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DISSERTAÇÃO DE MESTRADO

EXPOSIÇÃO À HIPERGLICEMIA MATERNA INDUZ DISFUNÇÃO ENDOTELIAL EM ARTÉRIAS DE RESISTÊNCIA DA PROLE ADULTA. PAPEL DAS PROSTAGLANDINAS DERIVADAS DA COX-2.

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RECIFE, 2012.

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ESTUDO DOS MECANISMOS ENVOLVIDOS NO EFEITO DO DIABETES MELLITUS MATERNO SOBRE A FUNÇÃO MICROVASCULAR DE RATOS ADULTOS: INFLUÊNCIA DA IDADE.

Dissertação apresentada ao Departamento de Fisiologia e Farmacologia do Centro de Ciências Biológicas da Universidade Federal de Pernambuco, para obtenção do grau de Mestre em Bioquímica e Fisiologia.

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RESUMO

O objetivo do presente estudo foi determinar como o diabetes durante a gestação afeta a função vascular na prole adulta, a influência da idade e se a ativação das vias das ciclooxigenases (COX) está envolvida neste efeito. O diabetes materno foi induzido por estreptozotocina no dia 7º de gestação em ratas Wistar. Estudos de reatividade vascular foram realizados para analisar o relaxamento à acetilcolina (ACh) e a contração à noradrenalina (NA) em artérias mesentéricas de resistência (AMR) de ratos machos provenientes de ratas controle (CON) e diabéticas (STZ) aos 3, 6 e 12 meses de idade (3M, 6M e 12M, respectivamente). A liberação de TxB_2, PGE_2 e $PGF_{2\alpha}$ foi determinada por imunoensaio enzimático. A expressão de COX-1 e COX-2 foi analisada por *Western blot*. A prole de ratas diabéticas desenvolveu hipertensão a partir dos 6 meses de idade comparada à prole de ratas controle. Em ratos STZ, o relaxamento à ACh foi reduzido em todas as idades estudadas enquanto à contração à NA foi aumentada no grupo STZ6M e STZ12M. Tanto a resposta à ACh, quanto à NA foram restauradas na presença do inibidor da COX-2 (NS-398). O bloqueio do receptor TP (SQ29548) restaurou o relaxamento à ACh nas artérias dos animais STZ3M, enquanto que no grupo STZ6M, somente o bloqueio dos receptores TP e EP (SQ29548+AH6809) foi capaz de restaurar o relaxamento à ACh e a contração à NA. No grupo STZ12M, as respostas à ACh e à NA foram restauradas quando os receptores TP, EP e FP foram bloqueados simultaneamente (SQ29548+AH6809+AL8810). A liberação de TxB_2 estimulada por ACh ou NA foi aumentada nos ratos STZ em todas as idades estudadas. O aumento da liberação de PGE_2 foi detectado nos vasos dos animais com 6 e 12 meses de idade, enquanto a produção de $PGF_{2\alpha}$ aumentou apenas no grupo STZ12M. A expressão da COX-2, mas não da COX-1, foi aumentada nas artérias dos ratos STZ comparadas ao grupo controle. Estes resultados indicam a influencia da idade sobre a regulação da COX-2 em artérias de resistências de ratos adultos provenientes de ratas diabéticas, associada a uma produção aumentada de prostanóides vasoconstritores. Este efeito desempenha um papel chave na patogênese da disfunção endotelial nestas artérias, que por sua vez poderia contribuir para a progressão da disfunção vascular nestes ratos.

Palavras chave: Diabetes; hipertensão; programação fetal; disfunção endotelial; ciclooxigenaase; prostanóides.

ABSTRACT

The present study was designed to investigate how diabetes during pregnancy affects vascular function in adult offspring, the influence of age and the role of cyclooxygenase (COX)-derived prostaglandins. Blood pressure, relaxation to acetylcholine (ACh) and vasoconstriction to noradrenaline (NA) were analyzed in the offspring of control (O-CR) and diabetic (O-DR) mothers at 3, 6 and 12 months of age. O-DR developed hypertension from 6 months of age compared to O-CR. ACh elicited relaxation that was impaired in mesenteric resistance arteries (MRA) from 3, 6 and 12-month old O-DR. Contraction to NA was increased in MRA from 6 and 12-month old O-DR. COX-2 inhibition restored ACh and NA responses in all O-DR groups. TP receptor blockade (SQ29548) restored ACh relaxation in MRA from 3-month-old O-DR. In 6-month-old O-DR TP and EP receptors blockade (SQ29548+AH6809) was required to restore the relaxation to ACh and the contraction to NA. In 12-month-old O-DR, these responses were restored only when TP, EP and FP receptors were blocked (SQ29548+AH6809+AL8810). ACh- or NA-stimulated TxB_2 were higher in all O-DR. Release of PGE_2 increased in MRA from 6- and 12-month-old O-DR while $PGF_{2\alpha}$ was increased only in MRA from 12-month-old O-DR. COX-2 expression was higher in MRA from O-DR than O-CR. In O-DR, COX-2 expression increased with age, reaching higher levels at 12 months of age. COX-1 expression was similar in all groups. Results indicate an age-dependent up-regulation of COX-2 coupled to an enhanced formation of vasoconstrictor prostanoids in MRA from O-DR. This increased formation of vasoconstrictor prostanoids plays a key role in the pathogenesis of endothelial dysfunction which in turn could contribute to progression of vascular dysfunction in these rats.

Keywords: Diabetes; hypertension; fetal programming; endothelial dysfunction; cyclooxygenase; prostanoids.

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SUMÁRIO

1. INTRODUÇÃO

Estudos epidemiológicos e experimentais têm fornecido evidências substanciais de que a exposição intrauterina à hiperglicemia materna tem efeitos sobre a descendência, incluindo aumento do risco de obesidade, de diabetes tipo 2 e de doenças cardiovasculares (CLAUSEN *et al*., 2009; SIMEONI & BARKER, 2009).

O conceito de "programação fetal" (*fetal programming*) sugere que o feto pode ser "programado" durante o período intrauterino para desenvolver doenças na vida adulta, e tem sido utilizado como uma das bases para o conhecimento da origem de algumas doenças crônicas, como é o caso das doenças cardiovasculares (BARKER *et al*., 1994; 1998). Trabalhos publicados pelo grupo do Dr. David Barker foram os primeiros a demonstrar o envolvimento de distúrbios ocorridos durante a fase intra-uterina no desenvolvimento de doenças cardiovasculares na vida adulta (BARKER *et al*., 1986; 1989; 1994; 1998). Barker e seus colaboradores observaram que a distribuição geográfica da taxa de 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). Além disso, 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). Vários estudos epidemiológicos atualmente suportam essa relação inversa entre peso corporal após o nascimento e a hipertensão arterial (LAW *et al*., 1996; HUXLEY *et al*., 2000). Além disso, uma elevação da pressão arterial também é encontrada em crianças com baixo peso corporal (LAW *et al*., 2002). Estas observações serviram como base de sustentação da "Hipótese de Barker" que postula que condições adversas ocorridas no útero, como a desnutrição, podem desencadear um retardo do crescimento e a uma "programação fetal" da hipertensão arterial e de outras doenças cardiovasculares. Portanto, a programação fetal pode ser definida como um

fenômeno que ocorre através estímulos ou insultos no ambiente uterino durante um período considerado “janela crítica de desenvolvimento” podendo trazer alterações na estrutura e função de órgaõs e tecidos ao longo da vida adulta (LEON *et al*., 1998; SIMEONI & BARKER, 2009).

Dentre as alterações mais comuns que podem resultar em programação fetal está o consumo de baixa quantidade de calorias (BLONDEAU *et al*., 2002) ou de proteínas (PINHEIRO *et al*., 2008) durante a gestação e/ou aleitamento, a insuficiência placentária (GATFORD *et al*., 2010), a exposição durante a gestação aos glicocorticódes (XU *et al*., 2011) e o diabetes gestacional (BLONDEAU *et al*., 2010; SILVERMAN *et al*., 1991).

2. FUNDAMENTAÇÃO TEÓRICA

2.1. Diabetes gestacional

Segundo a *American Diabetes Association* (ADA), o diabetes mellitus gestacional (DMG) é comumente identificado como o surgimento de intolerância à glicose que inicia, ou é reconhecido pela primeira vez, durante a gravidez. O resultado desse processo, quando não tratado, é o aparecimento de efeitos adversos sobre o feto, que incluem macrossomia, sofrimento fetal, restrição de crescimento intrauterino e prematuridade (MANDERSON *et al*., 2002; ROGERS & VELTEN, 2011).

O DMG é uma doença que afeta de 1 a 14% das mulheres, variando de acordo com a população estudada e os critérios diagnósticos utilizados (BRODY *et al*., 2003, RIBEIRO *et al*., 2011). No Brasil, os dados referentes a esta doença referem-se à última década, onde a estimativa era de que cerca de 3 a 4% das gestantes desenvolveriam DMG, o que significa mais de 200.000 novos casos por ano (MAGANHA *et al*., 2003; RIBEIRO *et al*., 2011; SCHMIDT *et al*., 2000). Devido ao aumento da prevalência de obesidade, estima-se que o número de mulheres com DMG cresça substancialmente nos próximos anos, tanto nos países desenvolvidos como naqueles em desenvolvimento (KIM *et al*., 2011b). Em países, como os Estados Unidos, aproximadamente 4-6% das gestações apresentam complicações relacionadas ao diabetes mellitus (ALBRECHT *et al*., 2011; KIM et *al*., 2011a). A maioria dos casos é diagnosticada pela primeira vez durante a gravidez e o restante dos casos é considerado como pré-gestacionais (DM tipo 1 ou tipo 2) (ALBRECHT *et al*., 2011).

A gestação consiste em um período no qual a fêmea é transitoriamente submetida a alterações metabólicas que são finamente reguladas a fim de fornecer suprimento adequado ao feto. Uma vez que a unidade feto-placentária encontra-se sob a ação de diversos hormônios envolvidos no metabolismo da glicose, como a prolactina e os esteróides, e é capaz de secretar substâncias como a leptina (AKERMAN *et al*., 2002), a resistina (LAPPAS *et al*., 2005), e os fatores de crescimento semelhantes à insulina (SFERRUZZI-PERRI *et al*., 2011), é possível observar que durante a gestação e a lactação ocorre uma complexa relação entre as alterações maternas e o controle da homeostase glicêmica no feto/ neonato.

Evidências de que a hiperglicemia materna é capaz de resultar em doenças na prole adulta foram inicialmente sugeridas através de estudos com uma população indígena da América do Norte, os índios Pima do Arizona (PETTIT *et al*., 1983). Esta população apresenta elevada prevalência de diabetes tipo 2 durante a infância e a vida adulta (FRANKS *et al*., 2006), sendo este fato associado à exposição aos altos níveis glicêmicos durante a vida intrauterina. Além disso, tem-se demonstrado que nesta população há risco aumentado de obesidade, hipertensão e dislipidemias na vida adulta (DABELEA *et al*., 1993; MANDERSON *et al*., 2002; SILVERMAN *et al*., 1991).

