da matriz extracelular, em todos os casos, contribuindo para o aumento da resistência vascular periférica, e dessa forma para a elevação da pressão arterial (Drummond *et al.*, 2011; Savoia & Schiffrin, 2007; Shepherd, 1990).

Como mencionado, danos causados à função endotelial estão ligados a fisiopatogênia do diabetes tipo-2, da resistência à insulina, da aterosclerose e

da hipertensão arterial. O endotélio pode ser definido como uma camada continua de células ao longo de todo o sistema cardiovascular, através do qual sintetiza uma variedade de mediadores químicos que participam da regulação do tônus do músculo liso vascular, do crescimento da parede vascular e do controle da adesão de leucócitos e plaquetas, contribuindo de maneira decisiva para a homeostase e para resposta a quadros inflamatórios. Ingram *et al.* (2008) recentemente demonstraram que as células progenitoras endoteliais da prole de mães diabéticas exibem uma função angiogênica diminuída e que esta disfunção está associada a uma redução da capacidade de auto-renovação e formação de novas colônias celulares, assim como uma senescência celular acelerada. Altas concentrações de glicose *in vitro* também foram responsáveis por efeitos similares (Ingram *et al.*, 2008). Devido ao papel central que o endotélio exerce na regulação do tônus vascular, muitos estudos sobre programação fetal têm focado na avaliação da função endotelial.

2.2 Fatores endoteliais reguladores do tônus vascular

O tônus vascular é fundamental para regulação do fluxo sanguíneo, sendo regulado por distintos fatores locais (músculo liso e endotélio) (Furchgott & Zawadzki, 1980), sistêmicos (sistema renina-angiotensina-aldosterona) (Touyz, 2005) e nervosos (sistema nervoso central e inervação perivascular) (Loesch, 2001). Dependendo do estimulo exercido sobre o vaso, fatores vasodilatadores, anti-proliferativos e anti-agregantes plaquetários ou fatores vasoconstrictores, promotores do crescimento celular e ativadores plaquetários são liberados em grande parte pelas células endoteliais regulando o processo da homeostase vascular (Rubanyi, 1993; Schiffrin, 1994; Schiffrin, 2001). O desequilíbrio entre esses fatores conduz ao quadro de disfunção endotelial observado nas doenças cardiovasculares, nos quais, estão envolvidas a redução de fatores vasodilatadores como o NO e o fator hiperpolarizante derivado do endotélio e o aumento na produção de fatores vasoconstritores

como as EROs, as prostaglandinas vasoconstritoras, a endotelina-1, e a angiotensina II (Drummond *et al*., 2011; Briones *et al*., 2000; Touyz, 2005).

2.2.1 Fatores vasodilatadores derivados do endotélio

Óxido Nítrico (NO)

O NO é considerado como o mais importante fator de origem endotelial. Ele é sintetizado a partir da oxidação do aminoácido L-arginina, por ação da enzima NO-sintase, que forma, além do NO, outra substância, a L-citrulina (Palmer *et al.,* 1988). Muitos tipos celulares são capazes de sintetizar NO. Até o presente foram identificadas três isoformas da NOS, que se diferenciam em sua expressão e atividade. Algumas isoformas se expressam de forma constitutiva nas células (cNOS) e outra se induz por estímulos imunológicos (iNOS ou tipo II) (Pu *et al*., 2011). Dentre as isoformas constitutivas encontra-se a isoforma endothelial (eNOS ou NOS III) e a isoforma neuronal (nNOS ou NOS I). A eNOS se expressa constitutivamente nas células endoteliais, embora também tenha sido encontrada em plaquetas; a nNOS se expressa em células neuronais do sistema nervoso central e periférico e em epitélios de traquéia e brônquios (Gyoda *et al*., 1995). A atividade das isoformas constitutivas é dependente de Ca^{2+}-CaM. A isoforma induzível (iNOS) é expressa em macrófagos, células endoteliais, neutrófilos ou células musculares lisas durante estados de inflamação ou depois de serem estimuladas com moléculas como o lipopolisacarídeo bacteriano (LPS) ou citocinas como a interleucina 1β (IL-1β) (Marin & Rodriguez-Martinez, 1997; Briones *et al*., 2000; Andreozzi *et al*., 2007; Pu *et al*., 2011).

Em condições fisiológicas, a produção de NO nas células endoteliais é estimulada por uma variedade de agentes químicos e pelas forças de atrito (estresse de cisalhamento) produzido pelo fluxo sanguiíneo (Tousoulis *et al*., 2012). À semelhança dos nitratos vasodilatadores, o NO também causa relaxamento da musculatura lisa vascular (Tousoulis *et al*., 2012); ele se difunde para a camada muscular, onde promove aumento da produção de monofosfato cíclico de guanosina (GMPc) e ativação da proteína quinase

dependente de GMPc (PKG). A ativação da PKG reduz o influxo de cálcio através da membrana plasmática e aumenta sua recaptação pelo retículo sarcoplasmático(Rapoport *et al.*, 1983). A PKG pode ainda ativar canais para K^+, levando à hiperpolarização das células musculares lisas (Figura 1) (Robertson *et al.*, 1993). Ademais, a PKG fosforila o receptor para o IP_3 da membrana do retículo sarcoplasmático, cuja função é promover a liberação de Ca^{2+} para o citoplasma, diminuindo, assim, a atividade deste. Por outro lado, a PKG fosforila a quinase de cadeia leve de miosina (MLCK), inibindo assim sua atividade, o que provoca diminuição da fosforilação da cadeia leve de miosina (MLC_{20}) e, portanto, inibe-se a contração muscular (Figura 1) (Marin & Rodriguez-Martinez, 1997).

O NO também atua como modulador do crescimento das células musculares lisas através da inibição da proliferação de células musculares lisas, da produção basal de colágeno, da divisão celular e da produção de matriz extracelular estimuladas pela endotelina-1 e/ou angiotensina II, além de estimular a apoptose, através de mecanismos dependentes do GMPc (Pollman *et al.*, 1996; Rizvi & Myers, 1997).

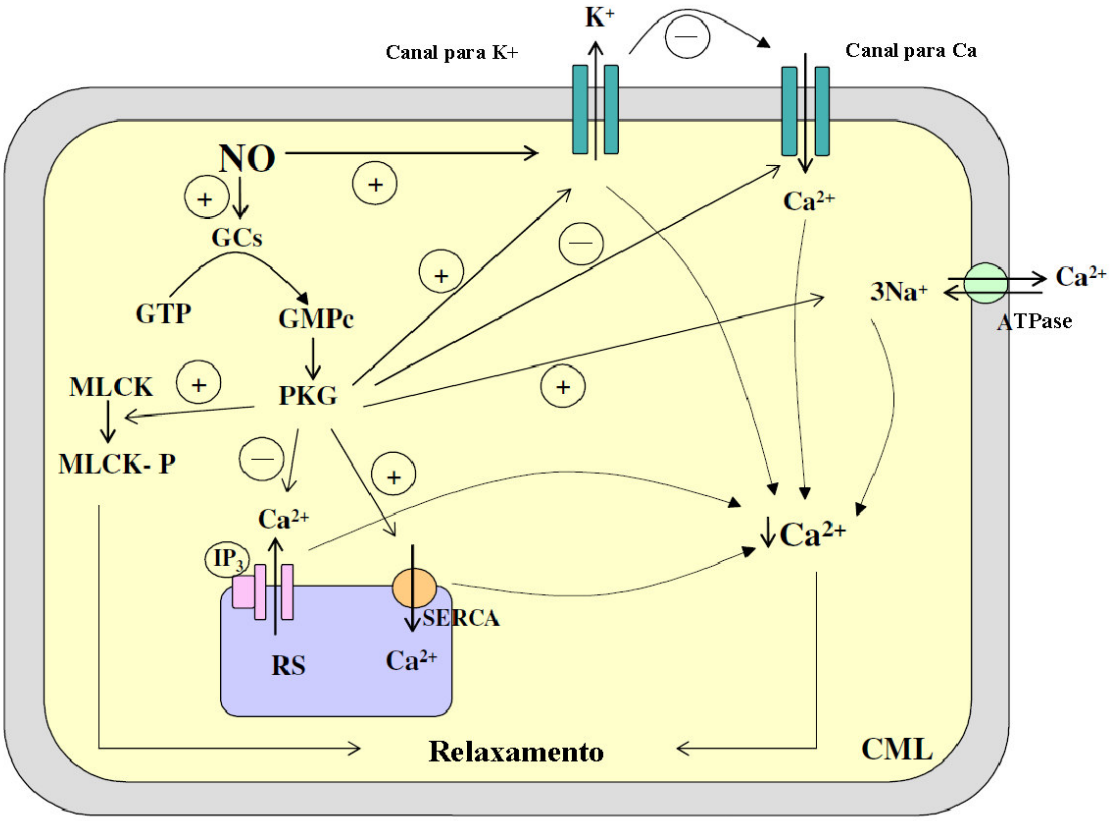

Figura 1. Mecanismos de relaxamento induzido pelo NO. GCs: guanilato ciclase solúvel, PKG: proteína quinase dependente de GMPc, RS: retículo sarcoplasmático, SERCA: Ca^{2+} ATPase do retículo sarcoplasmático, MLCK: quinase da cadeia leve da miosina, IP_3: inositol 1,4,5-trifosfato, CML: célula muscular lisa.

Fator hiperpolarizante derivado do endotélio (EDHF)

A identidade molecular e as vias de sinalização de EDHF ainda são objetos de muita discussão. Na verdade, as respostas vasodilatadoras do EDHF têm sido atribuídas a uma variedade de candidatos a este fator endotelial, como: os derivados da via do citocromo P450, o ácido epoxieicosatrienóico (EET), os produtos da lipoxigenase, o próprio NO, o peróxido de hidrogênio (H_2O_2), dentre outros (Feletou & Vanhoutte, 2006). A vasodilatação induzida por esse fator ocorre sem elevação dos níveis intracelulares de GMPc ou AMPc. A hiperpolarização, fenômeno correlato ao relaxamento, ocorre devido à ativação de canais para potássio sensíveis ao ATP ou ativados por Ca^{2+} e da ativação da Na^+, K^+-ATPase da membrana das células musculares lisas (Chen & Suzuki, 1989) Esse mecanismo leva à

inibição da entrada de Ca^{2+} através de canais para Ca^{2+} dependentes de voltagem e conseqüente relaxamento da musculatura lisa (Chen & Suzuki, 1989). Os efeitos do EDHF são mais evidentes nos vasos de resistência do que nas grandes artérias (Takamura *et al.*, 1999).

Prostaciclina (PGI_2)

A prostaciclina é um eicosanóide derivado do acido araquidônico, que é liberado dos fosfolipídios da membrana endotelial pela fosfolipase A_2. Através da reação catalizada pela ciclooxigenase, formam-se os endoperóxidos PGG_2 e PGH_2. Este último, através da ação da prostaciclina sintetase origina a PGI_2 (Figura 3) (Needleman *et al.*, 1986). Esta prostaglandina apresenta atividade vasodilatadora e antiagregante plaquetária (Busse *et al.*, 1987; Williams *et al.*, 1994); Este prostanoide é muito instável transformando-se espontaneamente em seu metabolito 6-ceto-$PGF_{1\alpha}$. Através da estimulação dos receptores IP, a PGI_2 promove a ativação da adenilato ciclase e aumento dos níveis de AMPc, este, por sua vez induz a ativação da PKA, a qual induz inibição dos processos contráteis mediados pelo complexo Ca^{2+}-calmodulina (Hathaway *et al.*, 1981). Entretanto, trabalhos recentes demonstram que a PGI_2 é capaz também de induzir vasoconstrição, a qual é mediada por receptores para o tromboxano A_2, os receptores TP (Gluais *et al.*, 2005; Xavier *et al.*, 2010) .

2.2.2 Fatores vasoconstritores derivados do endotélio

Os fatores vasoconstritores sintetizados pelo endotélio são classificados basicamente em três categorias: 1) metabólitos do ácido araquidônico (PGH_2, TXA_2, $PGF_{2\alpha}$), 2) espécies reativas do oxigênio e 3) peptídeos vasoativos, como a endotelina-1 e a angiotensina II.

Prostaglandinas vasoconstritoras

Após o estudo de Robert Furchgott (1980) demonstrando nas células endoteliais a liberação de fatores relaxantes derivados do endotélio (EDRF), em resposta à acetilcolina (Furchgott & Zawadzki, 1980) outro estudo realizado em veias caninas isoladas, demonstrou um aumento da tensão em contrações à norepinefrina, induzidas por acido araquidônico e trombina exógena, ao invés do relaxamento observado nas artérias correspondentes (De Mey & Vanhoutte, 1982; Furchgott & Zawadzki, 1980). Este resultado demonstrou a capacidade do endotélio de iniciar contrações do músculo liso subjacente, a qual era dependente da liberação de substâncias difusíveis denominados fatores vasoconstritores derivados do endotélio (EDCF). Estas contrações induzidas pelo acido araquidônico eram prevenidas por inibidores da ciclooxigenase (COX), sugerindo uma relação entre os EDCFs e a via metabólica desta enzima (Miller & Vanhoutte, 1985).

A atividade da ciclooxigenase é capaz de regular o tônus vascular momento a momento. Existem duas isoformas da ciclooxigenase denominadas COX-1 e COX-2 (Feletou *et al*., 2011). Ambas é heme-proteínas que apresentam a mesma potência para oxidar o acido araquidônico em endoperóxido (PGH_2), o precursor de todas as demais prostaglandinas (Figura 3) (Garavito & DeWitt, 1999). Nas contrações dependentes do endotélio em aorta de ratos espontaneamente hipertensos (SHR), o inibidor da COX-1 (valeril salicilato) é capaz de abolir essas contrações, enquanto que os inibidores de COX-2, como o NS-398, apenas reduzem essa resposta (Ge *et al*., 1995; Yang *et al*., 2002), sugerindo que as contrações dependentes do endotélio nesses ratos são mediadas pela ativação da COX-1. Em outro estudo (Tang *et al*., 2005), utilizando camundongos *knockout* para a COX-2 observou-se os efeitos da contração dependente do endotélio se mantiveram, enquanto que nos camundongos *knockout* para a COX-1 esse efeito não foi observado, o que sugere a participação indispensável da COX-1 nessa resposta contrátil.

Por outro lado, sob determinadas condições como, por exemplo, no processo de envelhecimento, ou por ação de citocinas e lipopolissacarideos (LPS) em células endoteliais e do músculo liso vascular (Vagnoni *et al*., 1999;

Yamagata *et al.*, 2001), a isoforma induzível, COX-2, pode ser expressa, participando em parte das contrações dependente do endotélio (Shi *et al.*, 2007). Alguns autores também têm encontrado a COX-2 expressa de forma constitutiva, podendo estar envolvida no desenvolvimento renal (Zhang *et al.*, 1997), produzindo prostanóides vasodilatadores e citoprotetores na mucosa gástrica de humanos e coelhos (Zimmermann *et al.*, 1998) ou participando na modulação da resposta vascular (Henrion *et al.*, 1997; Adeagbo *et al.*, 2003)

As prostaglandinas estão envolvidas em várias funções chave do sistema vascular, que vão desde processos inflamatórios à regulação da pressão arterial. Como já mencionado anteriormente, o acido araquidônico é o mais comum precursor das prostaglandinas. Estímulos como estiramento da parede vascular e agonistas elevam a concentração de cálcio intracelular no endotélio, esse aumento cálcio estimula a liberação de acido araquidônico pela fosfolipase A_2, que quando metabolizado pela ciclooxigenase gera prostanóides vasoconstritores derivados do endotélio (EDCF) (Garavito & DeWitt, 1999; Giles *et al.*, 2012). Esses prostanóides podem ativar os receptores para o tromboxano (TP) na membrana no músculo liso vascular induzindo contração (Vanhoutte *et al.*, 2005) Ao longo dos anos, EROs (Yang *et al.*, 2003), tromboxano A_2 (Xavier *et al.*, 2010), endoperóxidos (Ge *et al.*, 1995), a prostaciclina (Gluais *et al.*, 2005) e prostaglandina $F_{2\alpha}$ foram identificados como fatores vasoconstritores dependente do endotélio derivados da ciclooxigenase.

Tromboxano A_2 (TxA_2)

O TxA_2 é sintetizado a partir da ação da enzima TxA_2-sintetase sobre a PGH_2 derivado do acido araquidônico. Ele é considerado um dos prostanoides vasoconstritores mais importantes produzido na parede vascular, o qual ainda apresenta ação agregante plaquetária (Buzzard *et al.*, 1993). Sua ação é mediada pela ativação do receptor para tromboxano (receptor TP), o qual eleva as concentrações de Ca^{2+} intracelular e ativa a PKC induzindo vasoconstrição e agregação plaquetária (Figura 2) (Mayeux *et al.*, 1989). A liberação do TxA_2,

como de outras prostaglandinas pelas células endoteliais, ocorre através da ação de agonistas vasoconstritores (noradrenalina, serotonina, fenilefrina, angiotensina II, endotelina-1, etc), vasodilatadores (acetilcolina, bradicinina, etc) e por estímulos mecânicos (Taddei & Vanhoutte, 1993).

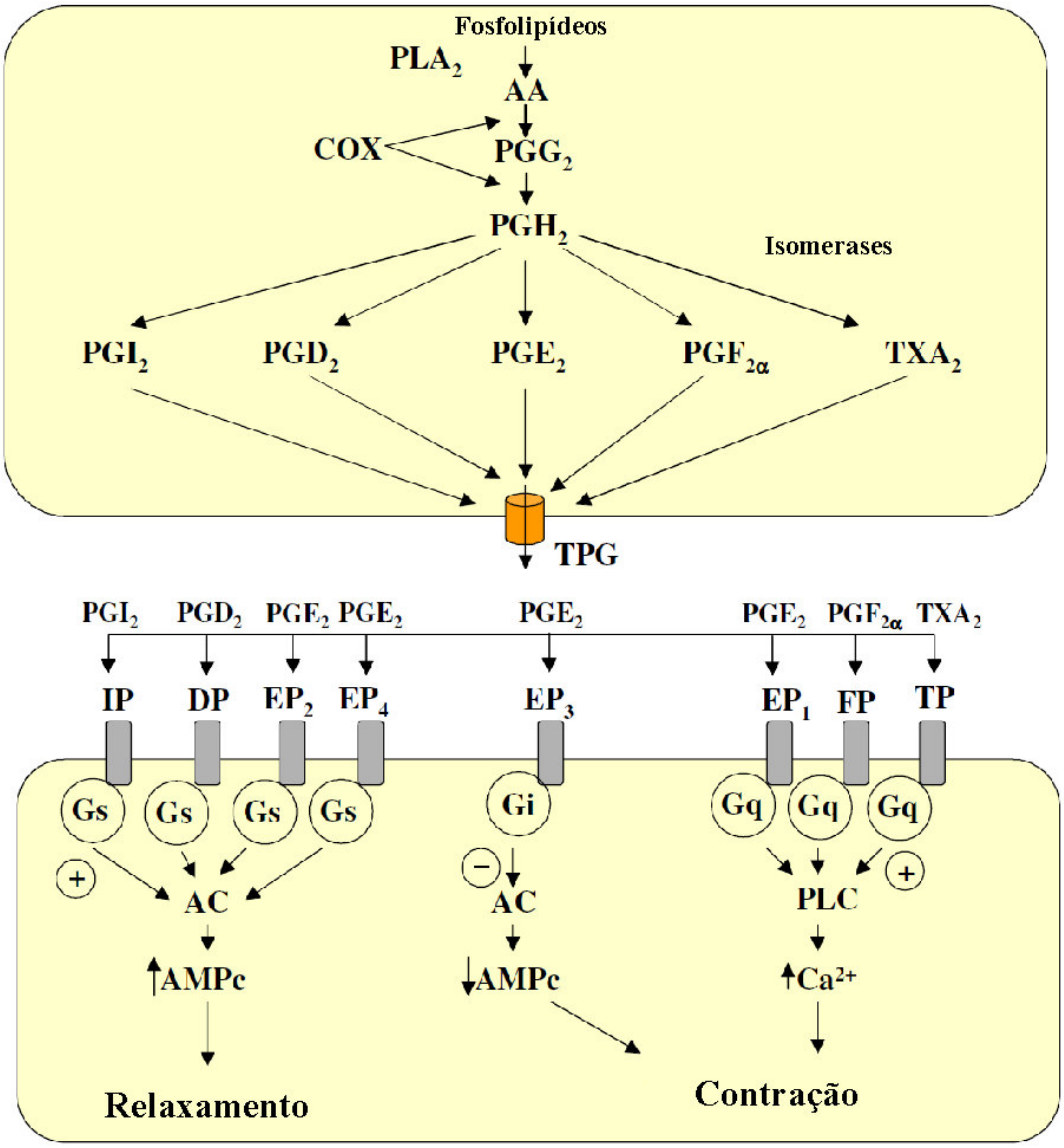

Figura 2. Síntese e mecanismos de ação de prostanóides. AA: ácido araquidônico, AC: adenilato ciclase, AMPc: Monofosfato cíclico de adonosina, DP: receptor da PGD_2, EP: receptor de PGE, FP: receptor de $PGF_{2\alpha}$, IP: receptor de PGI_2, TP: receptor de TxA_2, PLA_2: fosfolipase A_2, PLC: fosfolipase C, TPG: transportador de prostaglandinas.