Além de fatores ambientais, fatores genéticos têm sido considerados como uma possível explicação para a associação entre exposição perinatal a altas concentrações de glicose e o aparecimento de doenças metabólicas e cardiovasculares. De acordo com essa hipótese, mães com início precoce de diabetes ou com DMG podem ter um genótipo específico que transmite alta suscetibilidade à diabetes mellitus tipo 2 para a prole (SIMEONI & BARKER, 2009). Entretanto, dados disponíveis indicam também um papel importante do ambiente intrauterino sobre estas alterações. Trabalhos realizados na população de índios Pima sugerem esta conclusão. A prevalência de diabetes mostrou ser semelhante em mães que desenvolveram o diabetes durante a gravidez em relação àquelas com diabetes pré-existente (PETTIT *et al*., 1998). Além disso, a frequência para o desenvolvimento de diabetes tem se mostrado maior em filhos de mãe diabética do que em descendentes de homens diabéticos (LINDSAY *et al*., 2000). Portanto, a susceptibilidade genética para o diabetes poderá agir apenas como um fator predisponente ou agravante na determinação do risco de diabetes sobre a geração, quando adulta (SIMEONI & BARKER 2009; STRIDE *et al*., 2002).

Mulheres com diabetes pré-existente apresentam risco aumentado para complicações da gestação, incluindo retardo no crescimento fetal e morte perinatal (DUNNE *et al*., 2003). Embora os mecanismos a cerca dessas associações não sejam completamente compreendidos, tem sido mostrado que o controle da glicemia no início da gestação é capaz de reduzir tais complicações (KITZMILLER *et al*., 2008). Por outro lado, mulheres com diabetes gestacional que se mantêm hiperglicêmicas durante toda a gestação apresentam complicações mais severas comparadas àquelas que apresentam o diabetes durante o segundo ou terceiro trimestre de gestação, (CASEY *et al*., 1997; KIM *et al*., 2011b). Dessa forma, admite-

se que o diabetes pré-existente e o diabetes gestacional podem promover efeitos distintos sobre o desenvolvimento fetal e em longo prazo da prole, sendo necessário investigar as associações entre estas duas condições separadamente. Além disso, na mãe, o diabetes durante a gestação promove um risco adicional para desenvolvimento de doenças cardiovasculares e renais como, a hipertensão arterial, os acidentes vasculares e a insuficiência renal (PARADISI *et al*., 2002; RADENKIVIC *et al*., 2009).

A glicose é o principal nutriente que passa para o feto através de difusão facilitada (RUDGE *et al*., 2011). Dessa forma, adaptações nas células de contato entre a circulação materna e a fetal podem ocorrer em resposta ao abundante suprimento de glicose. Como demonstrado por Taricco *et al*., (2003), placentas de mães diabéticas mostram diversas modificações estruturais quando comparadas com placentas de mães não diabéticas, além disso, em outros estudos é possível observar imaturidade das vilosidades (DASKALAKIS *et al*., 2008), aumento de peso placentário e endoarterite quando em condições de diabetes (RUDGE *et al*., 2011; SALOMÓN *et al*., 2012).

Embora o mecanismo da relação entre a condição metabólica materna e o metabolismo da glicose na prole não esteja completamente esclarecido, pelo menos uma parte do efeito é atribuído à exposição intrauterina à elevada glicose materna. A glicose cruza livremente a placenta e estimula o pâncreas fetal a produzir insulina (FREINKEL, 1980) acarretando alterações potenciais sobre este órgão e sobre a sensibilidade dos tecidos à insulina. Estudos *in vitro* com fetos humanos oriundos de mães diabéticas demonstraram redução da secreção de insulina regulada por estimulação adrenérgica (REIHER *et al*., 1983). Em ratos, a exposição à hiperglicemia materna tem sido associada ao desenvolvimento anormal das células beta-pancreáticas e redução da absorção de glicose pelo músculo esquelético (BOLOKER *et al*., 2002).

Nesse sentido, com a finalidade de estudar a programação do desenvolvimento intrauterino durante a exposição à hiperglicemia materna, modelos animais têm sido desenvolvidos e têm sido relacionados ao aparecimento de alterações metabólicas, cardiovasculares e renais (GILL-RANDALL *et al*., 2004; FETITA *et al*., 2006; WEST *et al.,* 2011). Estudos deste porte, em humanos são limitados por questões éticas e pelo aparecimento de variáveis não controladas que podem modificar o ambiente intrauterino como, o comportamento alimentar e

socioeconômico, o *status* nutricional e os fatores genéticos (MOSES, 2012; NOMURA *et al*., 2012). Um método comumente utilizado para induzir hiperglicemia durante a gravidez é a injeção de estreptozotocina (STZ) (WARD *et al*., 2001) que é uma glicosamina-nitrosuréia capaz de promover efeitos tóxicos sobre as células β-pancreáticas (JUNOD *et al*., 1967). Nesta situação, observa-se risco aumentado de complicações sobre o feto, como má formação congênita, aborto e morte perinatal (CARRAPATO & MARCELINO, 2001; COX, 1994; YESSOUFOU & MOUTAIROU, 2011).

Quanto às alterações sobre o peso corpóreo da prole de ratas diabéticas pela injeção de STZ, observa-se tanto microssomia, na qual se observa baixo peso e retardo no crescimento (AERTS *et al*., 1990), macrossomia (OH *et al*., 1990; GELARDI *et al*., 1990), caracterizada por excesso de peso, ou ainda peso e crescimento normais (GERBER *et al*., 2000). O baixo peso ao nascimento, como consequência de alterações do ambiente intrauterino pode resultar tanto da desnutrição materna induzida por restrição protéica, como do aporte inadequado de nutrientes para o feto (HUXLEY *et al*., 2000; LAW *et al*., 1996). Neste sentido, Canavan & Goldspink (1988) demonstraram que a hiperglicemia durante a gestação induz supressão do crescimento fetal, o qual está associado com redução da síntese de proteína. Entretanto, este efeito está diretamente relacionado ao grau de elevação da glicemia materna. Assim, como consequência de uma hiperglicemia leve observa-se filhos com maior peso, enquanto que em um processo hiperglicêmico mais grave a prole apresenta baixo peso ao nascimento como conseqüência da maior redução na síntese proteica (SEGAR *et al*., 2009).

Portanto, estes modelos têm permitido investigar, em longo prazo, os efeitos da exposição intrauterina à hiperglicemia e suas conseqüências sobre a prole adulta. Embora, este tipo "programação fetal", no que se refere às doenças cardiovasculares tenha atraído muita atenção, os mecanismos envolvidos ainda não foram completamente elucidados.

2.2. Fisiopatologia das alterações cardiovasculares associadas à exposição fetal à hiperglicemia materna.

As doenças cardiovasculares são as principais causas de morte no mundo ocidental e o diabetes mellitus representa o maior fator de risco para o desenvolvimento destas doenças (CUGNET-ANCEAU *et al*., 2009).

Há diversos indícios epidemiológicos e estudos em animais indicando que a exposição perinatal ao diabetes materno está associada com o desenvolvimento de diabetes tipo 2 e de doenças cardiovasculares durante a vida adulta (METZGER *et al.,* 2002; SIMEONI & BARKER 2009). Além disso, tem-se verificado que o prejuízo no metabolismo da glicose adquirido durante a "janela de desenvolvimento" pode ser transmitido às gerações subseqüentes, possivelmente através de alterações epigenéticas sobre a expressão gênica (PINHEIRO *et al*., 2008).

Sabe-se que as consequências citadas anteriormente são resultados da combinação entre mecanismos que atuam em níveis molecular, celular e tecidual. Órgãos ou sistemas, como o rim ou os vasos sanguíneos, alcançam o desenvolvimento total no período perinatal, estando mais susceptíveis aos danos ocorridos durante este período (SIMEONI & BARKER, 2009). Amri *et al*. (1999) demonstraram que em ratos a exposição do feto à hiperglicemia materna diminui a nefrogênese, fato que contribui para o desenvolvimento de insuficiência renal crônica e hipertensão arterial na vida adulta. Estudos de Rocha *et al*. (2005) e Cavanal *et al*. (2007) demonstraram que ratos adultos normoglicêmicos provenientes de ratas diabéticas apresentam prejuízo da função renal e hipertensão arterial. Outros trabalhos têm ainda mostrado que filhos de mães diabéticas apresentam elevada incidência de intolerância à glicose, resistência à insulina e obesidade (BOLOKER *et al*., 2002; BLONDEAU *et al*., 2010), o que também predispõe o indivíduo ao desenvolvimento de diabetes e de doenças cardiovasculares na vida adulta.

Utilizando modelos de diabetes materno severo, estudos mostram que descendentes de ratas hiperglicêmicas apresentam disfunção endotelial quando adultos. Segar *et al*., (2010) desenvolveram um modelo de hiperglicemia em ratos durante a segunda semana de gestação (13º dia), onde foi possível observar disfunção endotelial e outras complicações cardiovasculares, tróficas e metabólicas na prole adulta. Utilizando modelo semelhante, Reinking *et al*. (2009) demonstraram que animais oriundos de ratas diabéticas apresentam cardiomiopatia hipertrófica, similar à encontrada em humanos descendentes de mães diabéticas. Além disso, Manderson *et al*. (2002) demonstraram que na prole de ratas diabéticas ocorre aumento plasmático na concentração de moléculas de adesão, incluindo a E-selectina, associado a uma maior predisposição a doenças vasculares.

Na última década, diversos trabalhos indicam que a hiperglicemia altera a angiogênese na prole em vários modelos experimentais, possivelmente através de uma redução na proliferação e aumento da apoptose de células endoteliais, estando estes mecanismos sob regulação do fator angiogênico VEGF (Fator de crescimento vascular) (LARGER *et al*., 2004; PINTER *et al*., 2001). Neste sentido, Ingram *et al*., (2008) descreveram que células progenitoras endoteliais isoladas de filhos de mães diabéticas apresentam funções angiogênicas alteradas, com redução da renovação e da capacidade de formação vascular. O estudo mostrou ainda que as células progenitoras endoteliais de descendentes de mães diabéticas apresentam um processo de senescência celular acelerado, o que pode predispor o indivíduo ao aparecimento de disfunção endotelial e de outras complicações vasculares (ACOSTA *et al*., 2011; INGRAM *et al*., 2008). Dessa forma, é possível concluir que uma disfunção endotelial precoce pode contribuir posteriormente para o surgimento de outras alterações vasculares e, conseqüentemente, de hipertensão arterial na descendência de mães diabéticas.

2.3. Sistema vascular e artérias de resistência na hipertensão e no diabetes

Hoje se sabe que existe uma grande heterogeneidade na reatividade dos diferentes territórios vasculares, a qual varia de acordo com os tipos e densidade de receptores farmacológicos e com os mecanismos de transporte de íons no músculo liso (MULVANY & ASLHJAEER, 1990). Além disso, há uma série de trabalhos demonstrando que a liberação de fatores endoteliais pode variar dependendo do tamanho e localização dos vasos sanguíneos (CLARK & FUCHS, 1997; LAGAUD *et al*., 1999).

A parede arterial é composta por três camadas claramente distintas: 1). A camada íntima, constituída por células endoteliais diferenciadas e arranjadas ao longo do eixo axial do vaso e na direção do fluxo sanguíneo, uma camada subendotelial contendo tecido conjuntivo e de uma membrana basal; 2). A camada média, constituída de células musculares lisas, as quais se encontram circunferencialmente arranjadas ao longo do eixo arterial e de uma matriz extracelular que inclui lâminas de fibras elásticas, fibras colágenas e proteoglicanos e 3). A camada adventícia, formada por tecido conjuntivo, contendo basicamente

fibras de colágeno, fibras elásticas e componentes celulares como fibroblastos e mastócitos (PEARSON, 1976).