Prostaglandina E_2 ($PGE_{2)}$

A PGE_2 é formada a partir da ação de sua sintetase sobre a PGH_2. Ela exerce efeitos em receptores específicos (receptores EP), os quais estão presentes no organismo na forma de quatro subtipos distintos: EP_1, EP_2, EP_3 e EP_4 (Bos *et al*., 2004). No sistema vascular, os subtipos EP_1 e EP_3, quando estimulados pela PGE_2, promovem vasoconstrição, a qual pode ser induzida pela ativação da via do IP_3/Ca^{2+} (receptor EP_1) ou através da diminuição dos níveis intracelulares de AMPc (receptor EP_3) por inibição da adenilato ciclase (Funk *et al*., 1993; Coleman *et al*., 1994). A ligação da PGE_2 aos receptores EP_2 e EP_4 promove vasodilatação através do aumento dos níveis de AMPc via ativação da adenilato ciclase (Figura 3) (Coleman *et al*., 1994) Tem sido descrito que a PGE_2 também é capaz de se ligar aos receptores TP, produzindo neste caso um efeito vasoconstritor (Bos *et al*., 2004).

Prostaglandina $F_{2\alpha}$ ($PGF_{2\alpha)}$

Esta prostaglandina é formada a partir da PGH_2 pela $PGF_{2\alpha}$-sintetase e representando uma das mais importantes prostaglandinas vasoconstritoras derivadas do ácido araquidônico. A $PGF_{2\alpha}$ participa da regulação do tônus vascular ao atuar elevando as concentrações de cálcio no músculo liso vascular promovendo contração (Yura *et al*., 1999). A $PGF_{2\alpha}$ age através dos receptores para $PGF_{2\alpha}$ (receptor FP), os quais são largamente distribuídos em vários tecidos, incluindo do sistema vascular. O receptor FP esta acoplado à proteína G e, quando estimulado ativa, a via do IP_3/ Ca^{2+} produzindo contração da musculatura lisa (Figura 2) (Pierce *et al*., 1999). Sua ação vasoconstritora também pode ser mediada através de sua ligação aos receptores TP (Cracowski *et al*., 2002). Em situações de estresse oxidativo, níveis de $PGF_{2\alpha}$ estão aumentados, os quais são gerados a partir de fosfolipídios da membrana plasmática pela ação da ciclooxigenase (Mervaala *et al*., 2001).

Espécies Reativas de Oxigênio e Nitrogênio (ERONs)

A geração das ERONs está associada ao metabolismo celular. No entanto, o aumento do estresse oxidativo tem sido associado a complicações cardiovasculares, como a hipertensão, o diabetes e a hipercolesterolemia (Cai & Harrison, 2000; Wolin, 2000). Nas membranas celulares, diversas enzimas realizam suas funções utilizando o oxigênio como aceptor de elétrons e, consequentemente, levam a formação de ânions superóxido ($\bullet O_2^-$) que é produzido pela redução de uma molécula de $\bullet O_2$. Estes ânions podem exercer sua ação diretamente no sistema vascular, servindo de substrato para a formação de outras ERONs. A partir do O_2^- se formam outras espécies reativas de oxigênio:

$$O_2 \xrightarrow{e^-} {}^{\bullet}O_2^- \xrightarrow{e^-} H_2O_2 \xrightarrow{e^-} {}^{\bullet}OH^- \xrightarrow{e^-} H_2O$$

As espécies reativas de oxigênio incluem o anion superóxido ($\bullet O_2^{-)}$, o radical hidroxila (OH^-) e o peróxido de hidrogênio (H_2O_2). Os dois primeiros possuem um elétron desemparelhado, situação que confere alta capacidade de reação.

O NO tem um elétron desemparelhado e assim podem reagir com moléculas que tenham também um elétron nesta forma (como o $\bullet O_2^-$), produzindo o peroxinitrito ($\bullet ONOO^-$) atavés de uma reação espontânea e irreversível (Wolin, 2000).

$$\bullet NO^- + \bullet O_2^- \longrightarrow {}^{\bullet}ONOO^-$$

Quando o O_2^- inativa o NO, o vasorrelaxamento se torna prejudicado e induz a apoptose nas células endoteliais, provocando uma falha na continuidade do endotélio que favorece o aparecimento de fenômenos trombóticos promovendo a adesão de diferentes células no endotélio. Adicionalmente, o produto desta reação, o $\bullet ONOO^-$, é um forte oxidante com importantes efeitos biológicos, como a nitrosilação de proteínas (Munzel *et al.*, 1997).

A manuntenção dos níveis de ERONs depende tanto de sua produção como de sua eliminação. Existem distintos sistemas enzimáticos e não

enzimáticos encarregados de eliminar os radicais livres produzidos em excesso. Assim, a superoxido dismutase (SOD) a partir do O_2^- produz H_2O_2, eliminando os ânions superoxido do meio e evitando a formação de peroxinitrito. O H_2O_2, por sua vez, é metabolizado a H_2O e O_2 pela ação da enzima catalase (Schiffrin, 2001). O $\bullet O_2^-$ e o H_2O_2 podem regular a atividade das metaloproteases da matriz do músculo liso vascular que degradam os proteoglicanos e o colágeno, produzindo mudanças na estrutura vascular (Wolin, 2000). Baixos níveis de EROs, estimulam o crescimento celular. Por outro lado, altos níveis de EROs produzem apoptose (Luczak *et al.*, 2004). Além disso, as EROs são capazes de modular as respostas contráteis. O $\bullet O_2^-$ e o H_2O_2 estimulam a contração mobilizando o Ca^{+2} armazenado nos depósitos intracelulares e ativam o trocador Na^+/H^+ também sendo capazes de produzir vasodilatação (Touyz, 2005) .

Endotelina-1

Yanagisawa *et al*. (1988) foram os primeiros autores a identificar esse potente peptídeo vasocostritor e vasopressor produzido pelas células endoteliais. A endotelina é um peptídeo composto por 21 aminoácidos, existente no ser humano em três isoformas: a endotelina-1 (ET-1), a endotelina-2 (ET-2) e a endotelina-3 (ET-3) (Inoue *et al.*, 1989). O endotélio vascular produz somente a ET-1, a qual é sintetizada a partir de um precursor, a pré-pró-endotelina, que sofre clivagem enzimática gerando uma forma intermediaria e inativa, a *big*-endotelina. Subseqüentemente, por ação da enzima conversora de endotelina (ECE), forma-se o peptídeo ativo, a endotelina-1.

A endotelina-1 atua em receptores específicos no músculo liso vascular, os subtipos ET_A e ET_B, e nas células endoteliais atuam através dos receptores ET_B (Patocka *et al*., 2005). No músculo liso vascular a ativação destes receptores leva ao aumento dos níveis intracelulares de cálcio e conseqüente, à vasoconstriçao. Esse aumento da concentração de cálcio se dá através da entrada deste íon por canais operados por voltagem e/ou operados por

receptor, além da sua liberação do retículo sarcoplasmático (Taddei & Vanhoutte, 1993; Patocka *et al*., 2005). Os receptores ET_A e ET_B são acoplados à proteínas G, e sua ativação induz aumento da atividade da fosfolipase C, com formação de IP_3 e DAG (Smith *et al*., 2003). Como conseqüência, tem-se vasoconstrição e proliferação das células musculares lisas. Já os receptores ET_B nas células endoteliais induz liberação de NO e PGI_2 (Patocka *et al*., 2005).

Angiotensina II

O sistema renina-angiotensina (SRA) é inicialmente ativado pela síntese de renina pelas células justaglomerulares. Nestas células a pré-prorenina é processada para a prorenina e, em seguida em renina ativa que é secretada na circulação sanguínea (Nguyen Dinh & Touyz, 2011). A liberação de renina renal é estimulada por estados de hipovolemia, elevadas concentrações de sódio nos túbulos distais, atividade nervosa simpática renal e reduzida perfusão renal. No sangue, a renina, um aspartil protease cliva o angiotensinogênio derivado do fígado para formar o decapeptídeo angiotensina I (Ang I) (Kumar *et al*., 2012; Nguyen Dinh & Touyz, 2011) .

No endotélio pulmonar encontra-se a enzima conversora de angiotensina (ECA), que hidrolisa o peptideo inativo Ang I no peptídeo biologicamente ativo, o octapeptídeo angiotensina II (ANG II). Além de clivar a Ang I, a ECA metaboliza o composto vasodilatador bradicinina, inativando-o em bradicinina 1-7 (de Mello & Frohlich, 2011). Assim, a ECA tem um papel duplo na vasculatura, por promover a produção de angiotensina II, um vasoconstritor potente, e degradar a bradicinina, um vasodilatador importante. Além disso, o SRA atua em diferentes órgãos de forma endócrina, atualmente, tornou-se evidente a existência de SRA locais ou teciduais definidas por meio da síntese de ANG II a partir do angiotensinogênio e enzimas produzidas localmente nos tecidos (De Mello & Frohlich, 2011; Nguyen Dinh & Touyz, 2011). O SRA local pode utilizar outras enzimas que não sejam a renina e a ECA para a síntese de ANG II, tais como as catepsinas e as quimases (Kumar *et al*., 2012). A ANG II

atua sobre as células vizinhas de maneira autócrina/parácrina, tendo sua função e regulação independente do SRA do sistema de circulatório (Kumar *et al.*, 2012) (Figura 3).

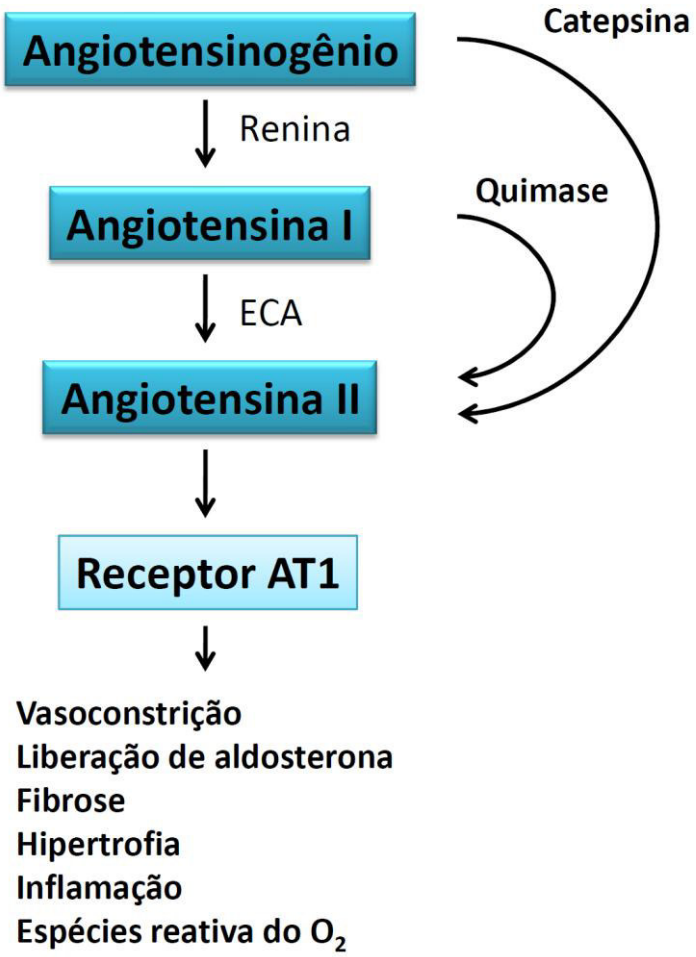

Figura 3. Na via clássica do sistema renina-angiotensina (SRA), a renina derivada do rim é secretada na circulação onde ela cliva o angiotensinogênio derivado do fígado em angiotensina I que será hidrolisada em angiotensina-II (ANG II) pela enzima conversora de angiotensina (ECA). O SRA local pode utilizar outras enzimas diferentes da renina e ECA para a sintese de ANG II, tais como, quimase e catepsina. A ANG II é capaz de se ligar ao receptor (AT1) para disparar diversas ações, tais como, vasoconstrição, liberação de aldosterona, fibrose , hipertrofia, indução de EROs e inflamação.

3. Papel da inervação perivascular na regulação do tônus vascular

As terminações nervosas perivasculares liberam distintos neurotransmissores vasoconstrictores e vasodilatadores que regulam o tônus vascular e o fluxo sanguíneo como resultado do equilibrio entre as inervações adrenérgica, colinérgica, nitrérgica e peptidérgica, cuja participação depende do leito vascular analisado (Loesch, 2002). Na artéria mesentérica de rato tem sido descrito as inervações: simpática ou noradrenérgica, nitrérgica e sensitiva (Burnstock & Ralevic, 1994; Blanco-Rivero *et al.*, 2011;Sastre *et al.*, 2012).

Inervação adrenérgica

No sistema nervoso periférico, o ramo simpático emerge da medula espinhal torácica e dos dois ou três primeiros segmentos lombares. Estes neurônios pré-glanglionares alcançam os distintos gânglios paravertebrais; entre estes últimos se encontram os gânglios mesentéricos que dão origem as fibras pós-ganglionares que formam os plexos ou nervos que inervam a região abdominal e pélvica (Costa & Robecchi, 1965). Estas terminações contem não somente noradrenalina (NA), como também cotransmissores como o neuropeptideo Y e o ATP (Donoso *et al.*, 1997). A NA é armazenada nas vesículas sinápticas e são liberadas por exocitose mediante processo dependente de cálcio. Uma vez liberado na fenda sináptica, a NA atua em dois tipos de receptores específicos, receptores α- e β-adrenérgicos tanto pré quanto pós-sinápticos (Nilsson, 1985).

Na artéria mesentérica de rato foram descritos os receptores α_1-adrenérgicos na musculatura lisa e no endotélio e os receptors α_2 nas células endoteliais (Nilsson, 1985; Buchholz *et al.*, 1998). Quando ativado esses receptores α_1-adrenérgicos, que estão acoplados a proteína G, a fosfolipase C (PLC) é ativada produzindo um aumento do inositol trifosfato (IP_3) e diacilglicerol (DAG), resultando em liberação de Ca^{2+} intracelular e vasoconstrição (Smith *et al.*, 2003). Os receptores α_2-adrenergicos se localizam nas terminações pré-sinápticas inibindo a liberação de NA (Buchholz *et al.*, 1998).

Inervação Nitrergica

O papel do NO como neurotransmissor durante muito tempo foi questionado. Entretanto, atualmente tem-se demonstrado sua existência em muitos leitos vasculares, dentre os quais se destacam os nervos craniais como artérias cerebrais e leitos vasculares viscerais como na artéria coronariana e do trato digestivo, renal e uterino, constituindo um importante mecanismo de controle do tônus vascular (Marin & Balfagon, 1998; Toda & Okamura, 2003).

A origem das fibras que formam esta inervação especialmente na artéria mesentérica não está clara, mas esta inervação desempenha um papel funcional no leito vascular mesentérico através do relaxamento do músculo liso vascular (Marín & Balfagón, 1998). Como já citado acima, o NO pode ser sintetizado por três enzimas eNOS, iNOS e nNOS. A nNOS é expressa, além de outros tecidos, no sistema nervoso central e periférico onde cataliza a reação de síntese de NO que irá atuar como molécula neurotransmissora (Fujiwara *et al*., 2012). Por se tratar de uma substancia gasosa, o NO é sintetizado de acordo com as necessidades exigidas pelo tecido e não pode ser armazenado em vesículas sinápticas nem ser liberado por exocitose; ele atua difundindo-se desde as terminações nervosas alcançando o músculo liso (Toda & Okamura, 2003; Hatanaka *et al*., 2006; Fujiwara *et al*., 2012), contradizendo uma ideia amplamente difundida de que os neurotransmissores são moléculas orgânicas de alto peso molecular, são armazenadas em vesicular sinápticas e atuam através de receptores de membrana (Li & Forstermann, 2000).

Inervação sensitiva

A inervação sensitiva da artéria mesentérica superior libera o peptídeo relacionado ao gene da calcitonina (CGRP) junto com a substância P como cotransmissor, tendo sua participação em processos fisiopatológicos pouco estudados no leito mesentérico (Wimalawansa, 1996; Wang & Li, 1999). O CGRP está localizado no sistema nervoso central onde participa em diferentes

atividades na função auditiva e olfativa, aprendizagem, alimentação e atividade motora e na inervação perivascular produz relaxamento (Nuki *et al*., 1994; Wimalawansa, 1996; Wang & Li, 1999). Sua liberação está regulada por fatores hormonais, endoteliais e neuronais (Angelucci *et al*., 2008). Além disso, existem evidencias indicando que o CGRP é autorregulado através de um mecanismo de retroalimentação negativa, estimulando receptores pré-sinapticos para CGRP (Nuki *et al*., 1994). Quanto ao mecanismo de ação nas artérias mesentéricas de ratos esse peptídeo atua em seu receptor CL (*calcitonin like*) produzindo uma resposta vasodilatadora provavelmente envolvendo a participação do AMPc e do GMPc diminuindo os níveis intracelulares de Ca^{2+} intracelulares no músculo liso vascular (Brain & Grant, 2004; Nuki *et al*., 1994).

4. Diabetes gestacional e os mecanismos envolvidos nas doenças cardiovasculares.

Como já destacado acima, o DMG está envolvido na programação de diversas doenças cardiovasculares na vida adulta, tais como infarto do miocárdio, acidentes vasculares cerebrais e hipertensão arterial sistêmica (Vrachnis *et al*., 2012; Ojeda *et al*., 2008). Uma grande quantidade de estudos clínicos e experimentais tem contribuído para o entendimento dos efeitos do diabetes materno na fase intrauterina e perinatal (Pedersen *et al*., 1968; Travers *et al*., 1989; Roberts & Pattison, 1990; Martinez-Friaz, 1994; Boloker *et al*., 2002; Fetita *et al*., 2006) e na vida adulta (Robinson *et al*., 1988; Holemans *et al*., 1999; Grill *et al*., 2001). Ross *et al*. (2007) sugeriram que um ambiente intra-uterino exposto a níveis elevados de glicose pode interferir no desenvolvimento fetal alterando os mecanismos de regulação homeostática em longo prazo. Essa exposição durante a gravidez leva a um aumento da susceptibilidade a complicações vasculares e metabólicas na fase adulta. Indivíduos submetidos ao diabetes gestacional podem ter consequências fisiopatológicas, como obesidade, hipertensão arterial, DM2 e complicações vasculares na fase adulta. Grill *et al*. (1991) e Fujisawa *et al*. (2007) demonstraram o surgimento de intolerância à glicose e resistência à insulina

em ratos com 4 e 6 meses de idade provenientes de ratas diabéticas. Manderson *et al*. (2002) demonstraram no plasma sanguíneo de ratos oriundos de ratas diabéticas um aumento na concentração de moléculas de adesão celular, alterações metabólicas e uma maior predisposição a doenças vasculares.

Além disso, Zandi-Nejad *et al*. (2006) demonstraram neste mesmo modelo experimental uma diminuição no número de néfrons, o que aumentaria nestes animais a susceptibilidade ao desenvolvimento de hipertensão arterial e outras complicações renais. Da mesma forma, Amri *et al*. (1999) demonstraram que em ratos, a exposição do feto à hiperglicemia materna diminui a nefrogênese, reduz o número de néfrons e predispõe a prole ao desenvolvimento de insuficiência renal crônica e hipertensão arterial na vida adulta. E em um estudo posterior, Cavanal *et al*. (2007) demonstraram que ratos adultos normoglicêmicos provenientes de ratas diabéticas apresentam prejuízo da função renal e hipertensão arterial.

A respeito dos danos causados pela hiperglicemia materna sobre a função vascular da prole, Holemans *et al*. (1999), demonstraram redução do relaxamento dependente do endotélio em artérias mesentéricas de ratos adultos. Da mesma forma, Rocha *et al*. (2005) demonstraram um prejuízo da função endotelial em artérias mesentéricas de ratos adultos provenientes de ratas com diabetes e sugeriram que este efeito poderia estar relacionado ao o desenvolvimento de hipertensão arterial. Em nenhum destes estudos os mecanismos responsáveis pela disfunção endotelial foram estudados.

Recentemente, nosso grupo ao analisar ratos adultos provenientes de mães diabéticas verificou a existência de intolerância à glicose, resistência à insulina e hipertensão arterial em animais com 6 e 12 meses de idade. Além disso, quando analisada as artérias mesentéricas de resistência desses animais, também observou-se alterações na função vascular decorrentes principalmente do aumento na produção de prostanoides vasoconstrictores derivados da COX-2, como o TxA_2, a PGE_2 e a $PGF_{2\alpha}$, reduzindo o relaxamento dependente do endotélio e alterando a regulação da

respossividade noradrenérgica no leito mesentérico (Ramos-Alves *et al*., 2012a; Ramos-Alves *et al*., 2012b).

A ANG II como já mencionado é um dos efetores do sistema-renina angiotensina e promove alterações funcionais, tanto agudamente quanto em longo prazo, principalmente por ativação do receptor (AT_1) localizado nas células musculares lisas vasculares. Além de ser um potente agente contrátil, a ANG II, atua como fator pró-inflamatorio, hipertrófico, fibrótico e metabólico, nos quais podemos incluir como efeitos, a produção de espécies reativas de oxigênio (ROS) (Schiffrin & Touyz, 2004; Cheng *et al*., 2005; Pauletto & Rattazzi, 2006), resistência à insulina, deposição de matriz extracelular e estimulação da proliferação celular. Tais consequências são bem descritas por contribuir para ocorrência de respostas inflamatórias vistas na hipertensão. A ANG II também estimula a liberação de prostaglandinas em uma variedade de tipos celulares, incluindo as células musculares lisas vasculares através da ativação da fosfolipase A_2. Além disso, a ANG II regula a expressão de COX-2 e produção de prostanóides em ratos normotensos pela ativação do receptor AT_1 (Ohnaka *et al*., 2002; Hu *et al*., 2002).

O aumento na expressão e atividade vascular da COX-2 tem sido bem descrito em vários modelos de hipertensão (Hernanz *et al*., 2004; Adeagbo *et al*., 2005;; Virdis *et al*., 2009) e seus efeitos normalizados pelo tratamento com antagonista do receptor AT_1 (Alvarez *et al*., 2007), dando suporte para a participação da ANG II nesses efeitos. Em experimentos *in vitro,* a ANG II induz a expressão e ativação da COX-2 e a produção de prostanóides, os quais, de maneira geral, apresentam um papel fundamental nas alterações vasculares associadas à hipertensão (Ohnaka *et al*., 2000; Wong *et al*.,2011). Nesse contexto, prostanóides contráteis produzidos pela COX-2 e COX-1 contribuem para a redução do relaxamento dependente do endotélio observado nos vasos de humanos e em modelos murinos (Adeagbo *et al*., 2005; Feletou et al., 2011) Alvarez *et al*., 2005). Os efeitos específicos dos prostanóides envolvidos nessas alterações dependeram dos leitos vasculares estudados. Assim, PGH_2, $PGF_{2\alpha}$ e TxA_2 são responsáveis pela disfunção endotelial na hipertensão via receptor TP (Gluais *et al*., 2006). Embora a prostaciclina seja o principal

prostanóide vasodilatador produzido pela COX-2, crescentes evidencias apontam para uma função vasoconstrictora via receptor TP na hipertensão e baixa responsividade ao IP nas células musculares lisas vasculares (Bos *et al*., 2004; Xavier *et al*., 2010).

Vale ressaltar ainda o papel da ANG II na formação de EROs. Inúmeras evidências sugerem papel fundamental da ANG II no aumento da atividade da NAD(P)H oxidase (Alvarez *et al*., 2008). Por outro lado, as EROs podem regular a expressão dos receptores AT_1 (Pernomiam *et al*., 2012). O tratamento com antagonistas do receptor AT_1 e AT_2 promove redução de EROs associada a uma melhora na função cardiovascular (Briones & Touyz, 2010). Nesse sentido parece haver uma correlação entre a ação da ANG II e a produção de EROs (Briones & Touyz, 2010; Drummond *et al*., 2011; Dikalov & Nazarewicz, 2013).

Inúmeros estudos demonstram que as complicações vasculares e renais decorrentes do diabetes têm relação com o SRA (Jaques, 2013; Fried *et al*., 2009). Uma vez que a hipertensão é frequentemente associada a alterações no metabolismo da glicose, como resistência à insulina e intolerância à glicose, estudos recentes têm demonstrado que drogas que reduzem a formação da ANG II podem reduzir a incidencia do diabetes. No músculo a ANG II inibe a fosforilação do receptor para o substrato insulina (IRS), prevenindo o aumento do fosfatidil-inositol 3 (PI_3) quinase e subsequente translocação do transportador da glicose (GLUT-4) para a membrana celular (Velloso *et al*., 1996; Andreozzi *et al*., 2004). Nesse sentido, inibidores de ECA e bloqueadores para receptor da angiotensina tem aumentado a translocação da GLUT-4 para a membrana e melhorando a entrada de glicose para o musculo esquelético em modelos animais (Henriksen *et al*., 2001; Shiuchi *et al*., 2002). Portanto, tal fato pode contribuir para as complicações cardiovasculares inter-relacionadas entre a hiperglicemia e a hipertensão.

A participação da ANG II também tem sido implicada na hiperativação do sistema nervoso simpático via receptores pré-sinapticos específicos e na inativação da liberação do NO pela inervação nitrérgica observada em alguns

leitos vasculares de modelos de hipertensão (Encabo *et al.*, 1994; Molderings *et al.*, 1988; Ferrer *et al.*, 2001). A inervação perivascular, especificamente, a inervação adrenérgica, nitrérgica e sensitiva desempenha um papel importante sobre a regulação do fluxo sanguíneo do leito mesentérico, afetando o fluxo sanguíneo sistêmico e na pressão arterial (Hobara *et al.*, 2005; Toda, 1995; Ferrer *et al.*, 2001). Além do relatado na hipertensão, alterações na inervação perivascular, tem sido implicadas na gênese e manutenção de alterações vasculares associadas ao diabetes, e envelhecimento, mediante o desequibrio entre fatores vasodilatadores e vasoconstritores (Marín & Balfagón, 1998; Tatchum-Talom, 2004; Xavier *et al.*, 2004; Haddock & Hill, 2009). Entretanto, os mecanismos relacionados ao envolvimento da inervação perivascular na programação de doenças crônicas ainda não foram estudadas em prole de ratas diabéticas.

Numa perspectiva geral, pode-se então compreender neste estudo que diabetes mellitus durante o desenvolvimento intrauterino representa um importante fator para o desenvolvimento de doenças cardiovasculares na vida adulta. Dessa forma, haja vista o papel plurifuncional da ANG II relacionados à modulação da pressão arterial, disfunção vascular, e ativação na produção de fatores vasoativos no leito vascular mesentérico em outros distúrbios crônicos, torna-se relevante investigar se a programação fetal induzida pelo diabetes poderia causar um quadro hipertensivo na vida adulta induzida pelo SRA.

Concomitantemente, a ausência de estudos relacionados aos impactos do diabetes materno sobre a inervação perivascular de origem adrenérgica, nitrérgica e sensitiva da prole e a importância do leito mesentérico na regulação do fluxo sanguineo, reforçam a importância de uma investigação sobre esta inervação em artérias mesentéricas, visando a elucidação de possíveis mecanismos que possam estar envolvidos na gênese e/ou manutenção da hipertensão arterial neste modelo experimental.

OBJETIVOS

2. OBJETIVOS

Analisar o efeito do tratamento crônico com losartan, um antagonista de receptores AT1, sobre a sensibilidade à insulina, a pressão arterial e a reatividade vascular em artérias mesentéricas de resistência (AMR) de ratos adultos provenientes de ratas diabeticas, bem como, os efeitos em longo prazo deste distúrbio sobre a função da inervação perivascular adrenérgica, nitrérgica e sensitiva em artéria mesentérica superior.

2.1. Objetivos específicos

2.1.1. Avaliar o efeito do tratamento com losartan sobre a pressão arterial de ratos adultos com 6 e 12 meses de idade provenientes de mães diabéticas;

2.1.2. Avaliar nestes ratos possíveis alterações sobre a tolerância à glicose e sobre a sensibilidade à insulina;

2.1.3. Avaliar se o tratamento com losartan é capaz de alterar o relaxamento dependente e independente do endotélio em AMR de ratos adultos com 6 e 12 meses de idade;

2.1.4. Estudar nestas artérias se a ANGII produz alterações na participação da COX-2 sobre a disfunção endotelial encontrada neste modelo;

2.1.5. Determinar a participação da inervação simpática, sensitiva e nitrérgica sobre artéria mesentérica superior de ratos adultos expostos ao diabetes materno;

2.1.6. Quantificar em artérias mesentéricas as inervações adrenérgica e nitrégica, a liberação de NA, de ATP e de NO neuronal, a produção de anions superóxido, a expressão da nNOS e da P-nNOS.

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Angiotensin II via AT_1 receptors modulates COX-2-dependent vascular dysfunction in mesenteric resistance artery from offspring of diabetic rats

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ABSTRACT

The concept of "fetal programming" suggests that an individual can be "programmed" during intrauterine and perinatal stages to develop diseases in adulthood. The literature shows that maternal diabetes cause important metabolic changes in adult offspring, predisposing them to the emergence of cardiovascular diseases. This study examined whether hypertension and vascular dysfunction observed in adult rats subjected to maternal diabetes would be mediated by the actions of angiotensin II via AT1 receptor activation and changes in perivascular innervation in mesenteric artery preparations. Maternal diabetes induced by streptozotocin in Wistar rats. Changes in glucose homeostasis, such as glucose intolerance and insulin resistance was observed in offspring diabetic rats (O-DR) and reversed when treated with losartan. Through direct measurement of BP, MAP of rats (O-DR Losartan) showed normotensive levels compared to offspring diabetic rats (O-DR). In group (O-DR losartan) we observed an increase in the endothelium-dependent relaxation and a reduction in the contraction to phenylephrine compared to the rats (O-DR). To assess the involvement of arachidonic acid derived metabolites, COX-1 and 2 (indomethacin) or COX-2 (NS-398) both did not alter the relaxation and contractility significantly in the group O-DR Losartan. These results suggest that diabetes mellitus during intrauterine and perinatal phase causes metabolic, cardiovascular changes in offspring diabetic rats and that these alterations can be explained by the involvement of the Renin-Angiotensin System in increased production of vasoconstrictors factors, COX-2 pathway on changes in endothelial function in resistance arteries of the offspring of diabetic rats.

Introduction

In recent decades, various studies have examined the hypothesis that an environmental stimulus experienced *in utero* during the critical period of development can induce structural and functional changes in adulthood [1,2]. This concept comes from epidemiological studies by Barker and colleagues who evidenced an inverse relationship between low weight at birth and development [3,4]. However, other maternal status that produces adverse environment to fetal development, including chronic hyperglycemia, also increases the risk of metabolic and cardiovascular diseases in the offspring [5,6].

We [7,8] and others [9-11] have demonstrated that adult offspring of streptozotocin-induced diabetic rats are insulin resistant, hypertensive and presented reduced endothelium-dependent relaxation in adulthood. Our previous results, concerning diabetic offspring model (DO) pointed to an interesting model in which, in 3-, 6- and 12-month-old rats, a decrease in endothelium-dependent relaxation and a hyperreactivity to noradrenaline may related to up-regulation of cyclooxigenase-2 (COX-2) [7,8]. These results are consistent with other showing that pathological states accompanied by insulin resistance, such as type-2 diabetes, are associated with a pro-inflammatory state of the vascular wall leading to endothelial dysfunction and hypertension [12].

In healthy blood vessels, most prostanoids are produced by the constitutive isoform of cyclooxigenase (COX-1). COX-2 is expressed under

pathological conditions in several organs and cell types including the vascular wall [13-15]. At the vascular wall, the COX-2 expression has been reported in various pathological states associated with cardiovascular risk, such as hypertension, diabetes and metabolic syndrome [16]. COX-2-derived prostanoids play a role in vascular changes observed in hypertensive, diabetic and insulin resistant animals [8,13,16].

In several tissues, including the vascular wall, angiotensin (Ang) II is a potent COX-2 expression inducer [17,18]. Jaimes *et al*. [19] demonstrated that Ang II increased PGI_2 and PGE_2 release in renal glomeruli and mesangial cells through a COX-2-dependent mechanism. Other authors also showed increased expression of COX-2 by Ang II in renal tissue [20], vascular smooth muscle cells [17,18], coronary arteries [21] and aortic fibroblasts [22]. The expression of COX-2 induced by Ang II decreases after treatment with AT1 receptor antagonists, indicating the involvement of this receptor on expression of COX-2 induced by Ang II [13,16].

An increase in the angiotensin converting enzyme (ACE) activity has been demonstrated in kidney, lung and heart of offspring of diabetic rats [22]. This increase has been correlated with the development of hypertension in this and other animal models. Sharifi *et al*. [23] demonstrated increased ACE activity in kidney, heart, lungs and aorta of two kidney-one clip hypertensive rats during the development of hypertension in this model, suggesting an important role of tissue renin-angiotensin system in the development of hypertension. Our results obtained in adult offspring from diabetic rats [7,8] showed hypertension associated with COX-2-dependent vascular changes. Whereas these animals

show increased ACE activity in various tissues and that Ang II induces COX-2 expression, we hypothesize that the vascular changes observed in the offspring of diabetic rats may involve the participation of Ang II. Furthermore, it has been shown that Ang II is a potent inducer of insulin resistance in several animal models, which is also associated with pro-inflammatory events.

Therefore, the aim of the present study was analyze whether Ang II, through the activation of AT_1 receptors, is implicated in the increased participation of COX-2-derived contractile mediators in acethylcholine and noradrenaline responses observed in mesenteric resistance arteries from offspring of diabetic rats.

Material and methods

All procedures used in this study were performed in accordance with guidelines of the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes of Health Publication No.85-23, revised 1996) and was approved by the Ethics Committee of the *Centro de Ciências Biológicas* of *Universidade Federal de Pernambuco*. Wistar rats from colonies maintained at the Animal Quarters of *Departamento de Fisiologia e Farmacologia* of *Universidade Federal de Pernambuco*. Rats were housed at a constant room temperature humidity and a light cycle (12:12h light-dark), with free access to standard rat chow and tap water.

Animals

On the 7th day of pregnancy, diabetes was induced by a single injection of streptozotocin (STZ, 50 $mg.kg^{-1}$; i.p.). The diabetes was confirmed by measuring plasma glucose concentrations (ACCU-CHEK®, Roche Diagnostics, Mannheim, Germany). After birth, each litter was reduced to six pups and restricted to male offspring only. When the male number was not enough to complete six, females were used but discarded at weaning. The offspring were divided into four groups: Rats were divided into four groups: O-CR (offspring of control rats), O-DR (offspring of diabetic rats), O-CR-los (O-CR treated losartan) and O-DR-los (O-DR treated with losartan). Losartan was administered in the drink water at the dose of 15 $mg.kg^{-1}$ during 12 weeks. This study used only rats with 12 months of age.

Glucose tolerance and insulin resistance

The oral glucose tolerance test was performed according to a standard protocol. After a 10h fast, a single oral dose (2 $g.kg^{-1}$ of body weight) of glucose was delivered. Blood glucose was then measured from the tail vein just before, and 30, 60, 90 and 120 min after glucose injection, using test strips and reader (ACCU-CHEK®, Roche Diagnostics). After 48h, the animals were subjected to a new 10h fast for assessment of insulin sensitivity by insulin tolerance test. For this, regular insulin was administered i.p. at the dose of 1.5 U.kg-1 body weight. Blood glucose was determined before and 15, 30, 45 and 60 min after insulin administration.

Arterial blood pressure measurement

The animals were anesthetized with a mixture of ketamine, xylazine and acetopromazin (64.9, 3.2, and 0,78 $mgkg^{-1}$, respectively, ip.) The right carotid artery was cannulated with polyethylene catheter (PE-50) filled with heparinized saline. After 24h, mean arterial pressure were measured in conscious animals by a pressure transducer (Model MLT844, ADInstruments Pty Ltd, Castle Hill, Australia) and recorded using an interface and software for computer acquisition (ADInstruments Pty Ltd, Castle Hill, Australia).

Vascular Reactivity Study

Rats were anaesthetized with ketamine, xylazine and acetopromazin mixture (64.9, 3.2 and 0.78 $mg \cdot kg^{-1}$, respectively, i.p.) and killed by exsanguination. The mesenteric vascular bed was removed and placed in cold (4°C) Krebs-Henseleit solution (KHS; in mM: 115 NaCl, 2.5 $CaCl_2$, 4.6 KCl, 1.2 KH_2PO_4, 1.2, $MgSO_4.7H_2O$, 25 $NaHCO_3$, 11.1 glucose and 0.03 EDTA). For reactivity experiments, the third-order branch of the mesenteric arcade was dissected and cut into segments of approximately 2 mm in length. Segments of mesenteric resistance arteries were mounted in a small vessel chamber myograph (Danish Myo Technology A/S, Aarhus, Denmark) to measure isometric tension accordding to Mulvany and Halpern (1977).

Experimental protocols

After a 45 min equilibration period, each arterial segment was exposed to potassium chloride (KCl, 75 mM) to assess its maximum contractility. After a washout period, the presence of the vascular endothelium was confirmed by the ability of 1 mM acetylcholine (ACh) to relax segments precontracted with Noradrenaline at a concentration that produced approximately 50–70% of the contraction induced by KCl. The segments were rinsed with KHS for 1h and then a cumulative ACh concentration-response curve (0.1 nM to 3 mM) was obtained in the noradrenaline pre-contracted segments. After 60 min, cumulative concentration–response curves for noradrenaline (10 nM - 0.1 mM) were generated.

The possible role of COX-derived metabolites was investigated in segments from O-CR and O-DR treated and nontreated with losartan. Arteries were pre-incubated with either indomethacin (a COX-1 and COX-2 inhibitor, 10 mM) or NS-398 (COX-2 inhibitor, 10 mM), before generating concentration-response curves to ACh and noradrenaline. All drugs were added 30 min before the concentration-response curve to ACh.

Statistical analysis

All the results are expressed as mean ± SEM of the number of rats used in each experiment. Differences were analyzed using Student's t-test, one way or two-way ANOVA. When ANOVA showed a significant treatment effect, Bonferroni's post hoc test was used to compare individual's means (GraphPad Prism Software, San Diego, CA, E.U.A). Differences were considered statistically significant at $P<0.05$.

Results

Glucose tolerance and insulin resistance

Blood glucose levels were higher in O-DR at 30 min compared with O-CR rats and remained increased until the time of 120 min (Figure 1A). After the treatment with the AT_1 receptor antagonist Losartan, the blood glucose levels were similar in both O-DR and O-CR in all time-point analised (Figure 1B).. Results from the insulin tolerance test demonstrated significant insulin resistance among the O-DR rats, as they presented a higher blood glucose from 30 min to 90 min after an insulin injection (Figure 1C). O-DR-Los and O-CR-Los presented similar glucose levels at all time-point estudied (Figure 1D).