As artérias de resistência podem ser definidas como vasos pré-arteriolares que contribuem passivamente e ativamente para a manutenção da resistência basal e para o controle do fluxo sanguíneo durante alterações hemodinâmicas (FEIH *et al*., 2008). A dissipação da energia necessária para superar a resistência vascular, evidenciada por um acentuado declínio na pressão arterial ocorre, principalmente, nas arteríolas pré-capilares e nas pequenas artérias, ou seja, nos vasos com diâmetro inferior a 350 µm (BORDERS & GRANGER, 1986; CHRISTENSEN & MULVANY, 2001; MULVANY & AALKJAER, 1990).

Alterações funcionais e estruturais nas artérias de resistência têm sido bem documentadas na hipertensão essencial e secundária (AGABITI, 2003; HEAGERTY, 2007) e também no diabetes mellitus tipos 1 e 2 (GREENSTEIN *et al*., 2009; LEVY *et al*., 2008). Estas alterações vasculares representam um dos principais fatores para o aumento da resistência vascular periférica, contribuindo, portanto, para o estabelecimento da hipertensão arterial. Essas alterações incluem espessamento da parede arterial e redução do diâmetro interno nas artérias de resistência (100 - 350 µm de diametro interno). Tais mudanças estruturais tem sido observadas em diversos leitos vasculares como em tecidos subcutâneo, omental, mesentérico e cerebral de ratos hipertensos (RIZZONI *et al*., 2001; RIZZONI *et al*., 2009). Entretanto, está cada vez mais evidente que as alterações nestes vasos não são ocasionadas apenas pela elevação crônica da pressão arterial. Outros fatores estão possivelmente envolvidos nas interações entre hipertensão e diabetes, incluindo a ativação do sistema nervoso simpático e do sistema renina-angiotensina, resistência à insulina, aumento dos níveis de leptina, disfunção endotelial e estresse oxidativo (BELIN DE CHANTEMÈLE *et al*., 2009; CHANG *et al*., 2012; GRASSI & DIEZ, 2009; NAKAJINMA *et al*., 2010).

A relação entre o diabetes e as complicações cardiovasculares é complexa, mas uma característica marcante consiste no prejuízo da função endotelial. A exposição crônica das proteínas plasmáticas e das membranas celulares à hiperglicemia implica em aumento da via dos polióis, glicosilação não-enzimática de proteínas, com aumento na produção dos produtos finais da glicação avançada (AGEs), sendo estes últimos conhecidos por inativarem o óxido nítrico, levando como conseqüência ao prejuízo da vasodilatação dependente do endotélio

(BUCALA *et al*., 1991). Nas proteínas como o colágeno, os AGEs causam ligações cruzadas entre os polipeptídios da membrana, levando ao aprisionamento das proteínas plasmáticas ou intersticiais (KALOUSOVÁ *et al*., 2002). Nos vasos de resistência e nos mais calibrosos, o aprisionamento da lipoproteína de baixa densidade (LDL) retarda seu efluxo a partir da parede vascular, acelerando a deposição do colesterol no endotélio e estimulando o processo aterogênico (JAKUS & RIETBROCK, 2004). Adicionalmente, nos capilares, a membrana basal glicosilada provoca a fixação de proteínas plasmáticas como a albumina, caracterizando a microangiopatia diabética (TAYLOR & POSTON, 1994).

Sabe-se que a hiperglicemia aguda ou crônica é capaz de causar inúmeras alterações sobre a função de pequenas e de grandes artérias, dentre elas: diminuição do relaxamento dependente do endotélio (DE VRIESE *et al*., 2000; OZYAZGAN *et al*., 2000; BROUWERS *et al*., 2010), aumento da resposta contrátil do músculo liso vascular (SHI *et al*., 2007; SHI & VANHOUTTE, 2008; XAVIER *et al*., 2003) e predisposição ao desenvolvimento de eventos inflamatórios, trombóticos e ateroscleróticos (SOWERS 1990; VILAHUR *et al*., 2009).

Um dos mecanismos responsáveis pela alteração vascular observada no diabetes inclui a reduzida disponibilidade do óxido nítrico (NO), seja por sua menor liberação/ biodisponibilidade (HAIDARA *et al*., 2006) ou redução da sensibilidade do músculo liso (FÉLÉTOU *et al*., 2010). Estas alterações normalmente, estão associadas com uma superprodução de espécies reativas de oxigênio (EROs), peroxidação lipídica e elevada produção de moléculas de adesão (DE VRIESE *et al*., 2000; HINK *et al*., 2001; MAZZONE *et al*., 2008). Além disso, a formação de produtos de glicação avançada e o diacilglicerol (DAG) juntamente com os ânions superóxidos contribuem para o desacoplamento da via da NOS (óxido nítrico sintase) endotelial através da depleção de tetrabiopterina (BH_4), um co-fator essencial para a síntese de NO (FÉLÉTOU *et al*., 2010).

Evidências crescentes sugerem que a disfunção endotelial em estados hiperglicêmicos é paralela ao estado de resistência insulínica, estado no qual há resposta inadequada dos tecidos à molécula, acompanhada de elevados níveis plasmáticos deste hormônio (HITOMI *et al*., 2011; VAZZANA *et al*., 2012). A insulina, em concentrações fisiológicas, atua como um vasodilatador e estimula a produção endotelial de NO (PESSIN & SALTIEL, 2000). Entretanto, na presença da resistência à insulina, é possível verificar prejuízo na captação periférica da glicose e na

vasodilatação dependente do endotélio mediada pelo hormônio (HITOMI *et al*., 2011; TAGUCHI *et al*., 2012). Diversos mecanismos inter-relacionados contribuem para a disfunção endotelial relacionada ao estado de resistência, tais como baixos níveis de colesterol HDL, aumento nos níveis de LDL e elevação de marcadores de inflamação (CHYU *et al*., 2011; FAGAN & DEEDWANIA, 1998; TOGASHI *et al*., 2012).

Diante dessa perspectiva, é possível deduzir que as alterações cardiovasculares observadas em animais expostos à hiperglicemia materna podem ser conseqüência de uma somatória de fatores, incluindo aqueles relacionados ao metabolismo da insulina. A maioria dos estudos em proles de ratas diabéticas demonstra hiperinsulinemia, a qual surge como um mecanismo adaptativo a fim de compensar o ambiente hiperglicêmico materno (BLONDEAU *et al*., 2002; BLONDEAU *et al*., 2010; GUZMÁN-GUTIÉRREZ *et al*., 2011). Entretanto, estes animais apresentam, além da hiperinsulinemia, resistência à insulina.

Estudos prévios realizados em nosso laboratório demostraram uma relação entre o aparecimento de desordens metabólicas e a hipertensão arterial em animais da prole de ratas diabéticas tipo 1. Os resultados desse estudo demonstraram que ratos adultos provenientes de ratas diabéticas desenvolvem alterações na homeostase da glicose, caracterizadas por intolerância à glicose e resistência à insulina. Além disso, observou-se que os animais com 6 e 12 meses de idade apresentaram hipertensão arterial, que foi acompanhada de disfunção endotelial. Os experimentos de reatividade vascular utilizando artérias de condutância (aorta e artéria mesentérica superior) revelaram um prejuízo do relaxamento dependente do endotélio aos 6 e 12 meses de idade e aumento da contratilidade à fenilefrina. Além disso, os resultados revelaram que esse desequilíbrio estaria associado a uma menor biodisponibilidade do óxido nítrico, em decorrência de uma maior produção de radicais livres acompanhada de uma liberação aumentada de substancias vasoconstritoras derivadas da ciclooxigenase (de QUEIROZ, 2010).

Sendo assim, as complicações vasculares em indivíduos expostos a estados hiperglicêmicos em fases iniciais da vida podem contribuir para o aumento da morbimortalidade das doenças cardiovasculares observado nas últimas décadas. Os mecanismos envolvidos no desenvolvimento destas doenças são complexos e parcialmente compreendidos, mas se sabe que são em grande parte desencadeados por um desequilíbrio na função do endotélio.

2.4. Controle endotelial do tônus vascular

As células endoteliais produzem diversas substâncias capazes de modular o tônus vascular. Dentre as substâncias vasoconstritoras se destacam os derivados do metabolismo do ácido araquidônico pela via das ciclooxigenases (SIMMONS *et al*., 2004; VIRDIS *et al*., 2010), os ânions superóxido (PARAVICINI & TOUYZ 2007; WENCESLAU *et al*., 2011), a endotelina-1 (BARTON, 2011) e a angiotensina II (YOU *et al*., 2005). Entre os fatores vasodilatadores, destacam-se o óxido nítrico (NO) (FURCHGOTT, 1983; GILL *et al*., 2007), a prostaciclina (PGI_2) (TRACHTE, 1986) e o fator hiperpolarizante derivado do endotélio (EDHF) (CHEN *et al*., 1991; FÉLÉTOU & VANHOUTTE *et al*., 2006).

A integridade endotelial é essencial para a regulação do tônus vascular.Em condições fisiológicas existe um equilíbrio entre a liberação de fatores vasorrelaxantes (EDRF) e vasoconstritores (EDCF). No entanto, em diversas condições patológicas, como na hipertensão arterial e no diabetes, esse equilíbrio é alterado, levando a um aumento da produção de EDCFs. Nos últimos anos, diversos estudos demonstram haver uma associação entre o aumento da produção de Prostaglandina H_2 (PGH_2) e tromboxano A_2, derivados do metabolismo do ácido araquidônico pela COX, e o estabelecimento da disfunção endotelial (ALFRANCA *et al*., 2006; FÉLÉTOU *et al*., 2010; PANNIRSELVAM *et al*., 2005).

2.4.1. Produtos derivados da via da ciclooxigenase

As prostaglandinas, potentes mensageiros bioativos, são produzidas a partir do ácido araquidônico (AA), e foram extraídas a partir de vesículas seminais por Goldblatt e von Euler em 1930, quando foi evidenciada a ação contratil destas substancias. A biossíntese das prostaglandinas depende basicamente da liberação do ácido araquidônico a partir da ação da fosfolipase A_2 sobre os fosfolipídeos da membrana (HAMBERG & SAMUELSSON, 1973; SIMMONS *et al*., 2004).

O ácido araquidônico pode ser oxidado pelas ciclooxigenases para produzir o endoperóxido cíclico (PGH_2) que servirá de substrato para a produção das prostaglandinas E_2 (PGE_2), $F_{2\alpha}$ (PGF_2), da PGI_2 e do tromboxano A_2 (TxA_2) (Figura 1). Além disso, ele pode ainda ser oxidado pelas lipoxigenases e originar o ácido 15-s-hidroxieicosatetraenóico (15-HETE), uma substância que é capaz de produzir contração vascular (VANHOUTTE *&* EBER, 1991). Adicionalmente, foi demonstrado por Miller & Vanhoutte (1985) que o próprio ácido araquidônico tem a capacidade de

induzir contração dependente do endotélio em veias femorais de cães e que esta resposta é bloqueada por inibidores das ciclooxigenases.