Assessment of mean arterial pressure

O-DR presented higher BP than O-CR (Figure 2). The treatment with losartan reduced blood pressure to a normotensive value in O-DR (O-DR-Los). The heart rate was similar in all groups studied (Results not shown).

Vascular function in adult offspring rats

KCl (75 mM) evoked similar contractions in vessels from all groups (O-CR: 2.06±0.06 vs. O-DR: 2.11±0.14 $mN \bullet mm^{-1}$ and O-CR-los: 2.01±0.07 vs. O-DR-los: 2.09±0.11 $mN \bullet mm^{-1}$; ANOVA, $P>0.05$).

ACh induced cumulative concentration and endothelium-dependent relaxation in noradrenaline-contracted arteries from all groups. This response

was impaired in arteries from O-DR which was normalized by the chronic treatment with Losartan (O-DR-los) (Figure 3A),.. The contractile response to noradrenaline was greater in arteries from O-DR than O-CR rats (Figure 3B). The treatment with losartan reduced this response to a similar level observed in O-CR. Losartan did not affect neither the relaxation to acetylcholine nor the vasoconstriction to noradrenaline in O-CR group (Figure 3A and 3B).

The selective COX-2 inhibitor NS-398 increase the vasoditatory response induced to acetylcholine in arteries from O-DR, but not in O-CR group (Figure 4A and 4B). In arteries from Losartan-treated O-DR and O-CR (O-DR-Los and O-CR-Los), NS-398 failed to produce any change in the acetylcholine-induced relaxation (Figure 4C and 4D).

Contractile reponse to noradrenaline was reduced by NS-398 in a greater extent in segments from O-DR than O-CR (Figure 5A and 5B). In arteries from O-DR-Los and O-CR-Los, NS-398 failed to produce any change in the noradrenaline response (Figure 5C and 5D)..

Discussion

The major finding of this study is that Ang II acting through AT_1 receptors produces long-lasting hypertension in male offspring of diabetic rats (O-DR) associated with changes in glucose homeostasis and increased participation of COX-2-derived products in the hyperreactivity to noradrenaline and endothelial dysfunction in mesenteric resistance arteries.

Exposure to diabetes *in utero* is a significant risk factor for development of metabolic syndrome components, including glucose intolerance, insulin resistance and hypertension [5,10,11]. In our study, O-DR exhibited changes in glucose metabolism, such as glucose intolerance and insulin resistance. However, treatment with losartan was able to reverse these changes in O-DR. Evidences suggest that Ang II modulates glucose homeostasis [24,25]. At the cellular level, Ang II induces insulin resistance by increasing oxidative stress, decreasing insulin receptor substrate (IRS)-1 phosphorylation and preventing glucose transporter (GLUT-4) translocation through cell membrane [26-28]. Ang II also induces oxidative stress, inflammation and apoptosis of pancreatic β-cells and it may indirectly impair insulin secretion by producing vasoconstriction and reducing islet blood flow [29,30]. Furthermore, ACE inhibitors or AT_1 receptor antagonists increase GLUT-4 translocation through cell membrane that may increase insulin sensitivity [27]. Thus, our results support that treatment with losartan decrease Ang II effect on glucose homeostasis changes in O-DR, improving glucose and insulin sensitivity in these animals.

RAS is a dynamic physiologic system that further affects glucose metabolism and plays a key role on blood pressure regulation and inflammation

process in several cardiovascular diseases, including diabetes and hypertension [30,31]. Wichi *et al*. [22] have reported increased angiotensin converting enzyme (ACE) activity in offspring of diabetic rats, which has been linked to increased blood pressure in these rats. In the present study, O-DR exhibited elevated blood pressure, without changes on heart rate, as previously reported by our group [7]. AT_1 receptor antagonist losartan significantly lowered blood pressure in O-DR, suggesting the involvement of Ang II on hyperglycemia-programmed hypertension. The hypertensinogenic effect of Ang II has been attributed various mechanism. Ang II stimulates hyperplasia and hypertrophy of vascular smooth muscle [32], induces vascular fibrosis [21,33], increases vasoconstriction and reduces vasodilator responses in hypertension [34]. Moreover, the role of endogenous Ang II in vascular inflammation has also been suggested [35].

The present and previous results [7,8] demonstrate that the participation of COX-2-derived contractile prostanoids in vasoactive responses to acetylcholine and noradrenaline is increased in O-DR. This effect contributed to impaired endothelium-dependent vasodilation and increased contractility to noradrenaline in mesenteric resistance arteries from O-DR. This is supported by the fact that the acetylcholine- or noradrenaline-induced responses were normalized by equally by both the COX-1/COX-2 inhibitor indomethacin and by the selective COX-2 inhibitor NS-398. In arteries from O-DR, an overepression of COX-2 and an increase in TxA_2, PGE_2 and $PGF_{2\alpha}$ were also demonstrated [7,8]

In several tissues, including the vascular system, Ang II increases prostanoids via COX-2 activity and/or expression [17,20,34]. Jaimes *et al*. [18] demonstrated increased production of COX-2-derived PGI2 and PGE2 in renal tissue after stimulation with Ang II. In vascular tissue Ang II also induces COX-2 expression [16], which decreases after treatment with antagonists of the AT_1 receptor [36]. To our knowledge, there are no reports analyzing the role of Ang II on the role of COX-2 on hyperglycemia-programmed vascular dysfunction.

Results obtained here suggest that AT_1 receptors activation by Ang II plays a role in the increased participation of contractile prostanoids in acetylcholine and noradrenaline responses in mesenteric reistance arteries from O-DR. This is based on fact that losartan treatment abolished the effect of indomethacin or NS-398 in acetylcholine-induced relaxation in arteries from O-DR, without effect in arteries from O-CR. Similarly, losartan treatment reduced the inhibitory effects of indomethacin or NS-398 in noradrenaline-induced vasoconstriction in arteries from O-DR but not in O-CR. After losartan treatment, no change in acetylcholine or noradrenaline responses in O-DR was observed.

It is known that inflammatory process itself is able to activate the RAS and contributes to vascular dysfunction and hypertension [14]. Numerous studies have shown evidence about the Ang II role in the increased vascular COX-2 expression on hypertension and diabetes [13,18,24]. In this study we demonstrated that the arachidonic acid-COX-2 axis is involved in vascular function regulating vasoconstrictor and vasodilator responses and that this effect is mediated by Ang II in offspring diabetic rats. It is worth mentioning the

role of ANG II in the generation of reactive oxygen species (ROS) in the vasculature from hypertensive rats [13,37]. A number of evidences suggest that Ang II is the main peptide involved in the activation of NADPH oxidase and consequently the production of ROS [14,34]. It has been demonstrated that proper expression of AT_1 receptors is modulated by ROS [36], a fact that creates a cycle of generation of products that act deregulating the vascular function. Furthermore, it is known that an up-regulation of COX-2 is already able to increase vascular oxidative stress [18]. Thus, arteries of O-DR could have endothelial dysfunction caused by an increased production of ROS due to increased activity of COX-2 and intense action of Ang II. However more studies should be conducted to investigate these mechanisms.

In conclusion, the results present here demonstrate that AT_1 receptor blockade normalized arterial pressure disturbances and insulin sensitivity in offspring of diabetic rats. Our results also show that Ang II acting through AT_1 receptor seems to play a role in the increased participation of COX-2-derived prostanoids on endothelial dysfunction and hyperreactivity to noradrenaline in resistance arteries from these rats. Thus, we suggest a reciprocal coupling between Ang II and insulin resistance, which may increased vascular COX-2 pathway and induce hypertension in offspring of diabetic rats.

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Figure legends

Figure 1. Blood glucose levels during intraperitoneal glucose and insulin test tolerance in O-DR, O-CR, O-DR-los and O-CR. Results are expressed as means±SEM. N=6-7 animals in each group. ANOVA (two way): *P<0.05 O-DR vs. O-CR.

Figure 2. Effect of treatment with losartan (Los) on mean arterial pressure (MAP) in offspring of diabetic (O-DR) and non-diabetic (O-CR) mothers. Results are expressed as means±SEM. N=6-7 animals in each group. ANOVA (one way): *P<0.05 O-DR vs. O-CR.

Figure 3. Concentration-response curves to acetylcholine and noradrenaline in mesenteric resistance arteries from O-CR and O-DR, untreated or treated (O-DR-Los and O-CR-Los) with Losartan.

Figure 4. Effect of 10µM NS-398 on the concentration-response curve to acetylcholine in mesenteric resistance arteries from untreated or Losartan-treated O-CR and O-DR.

Figure 5. Effect of 10μM NS-398 on the concentration-response curve to noradrenaline in mesenteric resistance arteries from untreated or Losartan-treated O-CR and O-DR.

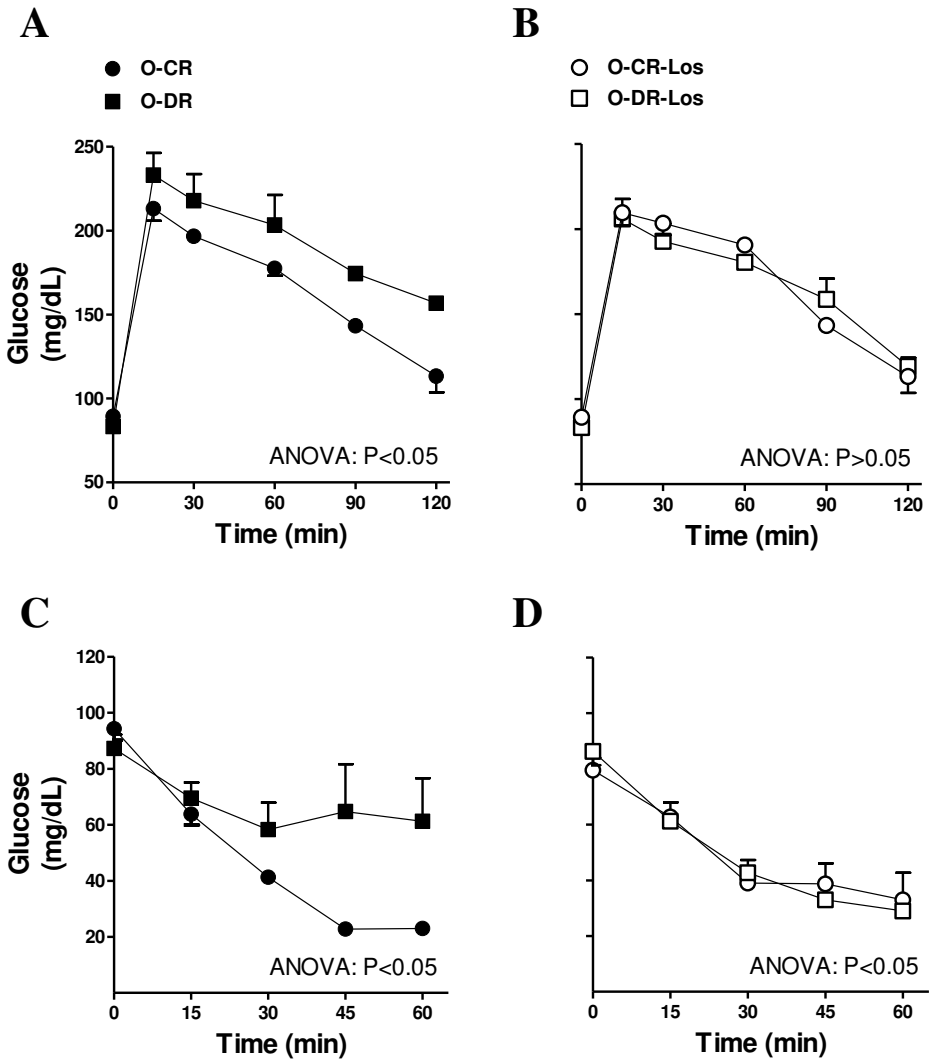

A
O-CR
O-DR
Glucose (mg/dL)
250
200
150
100
50
0
30
60
90
120
Time (min)
ANOVA: P<0.05
B
O-CR-Los
O-DR-Los
ANOVA: P>0.05
Time (min)
C
Glucose (mg/dL)
120
100
80
60
40
20
0
15
30
45
60
Time (min)
ANOVA: P<0.05
D
ANOVA: P<0.05
Time (min)

Figure 1

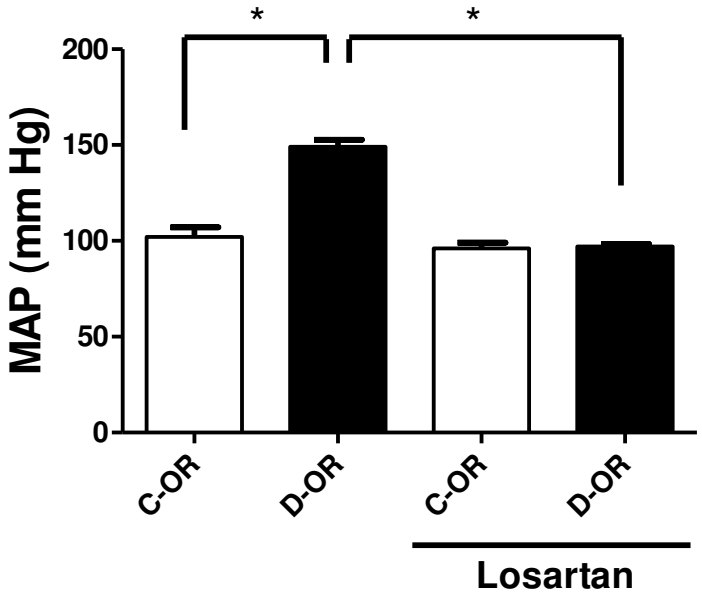
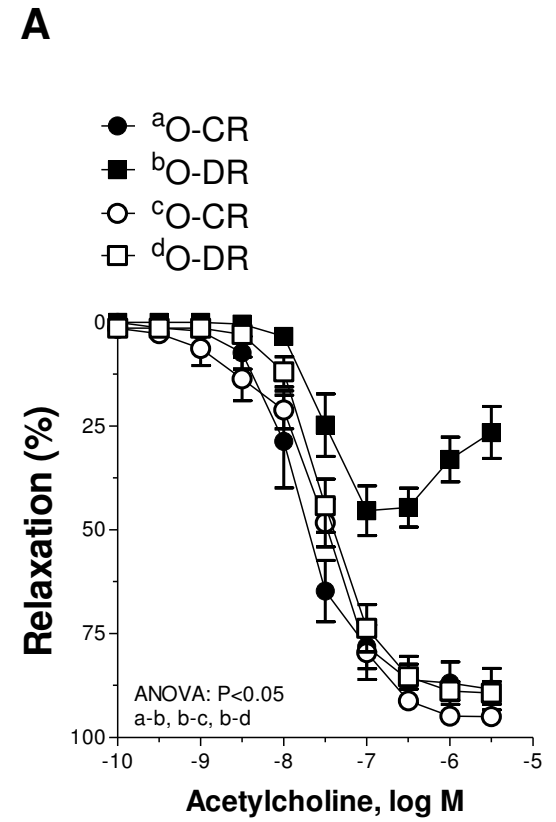


Figure 2


A
aO-CR
bO-DR
cO-CR
dO-DR
Relaxation (%)
0
25
50
75
100
ANOVA: P<0.05
a-b, b-c, b-d
-10
-9
-8
-7
-6
-5
Acetylcholine, log M

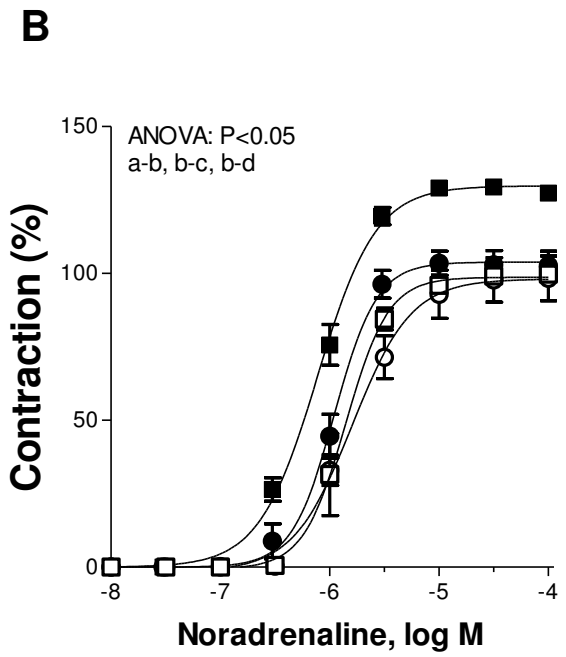

B
150
100
50
0
Contraction (%)
ANOVA: P<0.05
a-b, b-c, b-d
-8
-7
-6
-5
-4
Noradrenaline, log M

Figure 3

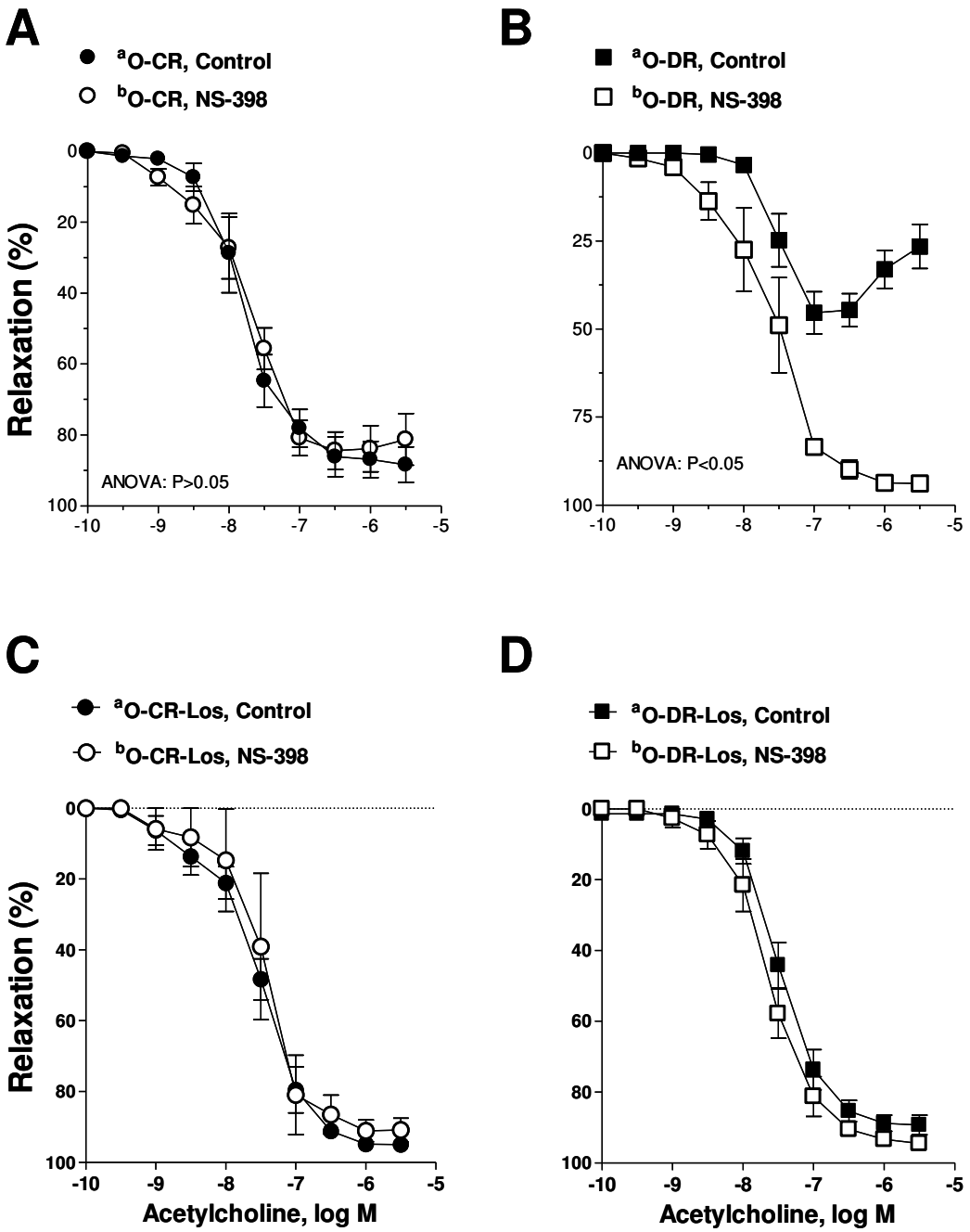

A
aO-CR, Control
bO-CR, NS-398
Relaxation (%)
ANOVA: P>0.05
B
aO-DR, Control
bO-DR, NS-398
ANOVA: P<0.05
C
aO-CR-Los, Control
bO-CR-Los, NS-398
Relaxation (%)
Acetylcholine, log M
D
aO-DR-Los, Control
bO-DR-Los, NS-398
Acetylcholine, log M