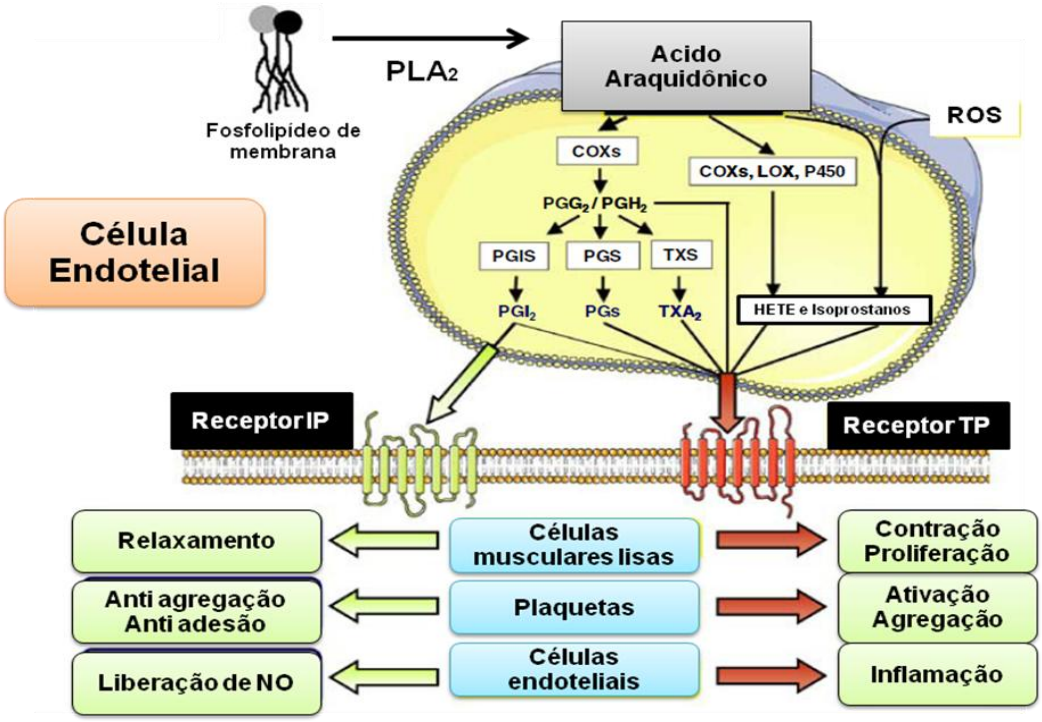

Figura 1: Metabolismo do ácido araquidônico e ação dos prostanóides sobre as células do músculo liso, plaquetas e células endoteliais. Envolvimento dos receptores IP e TP sobre a disfunção vascular. COX 1 e 2 catalisam a conversão do ácido araquidônico a endoperóxido cíclico PGH_2 e PGG_2 através de reações sequenciais de ciclooxigenação e hidroperoxidação. PGH_2 é metabolizado pelas sintases específicas para produzir prostanoides: *PGS* Sintases de prostaglandinas, *PGIS* Prostaciclina sintase, *TXS* Tromboxano sintase, *PGs* prostaglandinas, PGG_2 prostaglandina G_2, PGH_2 prostaglandina H_2, PGI_2 prostaglandina I_2 (prostaciclina), TxA_2 tromboxano A_2. O ácido araquidônico também pode ser oxidado pelo citocromo P450, e pelas Lipooxigenases. PGH_2 prostaglandina H_2, PGI_2 prostaglandina I_2 (prostaciclina), TxA_2 tromboxano A_2, *HETE* ácido hidroxieicosatetraenóico (Adaptado FÉLÉTOU *et al*., 2010).

Além das prostaglandinas citadas, a ativação da COX também pode levar à formação de espécies reativas de oxigênio, como os ânions superóxido, e de isoprostanos, já que estas enzimas são capazes de co-oxidar outras substâncias como a molécula de NADPH (nicotinamida-adenina dinucleotídeo fosfatado) (TANG *et al*., 2007; WATKINS *et al*., 1999), e de modificar através de oxidação não enzimática, ácidos graxos poliinsaturados. Espécies reativas de oxigênio diminuem a biodisponibilidade de óxido nítrico (RUBANYI & VANHOUTTE, 1986) e como um ciclo de retroalimentação positiva, há estimulação da COX produzindo mais prostanóides (MORITA, 2002). Tanto os ânions superóxido, como os isoprostanos

podem ser considerados biomarcadores de estresse oxidativo e têm sido associados a diversas doenças cardiovasculares (HAMILTON *et al*., 2001; PRATICO *et al*., 2001).

Existem duas isoformas da ciclooxigenase: a COX-1 e a COX-2. Ambas são heme-proteínas que apresentam potência semelhante para oxidar o ácido araquidônico em endoperóxido (GARAVITO & DE WITT, 1999), embora, baixas concentrações de ácido araquidônico são preferencialmente metabolizadas pela COX-2, enquanto que em níveis mais elevados o AA é metabolizado pela COX-1 (MORITA, 2002). Além disso, a COX-2 pode metabolizar outros substratos como, os ácidos graxos e o 2-araquidonil glicerol (SMITH & SONG, 2002) levando, portanto, a uma diferenciada produção de metabólitos entre as duas isoformas.

Considera-se geralmente que a COX-1 na maioria dos tecidos é expressa constitutivamente enquanto que a COX-2 é induzida principalmente nos locais de inflamação (FELETOU *et al.,* 2010). Alguns pesquisadores consideram que as prostaglandinas derivadas através do metabolismo da COX-1 apresentam papel positivo, visto que possuem funções fisiológicas importantes como controle da hemostasia, integridade da mucosa gastrointestinal e fluxo sanguíneo renal. Já as originadas da COX-2 têm papel negativo, pois estão envolvidas em reações inflamatórias e são responsáveis por fenômenos como dor, rubor, edema capilar e vasodilatação (PARENTE & PERRETTI, 2003). No entanto, a COX-2 também é expressa constitutivamente em vários tipos celulares, incluindo nas células endoteliais onde sua expressão é regulada pela tensão de cisalhamento (TOPPER *et al*., 1996), e em diversas partes do néfron (ZHANG *et al*., 1997). Na parede vascular, tanto células endoteliais como células musculares lisas contêm COX, porém, em vasos sanguíneos saudáveis, a proporção é maior nas células da camada íntima (DE WITT *et al*., 1983). Sob determinadas condições como, por exemplo, no processo de envelhecimento e inflamação, a COX-2, pode ser expressa em células endoteliais e do músculo liso vascular, participando das contrações dependentes do endotélio (SHI *et al.*, 2008).

Uma relação entre o aumento da expressão das ciclooxigenases e o estabelecimento de disfunção endotelial tem sido demonstrada em modelos de hipertensão e diabetes. Em aortas de animais espontaneamente hipertensos (SHR), as contrações dependentes do endotélio são associadas ao aumento na expressão de COX-1, e a uma maior liberação de prostaciclina e tromboxano A_2 (FÉLÉTOU *et*

al., 2009). Adicionalmente, em modelos de diabetes tipo 2, o aumento do tônus arteriolar tem sido atribuído a uma maior expressão de COX-2, levando a uma produção aumentada de prostanóides vasoconstritores (BAGI *et al*., 2005; GUO *et al*., 2005). Estes agentes contráteis interagem com receptores acoplados à proteína G, que são classificados em três subtipos: EP, FP, e TP, de acordo com a sensibilidade às prostaglandinas E_2, $F_{2\alpha}$ e tromboxano A_2, respectivamente (ALFRANCA *et al*., 2006; FELETOU *et al*., 2010; TSUBOI *et al*., 2002) (Figura 2).

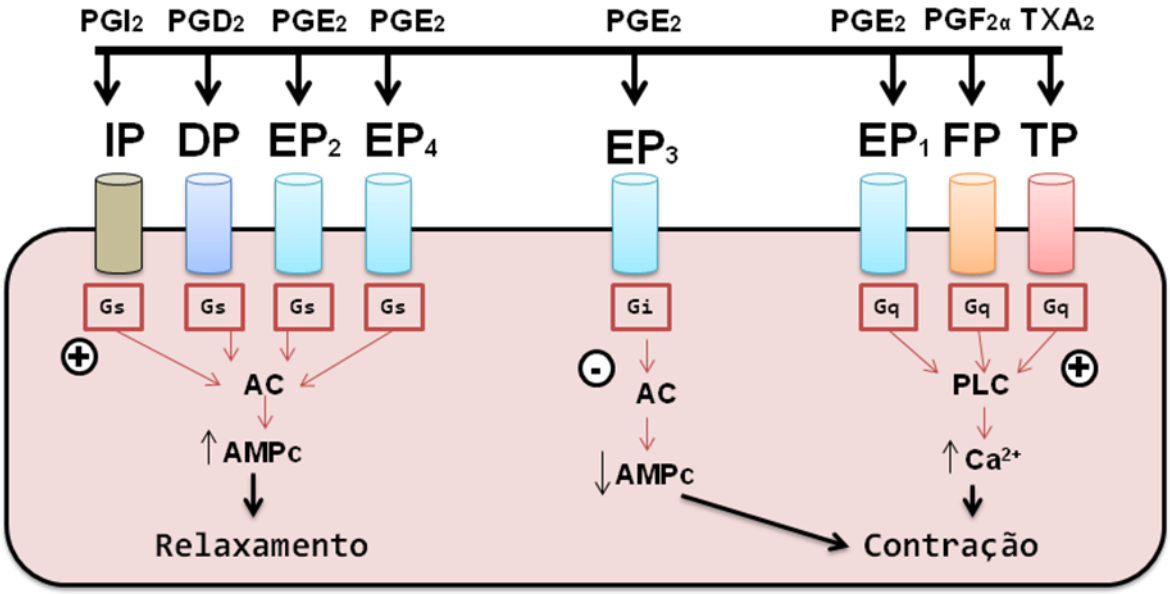

Figura 2. Mecanismos de ação dos prostanóides. AC: adenilato ciclase, AMPc: Monofosfato cíclico de adonosina, DP: receptor da PGD_2, EP: receptor de PGE, FP: receptor de $PGF_{2\alpha}$, IP: receptor de PGI_2, TP: receptor de TxA_2, PLC: fosfolipase C. (Adaptado BREYER *et al*., 2011; YAGAMI, 2006).

Tromboxano A_2 (TxA_2)

O TxA_2 é sintetizado a partir da ação da enzima TxA_2-sintetase sobre o PGH_2. Ele é considerado um dos prostanoides vasoconstritores mais importantes, também apresenta ação agregante plaquetária, promove a expressão de moléculas de adesão e favorece a infiltração de macrófagos através da modulação de receptores do tipo tirosina quinase (FONLUPT *et al*., 1991; NAKAHATA, 2008). Sua ação é mediada através do receptor para tromboxano (receptor TP) nas células musculares lisas, onde a transdução do sinal envolve uma proteína Gq resultando em estimulação da fosfolipase C, o que promove aumento na concentração intracelular

de Ca^{2+} induzindo vasoconstrição (MAYEUX *et al*., 1989) (Figura 2). Em humanos, mas não em roedores, duas isoformas para o receptor TP têm sido descritas, TPα e TPβ, as quais diferem apenas nas suas porções C-terminais (HIRATA *et al*., 1996).