Figure 4

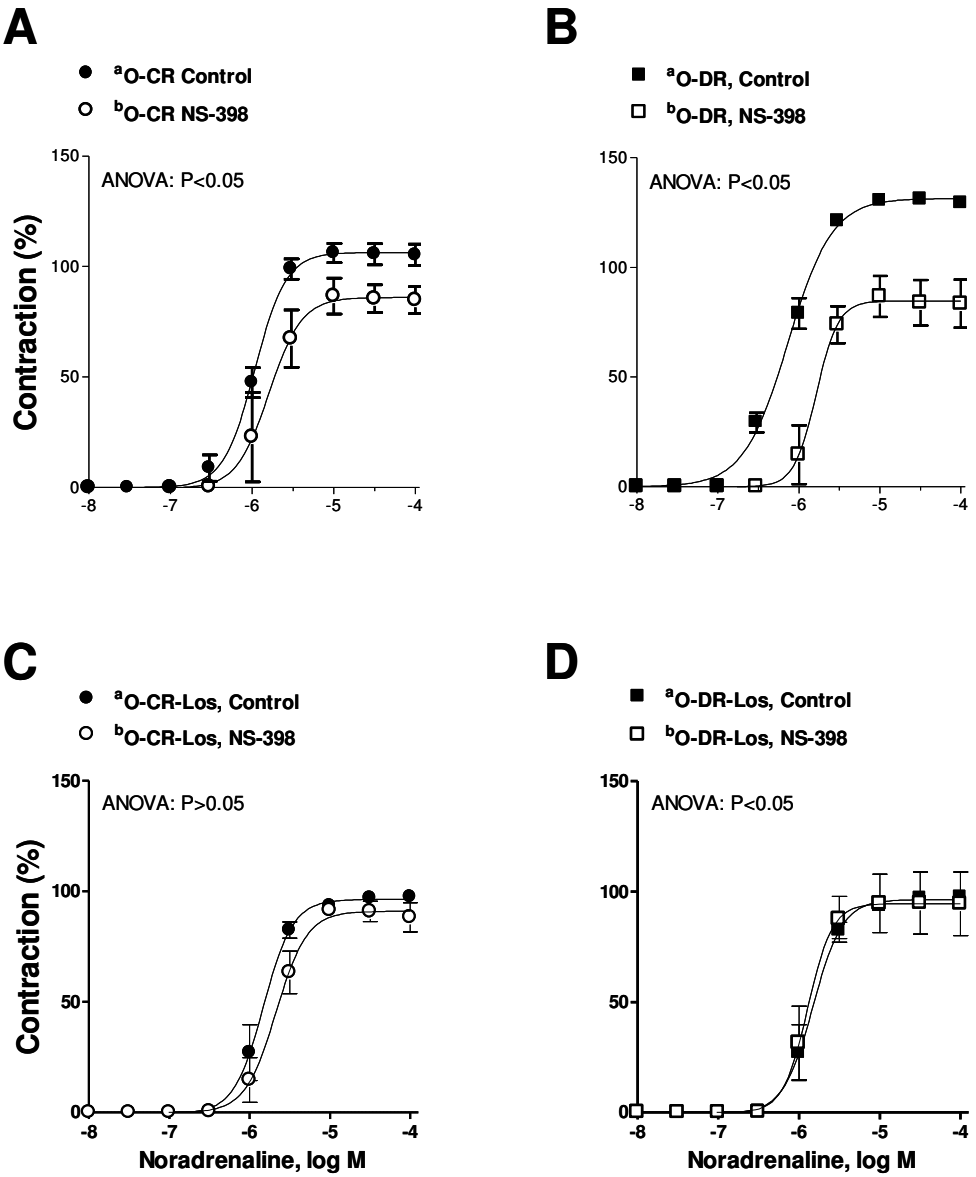

A
[a]O-CR Control
[b]O-CR NS-398
ANOVA: P<0.05
Contraction (%)
150
100
50
0
-8
-7
-6
-5
-4
B
[a]O-DR, Control
[b]O-DR, NS-398
ANOVA: P<0.05
C
[a]O-CR-Los, Control
[b]O-CR-Los, NS-398
ANOVA: P>0.05
Noradrenaline, log M
D
[a]O-DR-Los, Control
[b]O-DR-Los, NS-398
ANOVA: P<0.05
Noradrenaline, log M

Figure 5

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Alterations of perivascular innervation function in mesenteric artery from offspring of diabetic rats

Running title: Vascular innervation in offspring of diabetic rats

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Abstract

Background and purpose: The present study was designed to analyze the effect of *in utero* exposure to maternal diabetes on sympathetic and nitrergic function in superior mesenteric artery (SMA).

Experimental approach: Diabetes was induced in female Wistar rats by a single injection of streptozotocin (50 mg/kg body weight) on day 7 of pregnancy. De-endothelized vascular rings from 6-month-old offspring from control (O-CR) and diabetic rats (O-DR) were used. Vasomotor responses to electrical field stimulation (EFS), noradrenaline (NA) and nitric oxide (NO) donor DEA-NO were studied. Neuronal nitric oxide synthase (nNOS) and phospho-nNOS (P-nNOS) expressions were studied, and NA, adenosine triphosphate (ATP) and NO releases were also determined.

Key results: Blood pressure was significantly increased in 6-month old O-DR animals compared with age-matched O-CR. The vasoconstrictor response to EFS was greater in O-DR animals. This response was decreased more by phentolamine in O-DR animals than their controls. L-NAME increased vasoconstrictor response to EFS more strongly in O-DR than in O-CR segments. Vasomotor responses to NA or DEA-NO were not modified in our experimental conditions. NA, ATP and NO releases were increased in segments from O-DR. nNOS expression was not modified, while P-nNOS expression was increased in O-DR.

Conclusions and implications: The results show increased sympathetic and nitrergic innervation functions in SMA from O-DR animals. The net effect

produces an increase in EFS-induced contraction in these animals. These effects may contribute to the increased blood pressure in offspring of diabetic rats.

Key words: Diabetes; Fetal programming; Rat mesenteric artery; Perivascular innervation; Sympathetic innervation; Noradrenaline; ATP; Nitrergic innervation; Nitric Oxide.

Abbreviations: 4, 5- diaminofluorescein (DAF-2); Control offspring (O-CR); Diabetic Offspring (O-DR); Diethylamine Nonoate Diammonium Salt (DEA-NO); Electrical Field Stimulation (EFS); Noradrenaline (NA); Nitric Oxide (NO); Superior Mesenteric Artery (SMA)

Introduction

A growing body of evidence during the last decade suggests that adverse environmental conditions during crucial periods of development may cause profound structural and biochemical changes in the body, resulting in modifications that, in later life, can become threats to health (Barker, 2004; Visentin *et al.,* 2014). In line with this, maternal diabetes is a common medical complication in pregnancy that has been rapidly increasing worldwide. Both epidemiologic investigations and animal studies have shown that intrauterine exposure to a hyperglycemic environment can affect embryonic and fetal life, predisposing the offspring to develop metabolic disorders, including diabetes and hypertension in adult life (Simeoni and Barker, 2009).

The molecular mechanism underlying the association between diabetes during pregnancy and elevated blood pressure in the offspring remains unclear, and the relatively few existing studies rarely address vascular function. Holemans *et al* (1999) showed a reduced relaxation to endothelium dependent dilators and enhanced constriction to NA in small mesenteric arteries in offspring of diabetic rats. Rocha *et al* (2005) in this same model found early life hypertension with renal function impairment and decreased endothelium-dependent vasodilation in mesenteric microvessels. We ourselves, in mesenteric resistance arteries from the offspring of diabetic rats, have recently described an enhanced formation of vasoconstrictor prostanoids that contributes to a hyperrreactivity to noradrenaline that may participate in their hypertension (Ramos-Alves *et al.,* 2012a, 2012b).

It is well known that the vascular tone is determined by equilibrium among several mechanisms in which perivascular innervation plays an important role. Its regulation involves sympathetic, cholinergic, nitrergic, peptidergic and/or sensory innervations that are specific to the vascular bed under consideration (Loesch, 2002; Sastre *et al.,* 2010). The superior mesenteric artery regulates around 20% of total blood flow; thus, changes in mesenteric vascular tone participate in total peripheral resistance. The application of electrical field stimulation (EFS) produces a vasoconstrictor response that is the integrated result of the release of different neurotransmitters, including the sympathetic vasoconstrictors NA and ATP, NO from the nitrergic innervation and calcitonin gene-related peptide (CGRP) from sensory nerves (Kawasaki *et al.,* 1988; Li and Duckles, 1992; Marín and Balfagón, 1998; Blanco-Rivero *et al*., 2013b). Alterations in the functional role of these components in rat mesenteric artery have been associated with several experimental and pathophysiological conditions including hypertension, a high fat diet intake, ageing and portal hypertension (Marín *et al.,* 2000; Ferrer *et al.,* 2003; Blanco-Rivero *et al.,* 2011a; Sastre *et al.,* 2012a). Additionally, we have previously reported that diabetes alters perivascular innervation function in rat mesenteric arteries (Ferrer *et al.,* 2000; del Campo *et al.,* 2011).

Taking into account this information, we considered it relevant to investigate whether the development of maternal diabetes during pregnancy could lead to alterations in perivascular innervation participation in vascular tone of the adult offspring of diabetic rats. For this purpose we analyzed the possible functional changes of perivascular innervation in mesenteric artery from

offspring of diabetic rats evaluating possible alterations on sympathetic, nitrergic or sensory innervation, as well as, the possible mechanisms implicated.

Methods

Ethics Statement

All animals were obtained from the Animal Quarters of the Universidad Autónoma de Madrid and housed in the Animal Facility of the Universidad Autónoma de Madrid (Registration number EX-021U) in accordance with guidelines 609/86 of the E.E.C., R.D. 233/88 of the Ministerio de Agricultura, Pesca y Alimentación of Spain, and Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health [NIH publication No. 85.23, revised 1985]. The experimental protocol was approved by the Ethics Committee of the Universidad Autónoma de Madrid.

Animals

Virgin female Wistar rats were kept in cages with male rats in a 3:1 ratio for mating. Evidence of copulation was confirmed by the presence of sperm in vaginal smear, 24 hours afterwhich was considered to be day one of gestation. Seven days after the onset of pregnancy, as has been reported (Wichi *et al*., 2005) and we have previously used (Ramos-Alves *et al*., 2012a, 2012b) diabetes was induced by a single injection of streptozotocin (50 mg / kg, ip) Control animals received an equal volume of vehicle (citrate buffer). Blood glucose was measured seven days after injecting either streptozotocin or citrate buffer, to confirm whether or not diabetes had been induced; only rats with a severe hyperglycemia above 200 mg/dl were used. During lactation, the offspring were restricted to six animals per rat. This study used only 6-month-

old male offspring from diabetic mothers (O-DR) and control offspring (O-CR). Rats were weighed and sacrificed by CO_2 inhalation followed by decapitation; superior mesenteric artery was carefully dissected out, cleaned of perivascular fat and connective tissue and divided into 2 mm-long segments using a micrometer eyepiece mounted on a Euromex Holland binocular lens; and placed in Krebs–Henseleit solution (KHS, in mmol/L : NaCl 115, CaCl2 2.5, KCl 4.6, KH2PO4 1.2, MgSO4 . 7H2O 1.2, NaHCO3 25, glucose 11.1, Na2 EDTA 0.03) at 4°C. Some samples were immediately frozen in liquid nitrogen and stored at -70 °C. Additionally, some segments were mounted on 100 µm wires in a small vessel myograph to measure internal diameter (1-1.2 mm diameter; Simonsen *et al.,* 1999). No differences were found between the experimental groups.

Glucose tolerance and insulin sensitivity

The oral glucose tolerance test was performed according to a standard protocol. After a 10 h fast, a single oral dose (2 g/kg of body weight) of glucose was delivered. Blood glucose was then measured from the tail vein just before, and 30, 60, 90 and 120 min after glucose injection, using test strips and reader (ACCU-CHEK®, Roche Diagnostics). After 48 h, they were subjected to a new 10 h fast for assessment of insulin sensitivity by insulin tolerance test. For this, regular insulin was administered i.p. at the dose of 1.5 U/kg body weight. Blood glucose was determined before and 15, 30, 45 and 60 min after insulin administration.

Arterial blood pressure measurement

The animals were anesthetized with a mixture of ketamine, xylazine and acetopromazin (64.9, 3.2, and 0.78 mg/kg, respectively, ip.). The right carotid artery was cannulated with polyethylene catheter (PE-50) filled with heparinized saline. After 24h, mean arterial pressure was measured in conscious animals by a pressure transducer (Model MLT844, ADInstruments Pty Ltd, Castle Hill, Australia) and recorded using an interface and software for computer acquisition (ADInstruments Pty Ltd, Castle Hill, Australia). Heart rate was calculated from the pressure signal recorded by a pressure transducer and connected to a computer measuring program (LabChat v7, ADInstruments Pty Ltd, Castle Hill, Australia).

Serum bioquemicals parameters

Serum concentrations of total cholesterol, triglycerides and high density lipoprotein (HDL) cholesterol were determined using specific quantitative enzyme assay (Vitros Chemistry Products) and measured with colorimetric spectrophotometer (Vitros Fusion 5.1 FS Chemistry System, Ortho-Clinical). The assays were performed following the manufacturers' instructions. Results were expressed as mg/dL. Serum concentrations of insulin were analyzed using Rat/Mouse Insulin ELISA Kit (EMD Millipore Corporation. MA. USA). The assay was performed following the manufacturers' instructions. Results were expressed as µmol/L

Vascular Reactivity Study

The method used for isometric tension recording has been described in full elsewhere (Nielsen and Owman, 1971; Marín and Balfagón, 1998). Briefly, two parallel stainless steel pins were introduced through the lumen of the vascular segment: one was fixed to the bath wall, and the other connected to a force transducer (Grass FTO3C; Quincy, Mass.,USA); this, in turn, was connected to a model 7D Grass polygraph. For EFS experiments, segments were mounted between two platinum electrodes 0.5 cm apart and connected to a stimulator (Grass, model S44) modified to supply the appropriate current strength. Segments were suspended in an organ bath containing 5 mL of KHS at 37°C continuously bubbled with a 95% O_2 -5% CO_2 mixture (pH 7.4). Some experiments were performed in endothelium-denuded segments to eliminate the main source of vasoactive substances, including endothelial NO. This avoided possible actions by different drugs on endothelial cells that could have led to result misinterpretation. Endothelium was removed by gently rubbing the luminal surface of the segments with a thin wooden stick. The segments were subjected to a tension of 4.95 mN, which was readjusted every 15 min during a 90-min equilibration period before drug administration. After this, the vessels were exposed to 75 mmol/L KCl to check their functional integrity. Endothelium removal did not alter the contractions elicited by KCl. After a washout period, the presence/absence of vascular endothelium was tested by the ability of 10 µmol/L acetylcholine (ACh) to relax segments precontracted with 1 µmol/L noradrenaline (NA). We consider endothelium-denuded arteries those unable to relax to ACh

Vasodilator response to ACh (0.1 nmol/L-10mmol/L) was tested in endothelium-intact arteries from all experimental groups. Frequency-response curves to EFS, (1, 2, 4, 8 and 16 Hz, a range considered to reproduce physiological situations), were performed in endothelium-intact and endothelium-denuded mesenteric segments from all experimental groups. The parameters used for EFS were 200 mA, 0.3 ms, 1–16 Hz, for 30 s with an interval of 1 min between each stimulus, the time required to recover basal tone. A washout period of at least 1 h was necessary to avoid desensitization between consecutive curves. Two successive frequency-response curves separated by 1-hour intervals produced similar contractile responses. To evaluate the neural origin of the EFS-induced contractile response, the nerve impulse propagation blocker, tetrodotoxin, (TTX, 0.1mmol/L) was added to the bath 30 min before the second frequency-response curve was performed.

To determine the participation of sympathetic innervation in the EFS-induced response in endothelium-denuded segments from O-CR and O-DR rats, 1mmol/L phentolamine, an α-adrenoceptor antagonist or phentolamine plus 0.1 mmol/L suramin, a nonspecific P2 purinergic receptor antagonist, was added to the bath 30 min before performing the frequency-response curve. Additionally, the vasoconstrictor response to exogenous NA (1 nmol/L-10mmol/L) was tested in segments from both experimental groups.

To study the possible participation of sensory innervation in EFS-induced response in endothelium-denuded segments from O-CR and O-DR rats, 0.5 μmol/L CGRP (8–37), a CGRP receptor antagonist, was added to the bath 30 min before performing the second frequency–response curve.

To analyze the participation of NO in the EFS-induced response in endothelium-denuded segments from O-CR and O-DR rats, 0.1 mmol/L Nω-nitro-L-arginine methyl ester (L-NAME), a non-specific inhibitor of nitric oxide synthase (NOS), was added to the bath 30 min before performing the second frequency–response curve. The vasodilator response to the NO donor, diethylamine NONOate, (DEA-NO, 0.1 nmol/L–0.1 mmol/L) was determined in NA-precontracted arteries from both experimental groups.

Noradrenaline and ATP Release

Endothelium-denuded segments of rat mesenteric arteries from O-CR and O-DR animals were preincubated for 30 minutes in 5 mL of KHS at 37°C and continuously gassed with a 95% O2–5% CO2 mixture (stabilization period). This was followed by two washout periods of 10 min in a bath of 0.4 mL KHS. Then the medium was collected to measure basal release. Next, the organ bath was refilled and cumulative EFS periods of 30 s at 1, 2, 4, 8 and 16 Hz were applied at 1 min intervals. Afterwards, the medium was collected to measure EFS-induced neurotransmitter release. Mesenteric segments were weighed in order to normalize the results. NA and ATP releases were measured using Noradrenaline Research EIA (Labor Diagnostica Nord, Gmbh and Co., KG, Nordhon, Germany) or an ATP Colorimetric/Fluorometric Assay kit (Abcam, Cambridge, UK). The assays were performed following the manufacturers' instructions. Results were expressed as ng NA/mL mg tissue, or nmol ATP/mL mg tissue.

Nitric Oxide Release

NO release was measured using fluorescence emitted by the fluorescent probe 4,5-diaminofluorescein (DAF-2), as previously described (Blanco-Rivero *et al.,* 2011a). Endothelium denuded mesenteric arteries from O-CR and O-DR rats were subjected to a 60-minute equilibration period in HEPES buffer (in mmol/L: NaCl 119; HEPES 20; CaCl2 1.2; KCl 4.6; MgSO4 1; KH2PO4 0.4; NaHCO3 5; glucose 5.5; Na2HPO4 0.15; (pH 7.4) at 37 °C. Arteries were incubated with 2 µmol/L DAF-2 for 30 min. The medium was collected to measure basal NO release. Once the organ bath was refilled, cumulative EFS periods of 30 s at 1, 2, 4, 8 and 16 Hz were applied at 1 min intervals. Afterwards, the medium was collected to measure EFS-induced NO release. The fluorescence of the medium was measured at room temperature using a spectrofluorometer (LS50 Perkin Elmer Instruments, FL WINLAB Software, Whaltmann, MA, USA) with excitation wavelength set at 492 nm and emission wavelength at 515 nm.

The EFS-induced NO release was calculated by subtracting basal NO release from that evoked by EFS. Also, blank sample measures were collected in the same way from segment-free medium in order to subtract background emission. Some assays were performed in the presence of 0.1mmol//L TTX or 1mmol/ L 1400W, the specific iNOS inhibitor. The amount of NO released was expressed as arbitrary units/mg tissue.

Superoxide anion production

Superoxide anion levels were measured using lucigenin chemiluminescence (Blanco-Rivero *et al.,* 2011b). Endothelium-denuded mesenteric segments from O-CR and O-DR animals were rinsed in KHS for 30 min, equilibrated for 30 min in HEPES buffer at 37°C, transferred to test tubes that contained 1 mL HEPES buffer (pH 7.4) with lucigenin (5 µmol/L) and then kept at 37°C. The luminometer was set to report arbitrary units of emitted light; repeated measurements were collected for 5 min at 10 s intervals and averaged. 4,5-Dihydroxy-1,3-benzene-disulphonic acid “Tiron” (10 mmol/L), a cell-permeant, non-enzymatic superoxide anion scavenger, was added to quench the superoxide anion-dependent chemiluminescence. Also, blank samples were collected in the same way without mesenteric segments to subtract background emission.

Inmunofluorescence staining of nerve fibers

Superior mesenteric artery was immediately placed in cold phosphate buffer solution (PBS, in g/L): NaCl 8, Na2HPO4 1.15, KCl 0.2, KH2PO4 0.2 (pH 7.2). The whole vessel was fixed [4% paraformaldehyde in PBS, 50 min, room temperature (RT)]. After three 10-min PBS washing cycles nonspecific binding was blocked by incubating the samples for 1 h in 5% bovine albumin PBS+0.3% Tween 20 (PBS-T). The vessels were cut in segments and incubated with primary antibodies: rabbit polyclonal anti dopamine beta hydroxylase (sigma 1:200) or rabbit polyclonal anti nNOS (Abcam1:100) diluted

in 2% bovine albumin PBS-T. Thereafter, tissues were stained with the nuclear dye DAPI (1:500 dilution, 15 min, RT) and, after two PBS-T washing cycles, incubation with Alexa 647 anti-rabbit fluorescent secondary antibodies (1:200 dilution, 1 h, RT) was carried out. Negative controls were performed by omitting primary antibodies. After four PBS-T washing cycles, tissue preparations were mounted on a single well filled with antifading agent (Citifluor AF-2, Citifluor LTD).

Preparations were visualized with a Leica SP5 lasser scanning confocal microscopy (LSCM) system (Leica Microsystems, Wetzlar, Germany) fitted with an inverted microscope (× 40 oil immersion lens). Stacks of 2µm-thick serial optical images were captured from the entire adventitial layer, which was identified by the shape and orientation of the nuclei (Arribas *et al.*, 1997). Image acquisition was performed always under the same laser power, brightness, and contrast conditions.

Two to three different regions were scanned along each mesenteric artery and the resulting images were reconstructed separately with ImageJ 1.48c software (National Institutes of Health) to generate extended focus images. After obtaining the value 0 for the background, area fraction parameter was set to determine the areas occupied by fibers, defined as percentage of pixels with nonzero. Data were presented as percentage of area occupied by nerve fibers.

nNOs and P-nNOS Expression

Western blot analysis of nNOS and phosphorylated nNOS (P-nNOS) expression was performed in endothelium-denuded mesenteric segments from O-CR and O-DR rats, as previously described (Blanco-Rivero *et al.,* 2011a, 2011b). For these experiments, we used mouse monoclonal nNOS antibody (1:1000, Transduction Laboratories), rabbit polyclonal P-nNOS antibody (1:2000, Abcam, Cambridge, UK), and monoclonal anti-ß-actin-peroxidase antibody (1:50000, Sigma-Aldrich, Spain). Rat brain homogenates were used as positive control.

Drugs Used

L-Noradrenaline hydrochloride, ACh chloride, diethylamine NONOate diethylammonium salt, CGRP (8–37), TTX, L -NAME hydrochloride, 1400W, phentolamine, suramin sodium salt, lucigenin, tiron and DAF-2 (Sigma-Aldrich, Madrid, Spain) were used. Stock solutions (10 mmol/L) of drugs were made in distilled water, except for NA, which was dissolved in a NaCl (0.9%)-ascorbic acid (0.01% w/v) solution. These solutions were kept at –20°C and appropriate dilutions were made in KHS on the day of the experiment.

Data Analysis

The responses elicited by EFS and NA were expressed as a percentage of the initial contraction elicited by 75 mmol/L KCl for comparison between O-

CR and O-DR rats. The relaxation induced by ACh or DEA-NO was expressed as a percentage of the initial contraction elicited by NA (Control: 10.36± 0.85 mg; O-DR: 10.81±0.55 mg; $P>0.05$). For concentration-response curves, non-linear regression was performed. Results are given as mean ± S.E.M. Statistical analysis was done by comparing the curve obtained in the presence of the different substances with the previous or control curve by means of repeated measure analysis of variance (ANOVA) followed by the Bonferroni post-hoc test. Some results were expressed as differences of area under the curve (dAUC). AUC's were calculated from the individual frequency-response plots. For dAUC, NO, NA and ATP release experiments, the statistical analysis was done using one-way ANOVA followed by Newman-Keuls post-hoc test. Glucose tolerance, insulin sensitivity, arterial blood pressure, heart rate, results of general characteristics and immunofluorescence staining were analyzed using t-test. $P<0.05$ was considered significant.

Results

Dams injected with streptozotocin had sever hyperglycemia on gestational days 14 (control 829 ± 31 vs. diabetic 4572 ± 337 mg/L, t-test: $P<0.05$) and 21 (control 872 ± 26 vs. diabetic 4806 ± 394 mg/L, t-test: $P<0.05$) compared with control dams. Blood glucose levels were similar in O-CR an O-DR at 3-month-old (O-CR 764 ± 37 vs. O-DR 743 ± 28 mg/L, t-test: $P>0.05$) and 6-month-old (O-CR 788 ± 62 vs. O-DR 792 ± 78 mg/L, t-test: $P>0.05$).

Oral glucose tolerance test revealed that blood glucose levels were higher in O-DR at 30 min compared with O-CR (Results not shown) and remained increased until the time of 120 min (O-CR, 1133 ± 97 vs. O-DR, 1568 ± 34 mg/L, t-test: $P<0.05$). Results from the insulin sensitivity test demonstrated significant insulin resistance in the O-DR, as they presented a higher blood glucose from 30 min to 60 min after an insulin injection (blood glucose 60 min after the insulin injection; O-CR, 230 ± 15 vs. O-DR, 612 ± 97 mg/dL, t-test: $P<0.05$).

O-DR presented higher blood pressure than O-CR (Mean arterial pressure: O-CR: 119 ± 6.5 vs. O-DR: 137 ± 0.5 mmHg, t-test, $P<0.05$). The heart rate was similar in O-DR as compared with O-CR (O-CR: 392 ± 21 vs. O-DR: 397 ± 32 bpm, t-test, $P>0.05$).

Body weight was decreased in O-DR group (Table 1). Serum insulin, total cholesterol, HDL and triglycerides were similar in both experimental groups (Table 1).

Vasomotor Response to KCl

In endothelium-intact mesenteric segments, the vasoconstrictor response to 75 mmol/L KCl was similar in both experimental groups (O-CR: 13.8 $\pm$ 1.1 mN; O-DR: 15.53 $\pm$ 1.18 mN; $P>0.05$). Endothelium removal did not alter KCl-induced vasoconstriction (O-CR: 15.21 $\pm$ 1.5 mN; O-DR: 14.59 $\pm$ 10.9 mN; $P>0.05$).

Vasodilator response to ACh

ACh induced cumulative concentration and endothelium-dependent relaxations in noradrenaline-contracted arteries (O-CR: 10.26 ± 0.84 mN; O-DR: 10.71 ± 0.55 mN; $P>0.05$) from both the O-CR and O-DR groups. However, the exposure to maternal diabetes decreased this vasodilator response as compared to the response in the O-CR group (Figure 1A).

Vascular Responses to EFS

The application of EFS induced a frequency-dependent contractile response in endothelium-intact mesenteric segments from both O-CR and O-DR groups. This vasoconstriction was greater in segments from O-DR rats compared to O-CR animals (Figure 1B). Endothelium removal increased EFS-induced contractile response similarly in segments from all experimental groups (Figures 1C and 1D). EFS-induced contractions were practically abolished in

segments from all experimental groups by the neurotoxin TTX (0.1 mmol/L), indicating the neuronal origin of the factors inducing this response (Table 2).

Participation of the sympathetic component of mesenteric vascular innervation

Preincubation with the α-adrenergic antagonist phentolamine (1 μmol/L) decreased the vasoconstrictor response induced by EFS in endothelium-denuded segments from both experimental groups (Figures 2A and 2B). This decrease was greater in mesenteric segments from O-DR animals (Figure 2C). Noradrenaline-induced vasoconstriction was similar in both experimental groups (Figure 3A). EFS-induced NA release was higher in mesenteric segments from the O-DR group than in segments from O-CR animals (Figure 3B). There was a remnant phentolamine-resistant contractile response, which was greater in mesenteric segments from O-DR animals (Figure 2D). Preincubation with phentolamine plus 0.1 mmol/L suramin, a non-specific P2 purinergic receptor antagonist, decreased EFS-induced contraction only in segments from O-DR group (Figures 2A and 2B). In line with this, EFS-induced ATP release was increased in segments from O-DR animals (Figure 3C).

LSCM allowed visualization of the sympathetic nerve network in the adventitial layer of mesenteric arteries through reconstruction of stacks of images with dopamine beta hydroxylase immunoreactivity (Figure 4A, 4B). Quantification of nerve fibers density confirmed no differences between both experimental groups (Figure 4C).

Participation of the sensory component in vascular responses to EFS

Preincubation with the CGRP receptor antagonist CGRP (8–37) (0.5 μmol/L) did not alter the EFS-induced contraction in any experimental group (Figure 5).

Effect of maternal diabetes on neuronal nitrergic component of vascular response

Preincubation with unspecific NOS inhibitor L-NAME (0.1 mmol/L) significantly increased the EFS-contractile response in endothelium-denuded segments from both experimental groups (Figures 6A and 6B). This increase was greater in segments from O-DR animals (Figure 6C). EFS induced NO release in segments from both groups. This release was higher in O-DR mesenteric segments (Figure 7A). TTX practically abolished EFS-induced NO release, while 1400W did not modify in either experimental group (Figure 7A). The expression of nNOS was not modified, while P-nNOS expression was increased in homogenates from O-DR arteries compared to expression in O-CR segment homogenates (Figure 7B). Maternal gestational diabetes altered neither the vasodilator response to DEA-NO (NA pre-contraction: Control: 10.33 ± 0.74 mN; O-DR: 10.46 ± 0.65 mN; $P>0.05$) nor superoxide anion release in the offspring (Figure 7C and 7D).

LSCM allowed visualization of the nitrergic nerve network in the adventitial layer of mesenteric arteries through reconstruction of stacks of images with nNOS immunoreactivity (Figure 8A, 8B). Quantification of nerve

fibers density confirmed no differences between both experimental groups (Figure 8C).

Discussion

This study provides the first evidence that maternal diabetes alters the participation of the different kinds of perivascular mesenteric innervation in adult offspring. The results presented here demonstrate that *in utero* exposure to maternal hyperglycemia increases EFS-induced vasoconstriction in adulthood. This increase is endothelium-independent, and is the net effect of an increased participation of sympathetic innervation, through increased NA and ATP releases, and of augmented nitrergic innervation function, associated to increase NO production.

O-DR showed significant lower body weight compared with O-CR. This result agrees with previous reports showing that maternal diabetes induced by STZ produces a decrease on the offspring weight (Grill *et al*., 1991; Holemans *et al*., 1997; Porto *et al*., 2010; Ramos-Alves *et al*., 2012a), which has been associated with reduced protein synthesis (Canavan & Goldspink, 1998). Meanwhile, several studies describe no changes or increases on offspring body weight in this experimental procedure (Rocha *et al*., 2005; Nehiri *et al*., 2008). This controversy can be due to differences on the severity of mother hyperglycemia during pregnancy, as previously suggested by Segar *et al* (2009).

Intrauterine exposure to maternal hyperglycemia is a significant risk factor for the development of metabolic and cardiovascular disorders in the offspring. In the current study 6-month-old male offspring of diabetic mothers manifest glucose intolerance, insulin resistance and elevated blood pressure, as we have described previously (Ramos-Alves *et al*. 2012a, 2012b) and others

(Simeoni and Barker 2009). Glucose and insulin levels and lipid profile were similar in both experimental groups, in agreement with Blondeau *et al*. (2013). The mechanisms of hyperglycemia-programmed hypertension are complex and involve renal, neural and vascular factors (Rocha *et al.,* 2005; Wichi *et al.,* 2005; Nehiri *et al.,* 2008; Segar *et al.,* 2009; Chen *et al.,* 2010). The analysis of vasoconstrictor response induced by EFS in endothelium-intact segments showed a frequency-dependent contraction in segments from both experimental groups. This vasoconstriction was greater in mesenteric segments from O-DR than O-CR animals. This increase was not attributable to changes in the intrinsic contractile machinery as was demonstrated by the similar vasoconstrictor response to KCl in all experimental groups. Endothelium has been described to affect the response to several substances including neurotransmitters like NA (Vanhoutte and Houston, 1985; Li and Duckles, 1992). Since we observed that the relaxation to Ach was impaired in O-DR compared to O-CR rats, as we and other groups previously reported (Holemans *et al.,* 1999; Rocha *et al.,* 2005; Segar *et al.,* 2009; Ramos-Alves *et al.,* 2012a), we expected differences in the influence of endothelium on the vasoconstrictor response to EFS in segments from both experimental groups. However, endothelium removal increased this vasoconstriction to the same extent in both experimental groups. These results indicate that endothelial dysfunction does not influence the EFS-induced response in diabetic offspring, similarly to described on this artery in rats feed with a high fat diet (Sastre *et al*., 2015). One possible explication could be that the vasoconstrictor response to alpha-adrenergic activation has been referred to be maintained in absence of endothelium (Xavier *et al.,* 2004a) independently of the modifications on the

vasodilator response to Ach. In endothelium-denuded segments EFS-induced vasoconstriction was almost abolished in the presence of the neurotoxin TTX, indicating a neuronal origin for this response. The remnant contractile response to high frequency-stimulation could be explained by a direct action potential produced in smooth muscle cells elicited by EFS. For that reason, we performed the following experiments in endothelium-removed mesenteric segments.

Sympathetic function is essential to blood pressure regulation, and sympathetic hyperactivity has an important role in the development of hypertension (Lohmeier, 2001). Sympathetic innervation mainly releases NA and ATP when electrically stimulated. This innervation function is altered in different physiological and pathological situations (Sastre *et al.,* 2010, 2012). Increased sympathetic activity has been reported in O-DR (Young and Morrison, 1998; Iellamo *et al.,* 2006; de Almeida Chaves Rodrigues *et al.,* 2013). This effect could be associated with an increased NA release and/or vasoconstrictor response to NA. To the best of our knowledge, although increased NA release in different tissues has been suggested (Morris, 1984; Iellamo *et al.,* 2006), as well as increases or no modifications in NA vasoconstriction (Holemans *et al.,* 1999; Ramos-Alves *et al.,* 2012b), an integrated study of both mechanisms has not yet been performed in arteries. Thus, our next objective was to determine possible differences in the function of sympathetic innervation between O-CR and O-DR experimental groups. The fact that the α-adrenergic antagonist phentolamine significantly diminished the vasoconstrictor response to EFS in mesenteric segments from both

experimental groups confirms that this response would be mediated mainly by the release of NA from sympathetic nerve terminals. Moreover, our study showed that phentolamine produced a more marked decrease in EFS vasoconstriction in segments from OD-R rats than O-CR, confirming an increase involvement by sympathetic innervation in this experimental group. This different participation can be produced due to modifications in NA vasoconstrictor response and/or release. Exogenous NA-induced vasoconstriction was similar in both experimental groups, similarly to the vasoconstriction described in endothelium-denuded mesenteric resistance arteries (Ramos-Alves *et al.,* 2012b), while EFS-induced NA release was higher in segments from O-DR animals, confirming that the increased adrenergic function in O-DR is associated to increased NA release.

It should also be mentioned that both groups showed a substantial phentolamine-resistant contractile response. This remnant vasoconstriction was higher in segments from O-DR animals. We have previously observed that ATP released from sympathetic nerves cause vasoconstriction on this vascular bed (Blanco-Rivero *et al.,* 2011a, 2013a, 2013b). Based on this information, we analysed EFS-induced contraction after simultaneous preincubation with phentolamine plus the non-specific P2 purinergic receptor antagonist suramin. In these conditions, the contractile response to EFS was reduced only in segments from O-DR group, indicating a contribution of ATP in this response. In line with these results, EFS-induced ATP release**,** which was higher in mesenteric segments from O-DR than O-CR rats, similarly to previously described (Rummery *et al*., 2007; Sousa *et al.,* 2014), suggesting an increased

contribution of ATP in neurovascular transmission in hypertension. Altogether, these observations indicate that the increase in the participation of sympathetic innervation observed in mesenteric segments from O-DR animals is due to an increase in NA and ATP releases from nerve terminals. Increased sympathetic neurotransmitters release could be due to an increased in nerve fibers density, thus, our next objective was to determine possible differences in the sympathetic nerve density. Results obtained by immunofluorescence staining for dopamine beta hydroxylase showed no differences between both experimental groups. These results rule out an increased in sympathetic nerve density and suggest that the augmented NA and ATP releases could be related to an increase in enzymatic activity implicated in the synthesis of both neurotransmitters. On the other hand, neurotransmitters release on sympathetic innervation is mediated by several subtypes of adrenoceptor (Sanchez-Merino *et al.,* 1990; Arribas *et al.,* 1991; Enero *et al.,* 1997; Kanagi, 2005), whereby presynaptic dysfunction cannot rule out.

We have previously reported that CGRP released from sensory innervation does not participate in the vasoconstrictor response to EFS (Blanco-Rivero *et al.,* 2011a, 2011c), although this release is increased in several pathological situations, such as hypertension and diabetes (Blanco-Rivero *et al.,* 2011c, del Campo *et al.,* 2011; Sastre *et al.,* 2012a). Preincubation with the CGRP receptor antagonist CGRP (8–37) did not modify EFS-induced vasoconstriction in either O-CR or O-DR mesenteric segments. This observation led us to conclude that maternal diabetes did not alter sensory innervation function in this vascular bed in adulthood.

A decrease in endothelial NO release has been reported to participate in the development of hypertension (Wu *et al.,* 1997; Bertanova 2014). Similar results have been observed in rat aorta and mesenteric resistance arteries from offspring of diabetic rats (Holemans *et al.,* 1999; Cavanal *et al.,* 2007; Ramos-Alves *et al.,* 2012a)**.** However, there are no data studying the possible role of neuronal NO in this model. The fact that previous studies have reported increases in endothelial NO release due to ATP-induced activation of P2X4 and P2Y receptors and augmented NO synthase activity due to endothelial α_2D-adrenoceptor activation by NA in rat mesenteric arteries (Boric *et al.,* 1999; Buvinic *et al.,* 2002; Codocedo *et al.,* 2013), as well as our earlier report of increased neuronal NO in both diabetes and hypertension (Ferrer *et al.,* 2000, 2003; Marín *et al.,* 2000; del Campo *et al.,* 2011), make it necessary to evaluate whether a hyperglycemic *intra utero* environment could also affect the synthesis of NO from nitrergic neurons. In our experimental conditions, the involvement of neuronal NO in the EFS-induced response was demonstrated by the fact that preincubation with the unspecific NOS inhibitor L-NAME increased the response to EFS in segments from both experimental groups. The greater effect of L-NAME observed in segments from O-DR rats suggests an increased role for neuronal nitric oxide in this experimental group, possibly related to increases in nitric NO and/or increases in smooth muscle sensitivity to NO.