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).

Estudos recentes tem mostrado que espécies reativas de oxigênio são capazes de aumentar a densidade de receptores TP na membrana de células musculares lisas (VALENTIN *et al*., 2004; WILSON *et al*., 2009), além disso, em células endoteliais, a ativação de receptores TP reduz a produção de óxido nítrico (LIU *et al*., 2009, XAVIER *et al*., 2009), fato que pode, portanto, contribuir para o estabelecimento de disfunção endotelial. Neste sentido, é possível compreender a importancia deste prostanóide no desenvolvimento de desordens vasculares.

Embora o tromboxano A_2 seja o ligante preferencial para o receptor TP, outras prostaglandinas, inclusive o PGH_2, também podem ativar este receptor (GLUAIS *et al*., 2005). Além disso, isoprostanos são também potentes agonistas endógenos dos receptores TP (WATKINS *et al*., 1999) (Figura 2).

Prostaglandina E_2 (PGE_2)

A PGE_2 corresponde ao prostanóide mais abundante do organismo (SERHAN & LEVY, 2003), sendo produzido por diversos tipos celulares, como fibroblastos e células endoteliais. A ampla capacidade de promover efeitos pró e antiinflamatorios consiste na ativação de diferentes tipos e receptores, denominados EP_1, EP_2, EP_3 e EP_4.

No sistema vascular, os subtipos EP_1 e EP_3 promovem vasoconstrição, a qual ocorre através da ativação da via do IP_3/Ca^{2+} (receptor EP_1) ou da diminuição dos níveis intracelulares de AMPc (receptor EP_3) por inibição da adenilil ciclase (COLEMAN *et al*., 1994; FUNK *et al*., 1993). A ligação da PGE_2 aos receptores EP_2 provoca vasodilatação através do aumento nos níveis de AMPc via ativação da adenilil ciclase (COLEMAN *et al*., 1994), enquanto que através do receptor EP_4 há ativação da via do NO (HRISTOVSKA *et al*., 2007) (Figura 2).

Portanto, uma diminuição na vasodilatação mediada pelos receptores EP_2/EP_4 ou um aumento da sinalização através dos receptores EP_1/EP_3 pode estar envolvido no desequilibrio da função vascular e na fisiopatologia da hipertensão. Guan *et al.* (2007) demonstraram que a ativação do receptor EP_1 contribui para a ação vasoconstritora da angiotensina II. Além disso, tem sido demonstrado que a PGE_2 desempenha um papel chave no desenvolvimento da doença renal hipertensiva (SUGANAMI *et al*., 2003).

Prostaciclina (PGI_2)

A prostaciclina é o principal metabólito do ácido araquidônico produzido pelas ciclooxigenases em células endoteliais (MONCADA & VANE, 1979). Ao ativar os receptores IP no músculo liso vascular produz relaxamento, quando em vasos saudáveis (EDWARDS *et al*., 2010). Dependendo da artéria, uma hiperpolarização pode ocorrer, a qual envolve a abertura de um ou mais tipos de canais para potássio (FÉLÉTOU & VANHOUTTE, 2006b).

Esta prostaglandina apresenta atividade vasodilatadora e antiagregante plaquetária (RAMLI *et al*., 2011). Através da estimulação dos receptores IP, a PGI_2 promove a ativação da adenilil cilase levando ao aumento dos níveis de AMPc, este, por sua vez induz a ativação da PKA, a qual induz inibição da contração (HATHAWAY *et al*., 1981) (Figura 2). No entanto, estudos recentes demonstram que a PGI_2 é capaz também de induzir vasoconstrição, a qual é mediada através dos receptores TP (GLUAIS *et al*., 2005, XAVIER *et al*., 2009) (Figura 1). Através de sua ação nesse receptor, este prostanóide tem sido identificado como um importante contribuinte para a disfunção endotelial em artérias de animais normotensos *Wistar Kyoto* e hipertensos SHR tratados com aldosterona (BLANCO-RIVERO *et al*., 2005; XAVIER *et al*., 2008). Assim, dependendo das circunstâncias, esta prostaglandina pode ser considerada um EDCF. Somado a isto, hoje se sabe que a prostaciclina é capaz de regular as ações cardiovasculares do tromboxano A2, graças a uma interrelação entre os receptores TP e IP ainda não elucidada (FETALVERO *et al*., 2007).

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

A PGF2α participa da modulação do tônus vascular elevando os níveis intracelulares de cálcio no músculo liso vascular promovendo contração

(ABRAMOVITZ *et al*., 1994). O receptor FP está acoplado à proteína G_q, e quando estimulado ativa a via do IP_3/ Ca^{2+} produzindo contração da musculatura lisa (PIERCE *et al*., 1999). Sua ação vasoconstritora também pode ser mediada através de sua ligação aos receptores TP (WONG *et al*., 2009) (Figuras 1 e 2).

Kimura *et al*. (1994) demonstraram um aumento da resposta contrátil induzida pela $PGF_{2\alpha}$ em artérias e veias mesentéricas de resistência, na condição de diabetes, sendo este efeito relacionado à microangiopatia nesses leitos. Corroborando com estes dados, Georgescu & Popov (2003) demonstraram que aumentos nos níveis plasmáticos de glicose, colesterol ou proteína C reativa estão associados ao aumento da resposta vasoconstritora ao prostanóide. Adicionalmente, um outro estudo (YU *et al*., 2009) demonstraram haver uma relação entre a ativação dos receptores FP e o surgimento de hipertensão arterial e aterosclerose. Portanto, é possível concluir que um aumento na produção deste eicosanóide pode estar envolvido na gênese do processo hipertensivo, bem como das comorbidades relacionadas.

2.5. Hipótese de trabalho

Inicialmente, grande parte dos estudos sobre a “programação fetal” dava maior ênfase ao efeito da desnutrição materna sobre o surgimento de algumas doenças na vida adulta (LAW *et al*., 2002; SILVERMAN *et al*.,1991). Entretanto, hoje existem inúmeros trabalhos demonstrando que a exposição fetal à hiperglicemia materna tem uma contribuição importante para o aparecimento de distúrbios metabólicos, neurais e principalmente cardiovasculares no período pós-natal (BRINCIOTTI *et al*., 2011; HAY, 2012; NOLD & GEORGIEFF, 2004).

Com o objetivo de investigar o impacto do diabetes materno sobre o sistema cardiovascular da prole, vários estudos têm sido realizados (RADENKOVIC *et al*., 2009; STANLEY *et al.*, 2009; WICHI *et al*., 2005). Embora tenha sido encontrada uma relação positiva direta entre o ambiente intrauterino hiperglicêmico e o aumento da susceptibilidade para complicações cardiovasculares na vida adulta (CAVANAL *et al*., 2007; ROCHA *et al*., 2005; SEGAR *et al*., 2010), estudos sobre a contribuição do sistema vascular, incluindo a participação de mecanismos endoteliais e componentes da maquinaria contrátil do músculo liso ainda são muito escassos.

Nos últimos anos, estudos com vasos de resistência têm obtido maior destaque, principalmente no que diz respeito aos processos envolvidos no

estabelecimento/ manutenção da pressão arterial elevada e da disfunção endotelial decorrentes de estados hiperglicêmicos (RIZZONI *et al*., 2011; SCHIFFRIN, 2012). Tal importância deve-se ao fato de que esse tipo de artérias representam, na patogênese de muitas doenças cardiovasculares e em particular na hipertensão arterial, um papel determinante para o desenvolvimento e progressão dos distúrbios observados (JIN *et al*., 2011; MULVANY, 1987), e alguns autores têm inclusive sugerido que o desenvolvimento de anormalidades nestes vasos poderia ser a causa primária do processo hipertensivo (DENG *et al*., 1995; MULVANY, 2012).

Sendo assim, o efeito da hiperglicemia materna sobre as artérias de resistência da prole adulta pode representar uma das causas de desenvolvimento de doenças cardiovasculares nesses indivíduos, fato que torna de grande relevância o entendimento dos mecanismos responsáveis por estas alterações.

3. OBJETIVOS

Avaliar o papel da idade e das prostaglandinas derivadas da ciclooxigenase sobre as possíveis alterações do relaxamento dependente do endotélio e da resposta contrátil induzida por estimulação alfa-adrenérgica em artérias de resistência da prole de ratas diabéticas tipo-1.

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5. ARTIGO 1

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RESEARCH PAPER

Effect of age and COX-2-derived prostanoids on the progression of adult vascular dysfunction in the offspring of diabetic rats

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BACKGROUND AND PURPOSE
The present study was designed to determine how diabetes in pregnancy affects vascular function in their offspring, the influence of age and whether COX activation is involved in this effect.

EXPERIMENTAL APPROACH
Relaxation responses to ACh were analysed in mesenteric resistance arteries from the offspring of control rats (O-CR) and those of diabetic rats (O-DR) at 3, 6 and 12 months of age. TxB_2, PGE_2 and $PGF_{2\alpha}$ release were determined by enzyme immunoassay. COX-1 and COX-2 expression were measured by Western blot analysis.

KEY RESULTS
O-DR developed hypertension from 6 months of age compared with O-CR. In O-DR, relaxation responses to ACh were impaired in all ages studied and were restored by COX-2 inhibition. TP receptor blockade (SQ29548) restored ACh relaxation in arteries from 3-month-old O-DR while TP and EP receptor blockade (SQ29548 + AH6809) was required to restore it in 6-month-old O-DR. In 12-month-old O-DR, ACh relaxation was restored when TP, EP and FP receptors were blocked (SQ29548 + AH6809 + AL8810). ACh-stimulated TxB_2 was higher in all O-DR. ACh-stimulated PGE_2 release was increased in arteries from 6- and 12-month-old O-DR, whereas $PGF_{2\alpha}$ was increased only in 12-month-old O-DR. COX-2, but not COX-1, expression was higher in O-DR than O-CR.

CONCLUSIONS AND IMPLICATIONS
The results indicate an age-dependent up-regulation of COX-2 coupled to an enhanced formation of vasoconstrictor prostanoids in resistance arteries from O-DR. This effect plays a key role in the pathogenesis of endothelial dysfunction, which in turn could contribute to the progression of vascular dysfunction in these rats.

Abbreviations
AH6809, 6-isopropoxy-9-oxoxanthene-2-carboxylic acid; AL8810, 9α,15R-dihydroxy-11β-fluoro-15-(2,3-dihydro-1H-inden-2-yl)-16,17,18,19,20-pentanor-prosta-5Z,13E-dien-1-oic acid; EP, prostaglandin E_2 receptors; FP, prostaglandin $F_{2\alpha}$ receptors; KHS, Krebs-Henseleit solution; NS-398, N-(2-cyclohexyloxy-4-nitrophenyl) methansulfonamide; O-CR, offspring of control rats; O-DR, offspring of diabetic rats; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; SC-560, 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole; SQ29548, [1S-[1a,2a(Z),3a,4a]]-7-[3-[[2-(phenylamino) carbonyl]hydrazino]methyl]-7-oxabicyclo [2.2.1] hept-2-yl]-5-heptanoic acid; TP, thromboxane A_2 receptors; TxA_2, thromboxane A_2; U46619, (15)-hydroxy-11,9 -(epoxymethano)prosta-5,13-dienoic acid

Introduction

In the past few decades, the incidence of cardiovascular diseases has been increasing worldwide. In the early 20th century, it was responsible for less than 10% of deaths around the entire world, while at the beginning of the 21st century it accounted for almost 50% and 25% of deaths in developed and developing countries, respectively [World Health Organization (WHO), 2002]. In most countries, the increased incidence of cardiovascular diseases has been attributed in part to environmental factors such as diet, smoking and reduced physical exercise (WHO, 2002). On the other hand, the susceptibility to cardiovascular diseases may also be acquired *in utero* through changes in the uterine environment. Fetal programming refers to the observations that disturbances during the critical period of development can cause lifelong changes in the structure and function of the organism leading to diseases in later life (Barker, 2004). This concept comes from epidemiological studies by Barker and colleagues who obtained evidenced for an inverse relationship between low weight at birth and development of cardiovascular diseases in adulthood (Barker, 1995; 2004; Barker *et al.*, 2002). However, other maternal conditions that produce an adverse environment to fetal development, including chronic hyperglycaemia, also increase the risk of metabolic and cardiovascular diseases in the offspring (Simeoni and Barker, 2009).

Fetal exposure to maternal hyperglycaemia can result in insulin resistance, changes in glucose metabolism and increased risk of hypertension in adult life (Nehiri *et al.*, 2008; Segar *et al.*, 2009; Simeoni and Barker, 2009; Chen *et al.*, 2010; Blondeau *et al.*, 2011). This hypertension has been partially attributed to renal mechanisms. Amri *et al.* (1999) demonstrated that exposure to maternal diabetes impairs the offspring's nephrogenesis, reducing the number of nephron, which could be a risk factor for the development of chronic renal disease and hypertension in adulthood. In addition, baroreflex dysfunction and increased activity of the renin-angiotensin system have also been reported in adult offspring of diabetic mothers (Wichi *et al.*, 2005).

Other studies have shown that maternal diabetes promotes alterations in vascular reactivity in their offspring. Rocha *et al.* (2005) demonstrated that offspring of streptozotocin-induced diabetic rats had reduced endothelium-dependent relaxation and hypertension in adulthood. Likewise, Holemans *et al.* (1999) observed impaired endothelial function in resistance arteries from offspring of diabetic rats, but without changes in BP. The reason for this discrepancy with regard to BP is not fully known, but the age of animals used may have influenced the results. Rocha *et al.* (2005) used 12-month-old rats while Holemans *et al.* (1999) used animals that were only 3 months old, which suggest that age may influence the development of hypertension in these animals. The results of Nehiri *et al.* (2008) confirm this hypothesis; these authors demonstrated an increased BP in the offspring of diabetic rats when they were only 6 months-old. The contribution of the vascular system to these changes in BP as well as the mechanisms involved remain to be elucidated.

Several studies have demonstrated that the balance between vasodilator and vasoconstrictor prostanoids is modified in hypertension (Alvarez *et al.*, 2005; Virdis *et al.*, 2009; Félétou *et al.*, 2011) and diabetes (Bagi *et al.*, 2005; Félétou *et al.*, 2011), and may involve the inducible form of the enzyme, COX-2. In healthy arteries, most prostanoids are produced by the constitutive isoform of COX (COX-1). However, these products may also be synthesized by the COX-2 that is usually expressed at undetectable levels in the vascular wall but can be up-regulated by inflammatory and physical stimuli (Félétou *et al.*, 2011). COX-2-derived prostanoids are associated with the development of vascular complications under conditions of insulin resistance and cardiovascular risk (Helmersson *et al.*, 2004; Bagi *et al.*, 2005; Elmarakby and Imig, 2010; Retailleau *et al.*, 2010). On the basis of these findings, we hypothesized that endothelial dysfunction in resistance arteries from the offspring of insulin-resistant diabetic rats is the result of enhanced COX-2 expression/metabolism, which increases the formation of contractile prostanoids.

In the present study, we tested our hypothesis and specifically investigated the effects of COX inhibitors on ACh-induced relaxation of mesenteric resistance arteries from the offspring of control and diabetic rats. Secondly, we identified the prostanoids released by ACh in arteries from the offspring of diabetic rats and compared their concentrations with those produced in arteries from the offspring of control rats. The influence of age on these parameters was also investigated.

Methods

Experimental animals

Male and female Wistar rats (250–300 g) were obtained from colonies maintained in the animal quarters of the Universidade Federal de Pernambuco (UFPE). Rats were housed at a constant room temperature, humidity and light cycle (12 h light/ dark) and had free access to tap water and standard rat chow *ad libitum*. This study was approved by the Institutional Animal Care and Use Committee of UFPE (approval reference number: 23076.015755/2008-96), and conforms to the *Guide for Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH publication 85-23, revised 1996). The results of all studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010;McGrath *et al.*, 2010).

On the 7th day of pregnancy, diabetes was induced by a single injection of streptozotocin (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. The unwanted pups were killed by CO_2 inhalation followed by cervical dislocation. When the number of males was not enough to complete six, females were used but discarded at weaning. The offspring were divided into two groups: (i) O-CR – offspring of control rats and (ii) O-DR – offspring of diabetic rats. In this study, we used O-CR and O-DR at 3, 6 and 12 months of age.

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^{-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 48 h, the animals 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 $U \cdot kg^{-1}$ body weight. Blood glucose was determined before and 15, 30, 45 and 60 min after insulin administration.

Arterial BP measurement

Rats were anaesthetized with a mixture of ketamine, xylazine and acetopromazin (64.9, 3.2 and 0.78 $mg \cdot kg^{-1}$, respectively, i.p.). The right carotid artery was cannulated with a polyethylene catheter (PE-50) that was exteriorized in the mid scapular region. Adequacy of anaesthesia was assessed by monitoring withdrawal reflexes. After 24 h, arterial pressure was measured in conscious, freely moving rats. The arterial cannula was connected to a transducer and pressure signals were recorded for a 60 min period using an interface and software for computer data acquisition (ADInstruments Pty Ltd, Castle Hill, New South Wales, Australia).

Vessel preparation

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 according to the method described by Mulvany and Halpern (1977).

Experimental protocols

After a 45 min equilibration period, each arterial segment was exposed to KCl (120 mM) to assess its maximum contractility. After a washout period, the presence of the vascular endothelium was confirmed by the ability of 1 μM 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 1 h and then a cumulative ACh concentration-response curve (0.1 nM to 3 μM) was obtained in the noradrenaline-precontracted segments. Endothelium-independent relaxation was studied by evaluating relaxation to sodium nitroprusside (1 nM–10 μM) in arteries previously contracted with noradrenaline. The possible role of COX-derived metabolites was investigated in segments from O-CR and O-DR rats. Arteries were pre-incubated with either indomethacin (a COX-1 and COX-2 inhibitor, 10 μM), SC-560 (a COX-1 inhibitor, 1 μM), NS-398 (COX-2 inhibitor, 10 μM), SQ29548 [TxA_2 receptor (TP) antagonist, 1 μM], AH6809 [PGE_2 receptor (EP_1, EP_2 and EP_3) antagonist, 30 μM] or AL8810 [$PGF_{2\alpha}$ receptor (FP) antagonist, 10 μM], before generating concentration-response curves to ACh. All drugs were added 30 min before the concentration-response curve to ACh.

In another set of experiments, the vasoactive responses to the TP receptor agonist U46619 (1 nM–10 μM), PGE_2 (10 nM–10 μM) or $PGF_{2\alpha}$ (1 nM to 3 μM), were analysed in quiescent arteries from all groups.

Prostanoid production

To measure the release of TxA_2, PGE_2, $PGF_{2\alpha}$ and PGI_2, we used specific enzyme immunoassay kits (Cayman Chemical Company, Ann Arbor, MI, USA) for each prostanoid. The second, third and fourth branches of mesenteric artery were pre-incubated for 45 min in 200 μL of gassed KHS at 37°C. Afterwards, three washout periods of 10 min in a bath of 200 μL of KHS were run before incubation with ACh (0.1 nM–3 μM). The different assays were performed following the manufacturer's instructions. Results are expressed as $pg \cdot mL^{-1}$ mg^{-1} wet tissue.

Western blot analysis of COX-1 and COX-2

Mesenteric resistance arteries from O-CR and O-DR rats were homogenized; proteins were electrophoretically separated (30 μg per lane) and transferred to polyvinyl difluoride membranes (GE Healthcare do Brasil Ltda, São Paulo, SP, Brazil). Membranes were incubated with antibodies (Cayman Chemical Company) against COX-1 (1:1000 dilution), COX-2 (1:500 dilution) or α-actin (1:3000 dilution) proteins and individual horseradish peroxidase-conjugated secondary antibodies (GE Healthcare do Brasil Ltda) in blocking buffer. Immunoreactive proteins were detected by chemiluminescence with ECL Plus (GE Healthcare do Brasil Ltda) and subjected to autoradiography (Hyperfilm ECL, GE Healthcare do Brasil Ltda). Signals on the immunoblot were quantified using a computer programme (NIH Image V1.56, Bethesda, MD, USA). Ram seminal vesicle microsomes and mouse macrophage microsomes protein (Cayman Chemical Company) were used as positive control for COX-1 and COX-2, respectively.

Drugs

Drugs used were noradrenaline hydrochloride, ACh chloride, sodium nitroprusside, indomethacin (Sigma, St. Louis, MO, USA), NS-398, SQ29548, AH6809, AL8810, PGE_2, $PGF_{2\alpha}$ (Cayman Chemical Company) and U46619 (Calbiochem-Novabiochem GmbH, Darmstadt, Germany). Stock solutions of ACh and sodium nitroprusside were made in distilled water; noradrenaline was dissolved in an NaCl (0.9%)–ascorbic acid (0.01% w v^{-1}) solution; indomethacin, SQ29548, PGE_2 and $PGF_{2\alpha}$ were dissolved in ethanol; and AH6809, AL8810 and U46619 were dissolved in dimethyl sulfoxide. These solutions were kept at –20°C and appropriate dilutions were made on the day of the experiment. Drug/molecular target nomenclature conforms to the *British Journal of Pharmacology's Guide to Receptors and Channels* (Alexander *et al.*, 2011).

Statistical analysis

Relaxation responses to ACh and sodium nitroprusside are expressed as a percentage of the maximum contractile response induced by noradrenaline. U46619, PGE_2 and $PGF_{2\alpha}$ contractile responses are expressed as a percentage of the maximum response produced by KCl.

All values are expressed as means ± SEM of the number of animals used in each experiment. Results were analysed using Student's *t*-test or by one-way or two-way ANOVA for comparison between groups. When ANOVA showed a significant treatment effect, Bonferroni's *post hoc* test was used to compare individual means. Differences were considered statistically significant at $P < 0.05$.

Results

Dams injected with streptozotocin had severe hyperglycaemia on gestational days 14 and 21 compared with control dams (control 852 ± 24 vs. diabetic 4820 ± 225 mg·L^{-1}, *t*-test: $P < 0.05$). Gestation occurred normally, and the rats delivered spontaneously at term (21 days of gestation). Diabetic dams gave birth to fewer pups than the controls (control: 10 ± 1 vs. diabetic 6 ± 2 pups per litter; *t*-test: $P < 0.05$). As shown in Table 1, mean body weight was significantly lesser in O-DR than O-CR. Blood glucose levels were similar in O-CR and O-DR (Table 1).