In our experimental conditions, we observed an increased EFS-induced NO release in mesenteric segments from O-DR animals. The fact that preincubation with TTX abolished EFS-induced NO release in segments from both groups of rats, and that preincubation with the specific iNOS inhibitor

1400W did not alter NO release, confirms the neural origin and rules out the inducible origin of the NO. We have previously demonstrated in this vascular bed that NO released from nerve endings is synthesized through nNOS (Blanco-Rivero *et al*, 2011a, 2011b, 2013a, 2013b). The increase in neuronal NO could be due to an increased in nitrergic nerve fibers density or an increased in the enzymatic activity of nNOS. Thus, our next objective was to determine possible differences in the nitrergic nerve density. Immunofluorescence staining for nNOS showed no differences between experimental groups, suggesting that the differences in NO release could be due to modifications in expression and/or activation of nNOS. We found that nNOS protein expression was similar in both experimental groups, whereas the active form P-nNOS was increased in O-DR rats, indicating that an increased nNOS activity is responsible for the increase in NO release observed in O-DR mesenteric artery. These results contrast to observations in endothelium-intact arteries (Holemans *et al.,* 1999; Rocha *et al.,* 2005; Segar *et al.,* 2009; Porto *et al.,* 2010), where a decreased NO released from eNOS was observed. Similar differences in this vascular bed have been previously described in several pathological situations (Wu *et al.,* 1997; Ferrer *et al.,* 2000, 2003; Favero *et al.,* 2012).

Several observations indicate that diabetic pregnant rats and their offspring are exposed to an increased oxidative stress induced by the absence of adequate free radical scavenger system production (Horal *et al.,* 2004; Li *et al.,* 2005; Katkhuda *et al.,* 2012). Thus, the involvement of reactive oxidative species in the vascular response to NO cannot be ruled out, since these

species could alter the metabolism and consequently affect neuronal NO bioavailability. Superoxide anion production was similar in both experimental groups, contrasting to previous studies (Horal *et al.,* 2004; Li *et al.,* 2005; Katkhuda *et al.,* 2012). These differences can be attributed to the different tissues analyzed, as well as to the method of inducing maternal diabetes. As was expected from the above results, the vasodilator response to the NO donor DEA-NO was similar in segments from both experimental groups, and similar to responses in aorta and mesenteric resistance arteries (Holemans *et al.,* 1999; Katkhuda *et al.,* 2012; Ramos-Alves *et al.,* 2012)**.** Altogether, these results confirm that the increased function of the nitrergic innervation is due to increased neuronal NO release and not to changes in the vasodilator response and/or metabolism of neuronal NO.

A reciprocal interaction between sympathetic and nitrergic innervation has been described in several vascular beds (Koyama *et al.,* 2010; Hatanaka *et al.,* 2006; Lee 2002). However in superior mesenteric artery we have described an increased in nitrergic function with no change (Xavier *et al.,* 2004b); decrease (Sastre *et al.,* 2012a; Sastre *et al.,* 2012b) and increase in (Marín *et al.,* 2000; Ferrer *et al.,* 2000; Ferrer *et al.,* 2003; del Campo *et al.,* 2011) adrenergic function as the current study. These facts indicate that modifications on adrenergic and nitrergic innervation functions in OD-R are independent and lead us to consider as primary changes.

In conclusion, maternal diabetes increases contractile response to EFS in superior mesenteric artery from adult offspring. This effect is endothelium-independent and is the net result of increased sympathetic vasoconstrictors NA

and ATP along with an augmented neuronal NO release, while sensory innervation function remains unaltered. This mechanism could be involved in the genesis of hypertension in the adult life of offspring from diabetic mothers, reinforcing the concept of fetal programming of chronic diseases.

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Competing interests

None

Author contributions

Conceived and designed the experiments: DBQ, FEX, JBR, GB. Performed the experiments: DBQ, ES, LC, MC. Analyzed the data: DBQ, ES, LC, MC, JBR. Contributed reagents/materials/analysis tools: FEX, JBR, GB. Wrote the manuscript: DBQ, ES, FEX, GB, JBR.

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Figure Legends:

Figure 1: (A) ACh-induced vasodilation in endothelium-intact mesenteric segments from O-CR and O-DR rats. Results (mean ± S.E.M.) were expressed as a percentage of the previous tone elicited by exogenous NA. n = 12 animals each group. (B) EFS-induced vasoconstriction in endothelium intact mesenteric segments from O-CR and O-DR rats. Results (mean ± S.E.M.) were expressed as a percentage of the initial contraction elicited by KCl. ANOVA $P<0.05$ O-CR vs. O-DR. *$P<0.05$ vs. O-CR animals at each frequency (Bonferroni test). n = 12 animals per group. Effect of endothelium removal on the vasoconstrictor response to EFS in mesenteric segments from O-CR (C) and O-DR (D) animals. Results (mean ± S.E.M.) were expressed as a percentage of the initial contraction elicited by KCl. ANOVA $P<0.05$ endothelium-intact vs. endothelium-denuded arteries. *$P<0.05$ vs. O-CR animals at each frequency (Bonferroni test). n = 12 animals per group. (E) Differences of area under the curve (dAUC) in the absence or presence of endothelium.

Figure 2: Effect of preincubation with 1 µmol/L phentolamine or phentolamine plus 0.1 mmol/L suramin on the vasoconstriction response induced by EFS in endothelium-denuded mesenteric segments from O-CR (A) and O-DR animals (B). Results (mean ± S.E.M.) were expressed as a percentage of the initial contraction elicited by KCl. ANOVA $P<0.05$ vs. conditions without phentolamine or suramin in both experimental groups *$P<0.05$ vs. conditions without phentolamine at each frequency (Bonferroni test). # $P<0.05$ vs. conditions without suramin at each frequency (Bonferroni test). n= 8 animals each group.

(C) Differences of area under the curve (dAUC) in the absence or presence of 1 μmol/L phentolamine. dAUC values are expressed as arbitrary units. (D) Representation of remnant vasoconstriction after preincubation with 1 μmol/L phentolamine, expressed as area under the curve (AUC, in arbitrary units).

Figure 3: (A) Vasoconstrictor response to NA in segments from O-CR and O-DR. Results (mean ± S.E.M.) were expressed as a percentage of the initial contraction elicited by KCl. n = 8 animals each group. EFS-induced NA (B) and ATP (C) releases in mesenteric segments from O-CR and O-DR animals. Results (mean ± S.E.M.) were expressed as ng NA/mL mg tissue or nmol ATP/mL mg tissue). n = 8 animals per group.

Figure 4: Dopamine beta hydroxylase immunoreactivity in the adventitia of mesenteric arteries from O-CR (A) and O-DR (B) animals. Tissues were stained with primary monoclonal dopamine β-hydroxilase (DBH) antibody and a species specific secondary Alexa 647 antibody. (C) Percentage of area occupied by sympathetic nerve fibers. n = 6 animals per group.

Figure 5: Effect of preincubation with 0.5 μmol/L CGRP (8-37) on the vasoconstrictor response induced by EFS in mesenteric segments from O-CR (A) and O-DR (B) animals. Results (mean ± S.E.M.) are expressed as a percentage of the previous contraction elicited by KCl. n = 8 animals each group.

Figure 6: Effect of preincubation with 0.1 mmol/L L-NAME on the vasoconstrictor response induced by EFS in mesenteric segments from O-CR (A) and O-DR (B) rats. Results are expressed as a percentage of the previous contraction elicited by KCl. ANOVA $P<0.05$ vs. conditions without phentolamine or suramin in both experimental groups. *$P<0.05$ vs. conditions without L-NAME for each frequency (Bonferroni test). n = 8 animals each group. (C) Differences of area under curve (dAUC) in the absence or presence of 0.1 mmol/L L-NAME, dAUC values are expressed as arbitrary units.*$P<0.05$.

Figure 7: (A) EFS-induced NO release in segments from O-CR and O-DR rats. Results (mean ± S.E.M.) were expressed as arbitrary (A.U.)/mg tissue; n = 8 animals per group. (B) Effect of exposure to maternal hyperglicemia on nNOS and P-nNOS expression. The blot is representative of eight separate segments from each group. Rat brain homogenates were used as a positive control. Lower panel shows relation between P-nNOS or nNOS expression and β-actin. Results (mean ± S.E.M.) are expressed as ratio of the signal obtained for each protein and the signal obtained for β-actin. (C) Vasodilator response to NO donor DEA-NO in segments from O-CR and O-DR rats. Results (mean ± S.E.M.) are expressed as a percentage of the previous tone elicited by exogenous NA. n = 8 animals each group. (D) Superoxide anion release in mesenteric segments from O-CR and O-DR rats. Results (mean ± S.E.M.) are expressed as chemiluminiscence units (U)/min mg tissue. n = 8 animals each group.

Figure 8: nNOS immunoreactivity in the adventitia of mesenteric arteries from O-CR (A) and O-DR (B) animals. Tissues were stained with primary polyclonal nNOS antibody and a species specific secondary Alexa 647 antibody. (C) Percentage of area occupied by nitrergic nerve fibers. n = 6 animals per group. bar = 50 µm. All images are reconstructions from 10 serial optical sections obtained by LSCM.

Table 1: Body weight and serum bioquemical parameters.

	O-CR	O-DR
Body weight (mg)	438.8±17.16	381.3±8.25*
Total Cholesterol (mg/dL)	80.11±6.65	73.25±4.05
HDL (mg/dL)	51.67±4.11	48±2.34
Triglycerides (mg/dL)	131.6±11.95	127.4±16.54
Insulin (µg/dL)	0.51±0.03	0.48±0.01

At 6-month-old body weight was measured, total Cholesterol, HDL-cholesterol, triglycerides and insulin in O-CR and O-DR animals. Data shown are means ± SEM. *P<0.05 vs. O-CR. n=8 animals per group.

Table 2. Effect of preincubation with tetrodotoxin (TTX, 0.1 µmol/L) on the frequency–contraction curves performed in mesenteric segments from O-CR and O-DR rats.

	1 Hz	**2 Hz**	**4 Hz**	**8 Hz**	**16 Hz**
O-CR	8.74±2.9	25.45±4.1	41.44±5.7	58.49±6.9	81.92±8.5
TTX	0	0	0	0.4±0.06	0.7±0.1
O-DR	21.61±6.8	37.92±6.4	35.53±6.9	74.05±7.1	100.49±8.5
TTX	0	0	0	0.5±0.04	0.9±0.02

Results (means ± S.E.M.) are expressed as percentages of the response elicited by 75 mMKCl; zeros are used when contraction was not detected. n = 7 animals each group.

Figure 1

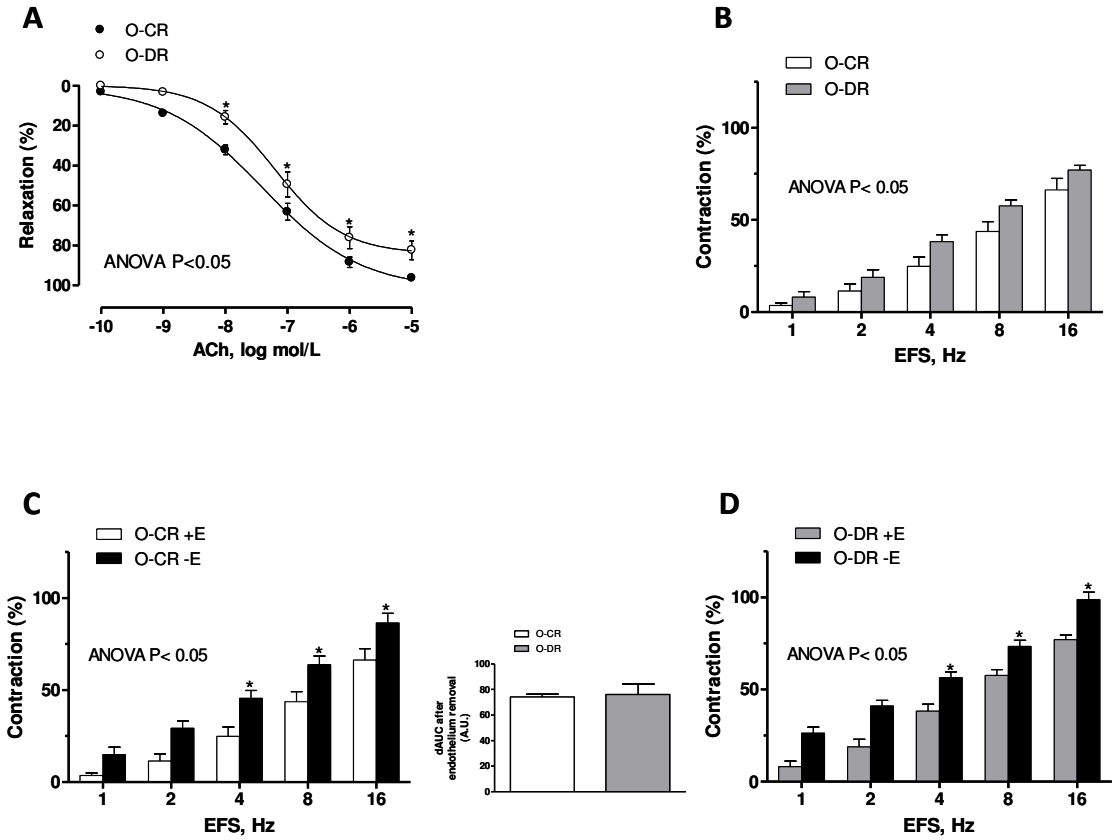

A
O-CR
O-DR
Relaxation (%)
ANOVA P<0.05
ACh, log mol/L
B
O-CR
O-DR
Contraction (%)
ANOVA P< 0.05
EFS, Hz
C
O-CR +E
O-CR -E
Contraction (%)
ANOVA P< 0.05
EFS, Hz
dAUC after endothelium removal (A.U.)
D
O-DR +E
O-DR -E
Contraction (%)
ANOVA P< 0.05
EFS, Hz

Figure 2

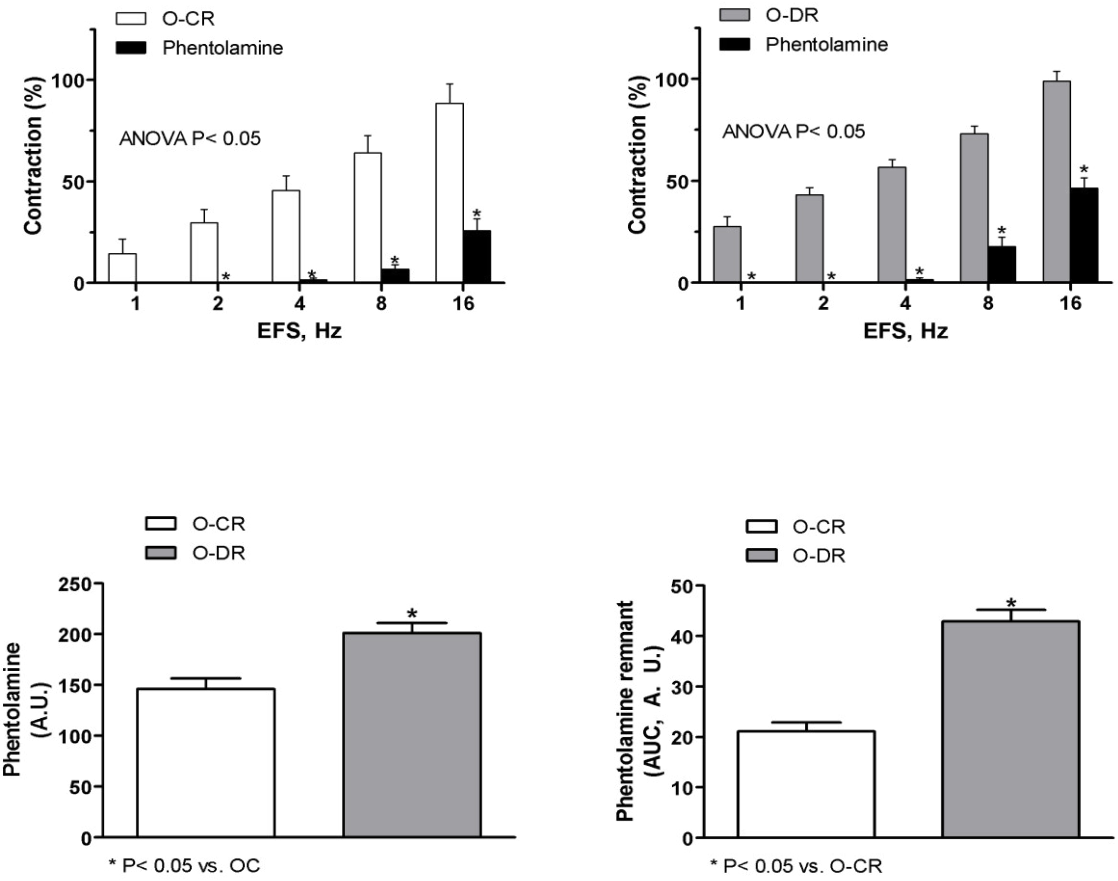

O-CR
Phentolamine
Contraction (%)
ANOVA P< 0.05
EFS, Hz
O-DR
Phentolamine
Contraction (%)
ANOVA P< 0.05
EFS, Hz
O-CR
O-DR
dAUC after Phentolamine (A.U.)
* P< 0.05 vs. OC
O-CR
O-DR
Phentolamine remnant (AUC, A. U.)
* P< 0.05 vs. O-CR

Figure 3

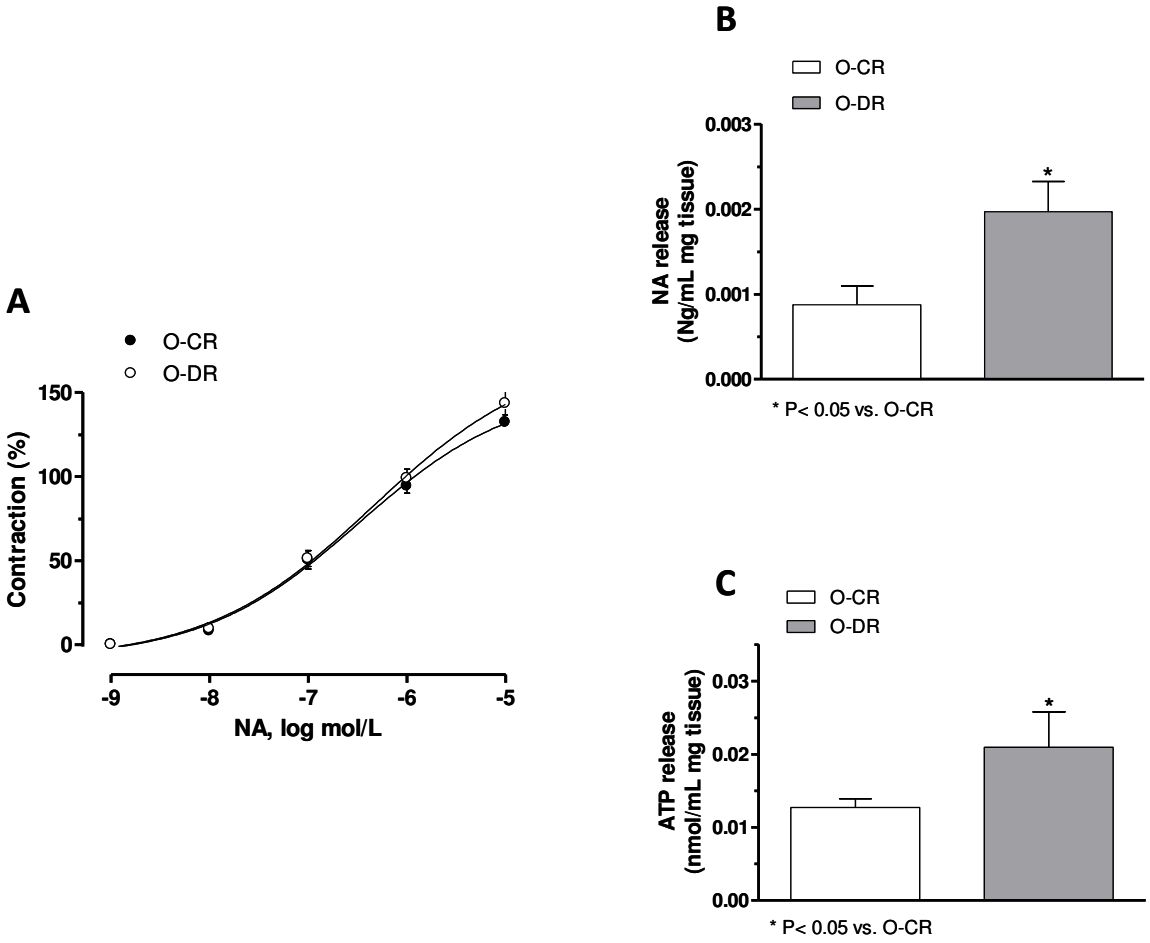

A
O-CR
O-DR
Contraction (%)
150
100
50
0
-9
-8
-7
-6
-5
NA, log mol/L
B
O-CR
O-DR
NA release
(Ng/mL mg tissue)
0.003
0.002
0.001
0.000
*
* P< 0.05 vs. O-CR
C
O-CR
O-DR
ATP release
(nmol/mL mg tissue)
0.03
0.02
0.01
0.00
*
* P< 0.05 vs. O-CR