Oral glucose tolerance test was performed at 3 and 12 months of age. Blood glucose levels were higher in both 3 and 12-month-old O-DR at 30 min compared with O-CR rats (results not shown) and remained increased until the time of 120 min (3-month-old rats: O-CR, 1050 ± 49 vs. O-DR, 1280 ± 45 mg·L^{-1}, *t*-test: $P < 0.05$; 12-month-old rats: O-CR, 1060± 32 vs. O-DR, 1420 ± 23 mg·L^{-1}, *t*-test: $P < 0.05$). Results from the insulin tolerance test demonstrated significant insulin resistance among the O-DR rats, as they presented a higher blood glucose from 15 min to 60 min after an insulin injection (blood glucose 60 min after the insulin injection; 3-month-old rats: O-CR, 350 ± 40 vs. O-DR, 470 ± 23 mg·L^{-1}, *t*-test: $P < 0.05$; 12-month-old rats: O-CR, 330 ± 15 vs. O-DR, 572 ± 32 mg·L^{-1}, *t*-test: $P < 0.05$).

O-DR presented higher BP in adulthood. Although the mean arterial pressure of 3-month-old rats was similar in both groups (O-CR: 97.5 ± 2.54 vs. O-DR: 104 ± 8.40 mmHg, *t*-test, $P > 0.05$), it was significantly increased in O-DR at both 6 (O-CR: 105 ± 4.70 vs. O-DR: 132 ± 5.30 mmHg, *t*-test, $P > 0.05$) and 12 months (O-CR: 102 ± 5.10 vs. O-DR: 149 ± 3.70 mmHg, *t*-test, $P > 0.05$) compared with O-CR. The heart rate was similar in all O-DR groups compared with their respective age-matched O-CR (results not shown).

Vascular function in adult diabetic offspring

KCl (120 mM) evoked similar contractions in vessels from both diabetic and age-matched control offspring rats (3-month-old rats, O-CR: 3.15 ± 0.04 vs. O-DR: 3.19 ± 0.07 mN·mm^{-1}; 6-month-old rats, O-CR: 3.28 ± 0.12 vs. O-DR: 3.19 ± 0.09 mN·mm^{-1}; 12-month-old rats, O-CR: 3.21 ± 0.02 vs. O-DR: 3.25 ± 0.13 mN·mm^{-1}; ANOVA, $P > 0.05$).

ACh induced cumulative concentration- and endothelium-dependent relaxations of noradrenaline-contracted arteries from 3-, 6- and 12-month-old O-CR (Figure 1). However, in mesenteric arteries from O-DR, ACh induced a biphasic response, characterized by a relaxing effect at concentrations equal or below 0.1 μM, which was lower than that in age-matched O-CR, and by a contractile response at concentrations above 0.3 μM that was absent in arteries from O-CR (Figure 1A). Further deterioration of this relaxation was noted with aging (compare Figure 1A–C). Mesenteric resistance arteries from 3-month-old O-DR relaxed 86% to ACh while 6- and 12-month-old O-DR showed 59% and 37% relaxation, respectively (ANOVA: $P < 0.05$). In arteries from O-CR, relaxation to ACh did not change with age (Figure 1). In all groups, relaxation induced by sodium nitroprusside was comparable (Figure 2).

Effects of COX-1 and COX-2 inhibition and TP, EP and FP receptor antagonism on endothelium-dependent relaxation

In 3-, 6- and 12-month-old O-CR, ACh-induced vasodilatation was not modified by indomethacin or NS-398 (Figure 3A,C,E). In contrast, the altered response to ACh in arteries from 3-, 6- and 12-month-old O-DR was normalized by either indomethacin or NS-398, but not by SC-560, (Figure 3B,D,F).

In vessels from 3-month-old O-DR, the response to ACh was also enhanced by SQ29548 to similar values to those obtained with COX-2 blockade (Figure 4A). When applied concomitantly with SQ29548, AH6809 or AH6809 plus AL8810 failed to further increase ACh-induced relaxation (Figure 4A). In 6-month-old O-DR, SQ29548 increased the relaxation to ACh, but its effect was reduced compared to that obtained with NS-398 (Figure 4B). When co-incubated with SQ29548, AH6809 increased the response to ACh to similar values to those obtained with NS-398. AL8810 failed to enhance the effect of SQ29548 plus AH6809 (Figure 4B). In arteries from 12-month-old O-DR, SQ29548 partly increased the response to ACh (Figure 4C). In arteries from this group, although co-incubation with AH6809 increased the effect of SQ29548, the normalization of the ACh response was achieved only when arteries were treated with SQ29548 in combination with AH6809 and AL8810 (Figure 4C).

Table 1

Body weights (BW) and blood glucose (BG) at the times of vascular testing

	3 months		6 months		12 months	
	BW (g)	BG (mg·L^{-1})	BW (g)	BG (mg·L^{-1})	BW (g)	BG (mg·L^{-1})
O-CR	318 ± 7.23	957 ± 34	422 ± 6.18	952 ± 27	467 ± 0.40	942 ± 41
O-DR	295 ± 9.40*	994 ± 13	375 ± 11.7*	925 ± 30	443 ± 2.50*	908 ± 21

Values are means ± SEM. ANOVA: *$P < 0.05$ compared with O-CR at the same time point.
O-CR, offspring of control rats; O-DR, offspring of diabetic rats.

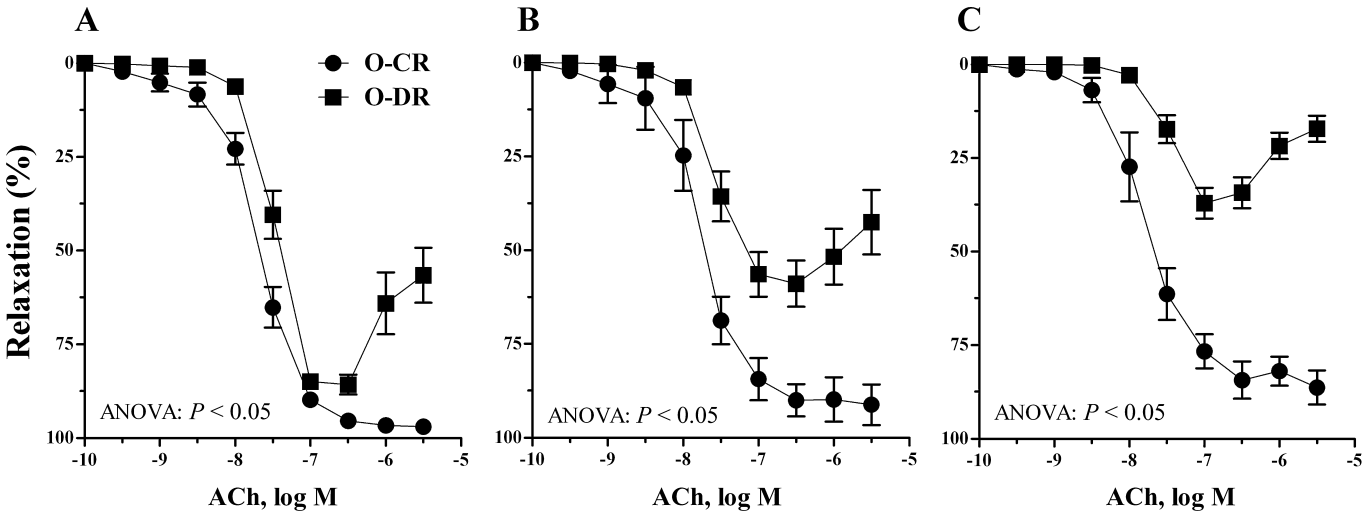

Figure 1

Endothelium-dependent relaxation induced by ACh in mesenteric resistance arteries from (A) 3-, (B) 6- and (C) 12-month-old offspring of control (O-CR) and diabetic (O-DR) rats. Each point represents the mean of 7–8 experiments ± SEM.

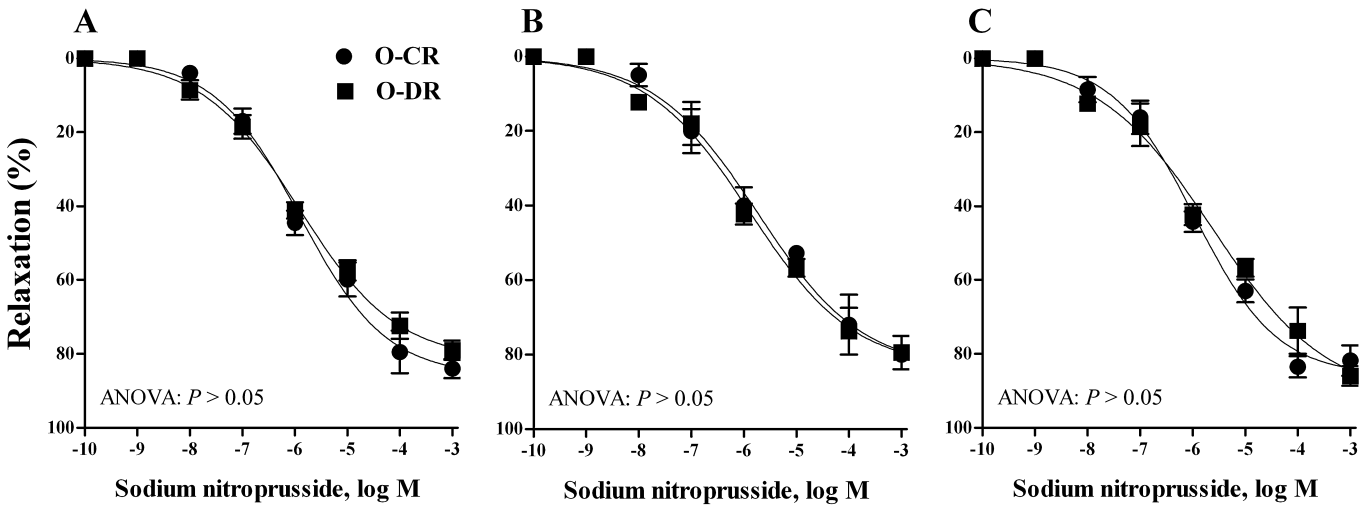

Figure 2

Endothelium-independent relaxation induced by sodium nitroprusside in mesenteric resistance arteries from (A) 3-, (B) 6- and(C) 12-month-old offspring of control (O-CR) and diabetic (O-DR) rats. Each point represents the mean of 7–8 experiments ± SEM.

Vascular response to U46619, PGE_2 and $PGF_{2\alpha}$

U46619, PGE_2 and $PGF_{2\alpha}$ caused cumulative concentration-dependent contractions in quiescent arteries. These contractions were not significantly different (ANOVA: $P > 0.05$) between preparations of O-CR and O-DR (results not shown).

Prostanoids production

In mesenteric resistance arteries from all groups, ACh increased the production of TxB_2, PGE_2 and $PGF_{2\alpha}$ (Figure 5). The ACh-stimulated TxB_2 levels were higher in arteries from both 3-, 6- and 12-month-old O-DR compared with their respective age-matched O-CR (Figure 5A). In arteries from 6- and 12-month-old O-DR, the ACh-stimulated levels of PGE_2 were higher, while in 3-month-old O-DR they were not modified (Figure 5B). The ACh-stimulated $PGF_{2\alpha}$ levels were higher only in arteries from 12-month-old O-DR (Figure 5C).