Figure 4

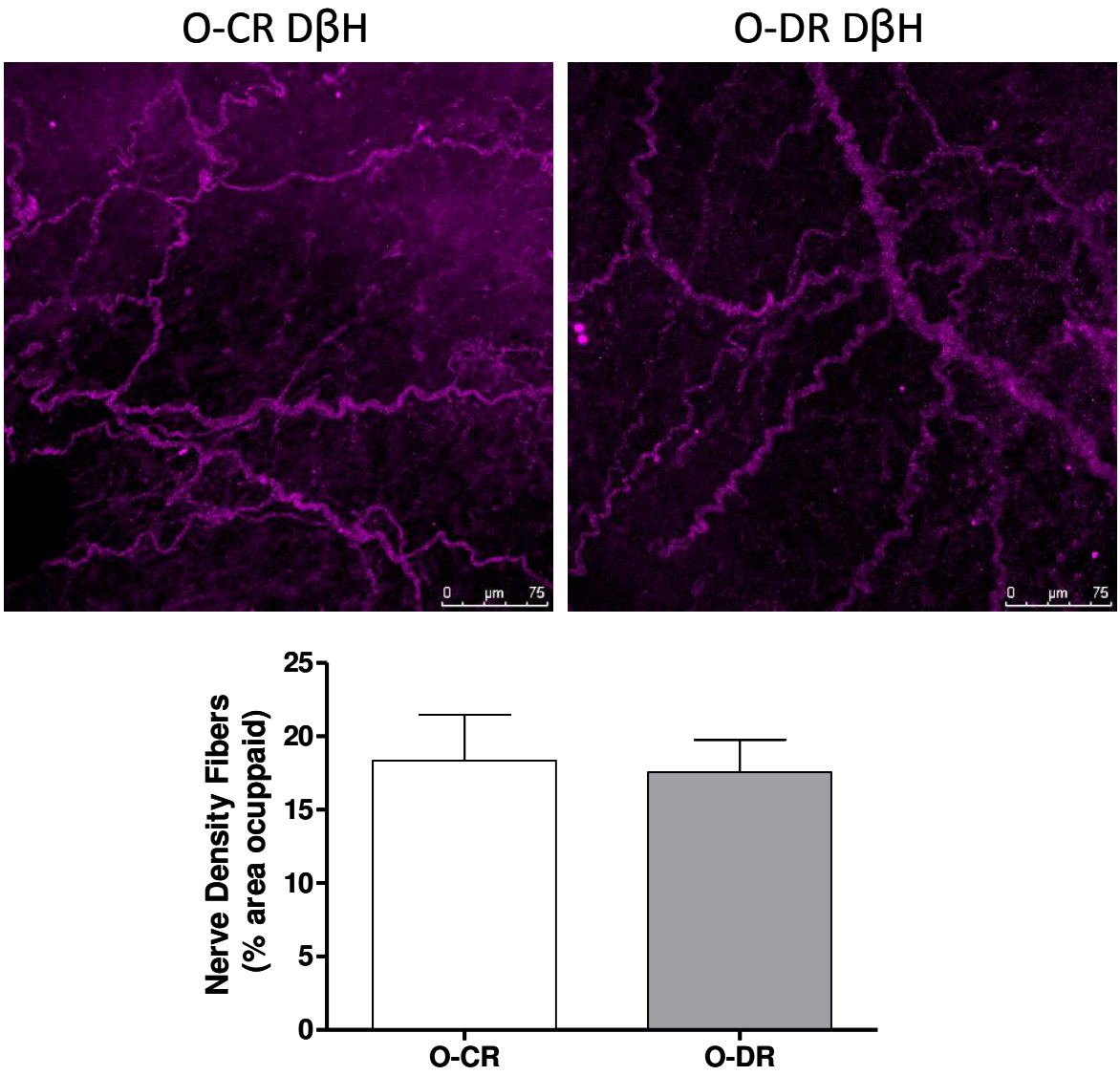

O-CR DβH
O-DR DβH
0 µm 75
0 µm 75
Nerve Density Fibers
(% area ocuppaid)
25
20
15
10
5
0
O-CR
O-DR

Figure 5

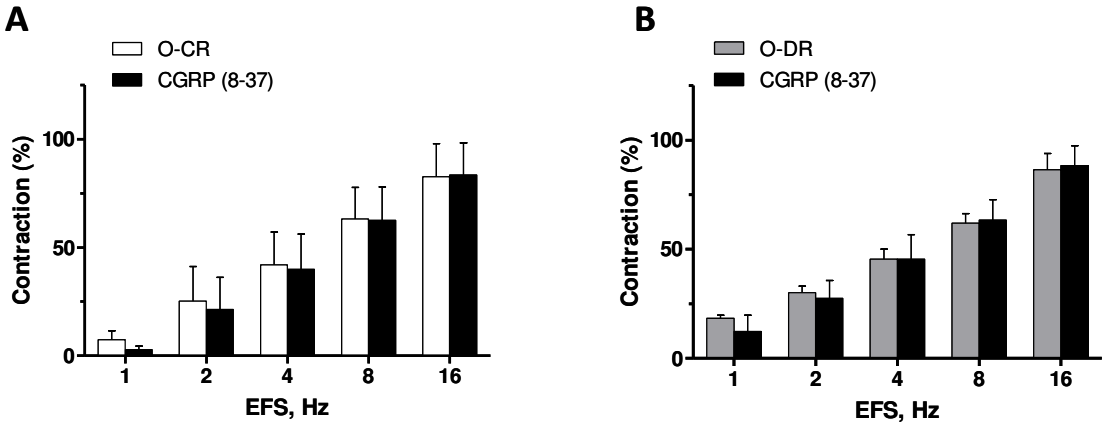

A
O-CR
CGRP (8-37)
Contraction (%)
100
50
0
1
2
4
8
16
EFS, Hz
B
O-DR
CGRP (8-37)
Contraction (%)
100
50
0
1
2
4
8
16
EFS, Hz

Figure 6

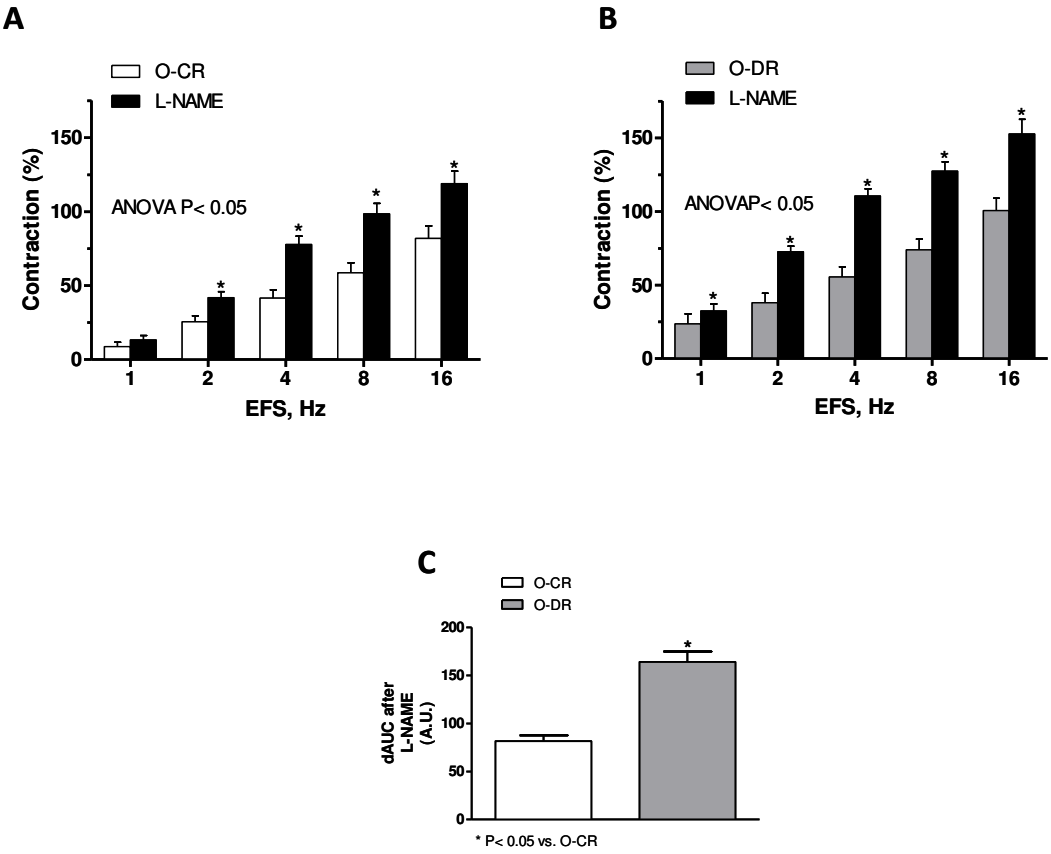

A
O-CR
L-NAME
Contraction (%)
150
100
50
0
ANOVA P< 0.05
*
1
2
4
8
16
EFS, Hz
B
O-DR
L-NAME
Contraction (%)
150
100
50
0
ANOVAP< 0.05
*
1
2
4
8
16
EFS, Hz
C
O-CR
O-DR
dAUC after L-NAME (A.U.)
200
150
100
50
0
*
* P< 0.05 vs. O-CR

Figure 7

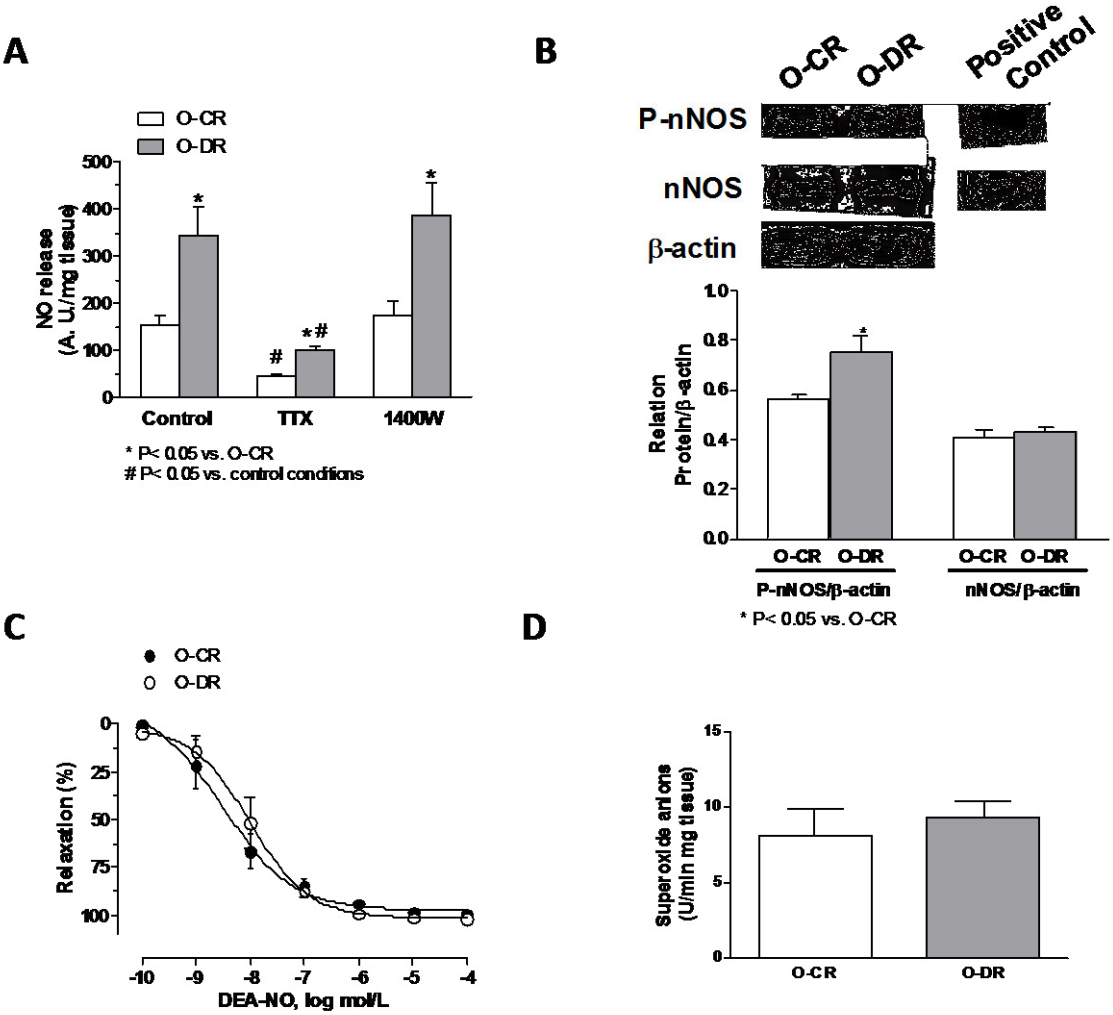
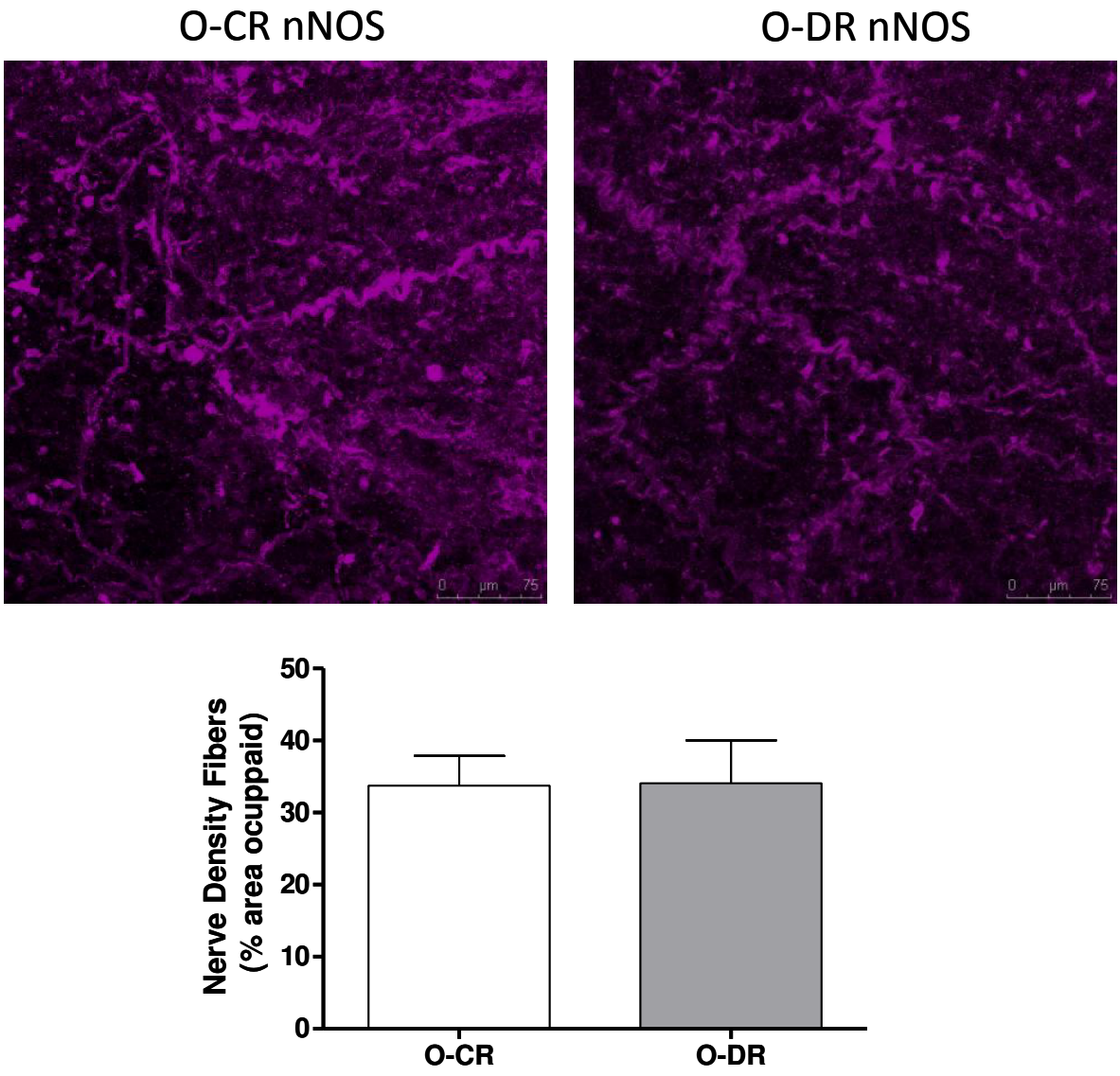

A
O-CR
O-DR
NO release (A. U./mg tissue)
500
400
300
200
100
0
Control
TTX
1400W
* P< 0.05 vs. O-CR
# P< 0.05 vs. control conditions
B
O-CR
O-DR
Positive Control
P-nNOS
nNOS
β-actin
Relation Protein/β-actin
1.0
0.8
0.6
0.4
0.2
0.0
O-CR O-DR
O-CR O-DR
P-nNOS/β-actin
nNOS/β-actin
* P< 0.05 vs. O-CR
C
O-CR
O-DR
Relaxation (%)
0
25
50
75
100
-10 -9 -8 -7 -6 -5 -4
DEA-NO, log mol/L
D
Superoxide anions (U/min mg tissue)
15
10
5
0
O-CR
O-DR

Figure 8


O-CR nNOS
O-DR nNOS
Nerve Density Fibers
(% area ocuppaid)
50
40
30
20
10
0
O-CR
O-DR

3. CONCLUSÕES

Os resultados obtidos nesta tese demonstram que a exposição ao diabetes gestacional na fase intrauterina e perinatal causa distúrbios metabólicos e cardiovasculares em longo prazo e que estes efeitos em parte tem a participação da ANG II e da inervação perivascular sobre o leito mesentérico vascular em ratos adultos. Esta conclusão geral emerge das conclusões obtidas pelos dois artigos científicos apresentados durante esta tese:

- O diabetes melitus gestacional severo proporciona efeitos cardiovasculares e metabólicos deletérios na prole adulta, sendo que estes efeitos parecem estar influenciados pela participação da ANG II, mas especificamente por ativação do receptor AT1;
- A ANG II parece ter papel chave no aumento da pressão arterial observado nos ratos adultos prole de diabéticos. Uma vez que, o tratamento com antagonista do receptor AT1 (Losartan) foi capaz de reverter esse efeito;
- O tratamento com losartan foi capaz de reduzir a contração induzida por noradrenalina e melhorar o relaxamento dependente do endotélio, sendo estas respostas pronunciadas nos ratos adultos prole de diabéticos. Ao inibir a ativação do receptor AT1 foi alterado o envolvimento dos prostanoides vasoconstritores derivados da ciclooxigenase-2 em modular a função vascular;
- A exposição à hiperglicemia durante a fase intrauterina e perinatal parece também ter efeitos em longo prazo na função da inervação perivascular em artéria mesentérica superior em ratos adultos.
- O aumento nos níveis de NA e ATP via inervação adrenérgica observado, parece contribuir para o quadro de hipertensão arterial sistêmico observado neste modelo.

- Concomitante ao efeito comentado acima, a inervação nitrérgica também parece estar aumentada podendo ser explicada como um mecanismo de resposta aos estímulos pró-vasoconstritores.
- As alterações observadas na inervação perivascular em artéria mesentérica superior podem estar relacionadas com a gênese da hipertensão arterial observadas nesse modelo de diabetes materno. Reforçando o conceito da programação fetal das doenças crônicas.

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