COX-1 and COX-2 protein expression

Western blot analysis showed a basal protein expression of COX-2 in mesenteric arteries from O-CR and O-DR (Figure 6). This expression was higher in arteries from O-DR and increased with age, reaching higher levels in 12-month-old O-DR (Figure 6). COX-1 expression was similar in all groups (Figure 6).

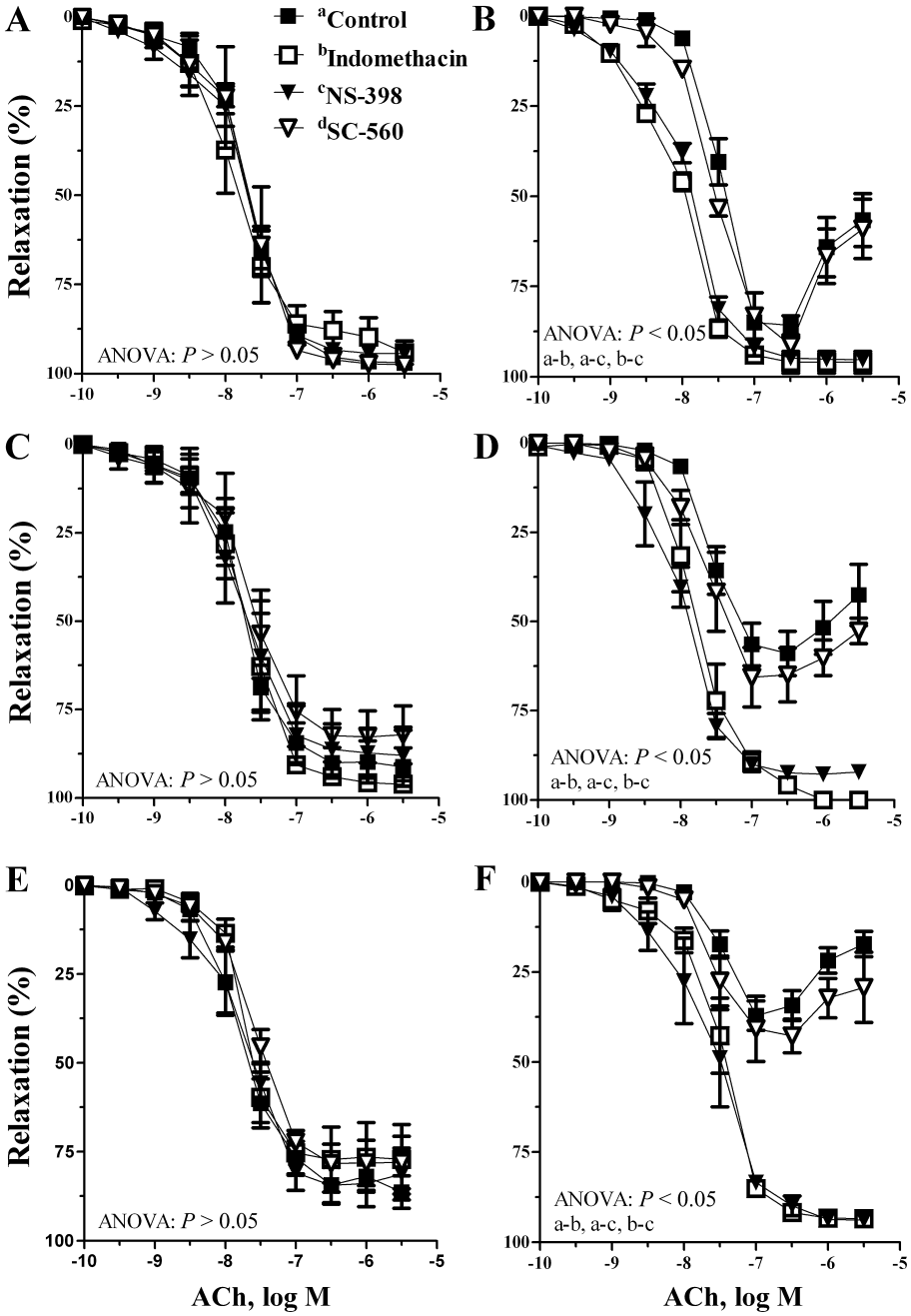

Figure 3

Effect of indomethacin (10 μM) or NS-398 (10 μM) on the concentration-dependent relaxation to ACh in segments of mesenteric resistance arteries from (A) 3-month-old O-CR, (B) 3-month-old ODR, (C) 6-month-old O-CR, (D) 6-month-old ODR, (E) 12-month-old O-CR and (F) 12-month-old ODR. Results are expressed as mean ± SEM; n = 7–8 animals in each group.

Discussion and conclusions

In the present study, we investigated the long-term vascular consequences of *in utero* exposure to maternal hyperglycaemia in rats. Our major novel finding is that this exposure is associated with an age-dependent COX-2 up-regulation, coupled with an enhanced formation of contracting prostanoids that contribute to the impairment of endothelial function in mesenteric resistance arteries from adult offspring of diabetic rats.

Hyperglycaemia in pregnancy leads to insulin resistance in the offspring (Segar *et al.*, 2009; Blondeau *et al.*, 2011), leading to vasoconstriction, inflammation and thrombosis, which occasionally produces hypertension (Chahwala and Arora, 2009). In the current study, although no offspring group developed pre-diabetes or diabetes during the

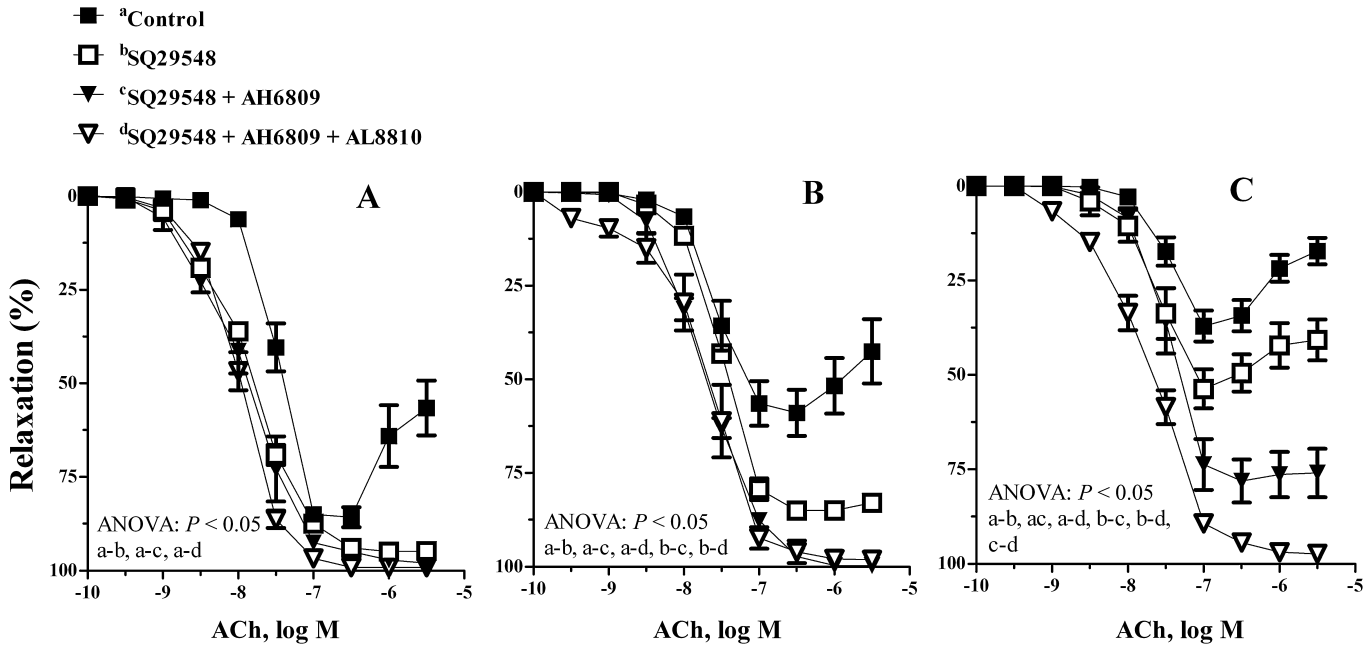

Figure 4

Effect of SQ29548 (1 μM) alone or in association with AH6809 (30 μM) or AH6809 plus AL8810 (10 μM) on the concentration-dependent relaxation to ACh in segments of mesenteric resistance arteries from (A) 3-, (B) 6- and (C) 12-month-old offspring of diabetic (O-DR) rats. Results are expressed as mean ± SEM; n = 7–8 animals in each group.

follow-up period, O-DR showed insulin resistance and glucose intolerance at 3 months of age and a further impairment of these parameters at 12 months of age. This effect did not seem to be related to a toxic effect of streptozotocin on the fetal endocrine pancreas as: (i) streptozotocin has a very short (30 min) half-life (Schein and Loftus, 1968); (ii) beta-cell development in the rat occurs mainly during the last week of fetal growth (Blondeau and Breant, 2005); (iii) streptozotocin presented no cytotoxic effect on fetal pro-islets (Liu *et al.*, 1994); and (iv) insulin resistance in O-DR occurs irrespective of the time of streptozotocin administration.

The mechanisms of the hyperglycaemia-programmed hypertension are complex and involve renal, neural and vascular effects (Amri *et al.*, 1999; Holemans *et al.*, 1999; Rocha *et al.*, 2005; Wichi *et al.*, 2005; Nehiri *et al.*, 2008; Segar *et al.*, 2009; Chen *et al.*, 2010). Previous studies have demonstrated a reduced endothelium-dependent relaxation response to dilators in both conductance (Segar *et al.*, 2009; Porto *et al.*, 2010) and resistance (Holemans *et al.*, 1999; Rocha *et al.*, 2005) arteries from O-DR compared with O-CR. This response has been accompanied, or not, by hypertension. Holemans *et al.* (1999) demonstrated impaired relaxation to ACh and bradykinin in arteries from 3-month-old offspring of diabetic dams, but without changes in BP. On the other hand, Rocha *et al.* (2005) demonstrated increased BP in 12-month-old diabetic offspring associated with impaired endothelial and renal functions. Therefore, it is possible to postulate that the phenotype of the offspring of diabetic rats tends to vary according to age at the time of analysis.

The results obtained here show that although the BP in 3-month-old rats was similar in both groups, it was increased in the 6- and 12-month-old rats in O-DR compared with those in the O-CR group, as previously reported by Nehiri *et al.* (2008). Our results also demonstrate that in mesenteric resistance arteries from 3-, 6- and 12-month-old O-DR, the relaxation to ACh, but not to sodium nitroprusside, was impaired compared with that observed in O-CR. In O-DR arteries, this relaxation was progressively impaired with age while in O-CR it remained unmodified. The importance of abnormal endothelium-dependent dilation, as observed in O-DR, lies in the possible pathological consequences. Reduced endothelial function may contribute to coagulation, inflammation and atherosclerosis, as well as to increased peripheral resistance and hypertension (Félétou and Vanhoutte, 2006). Our results also revealed that the endothelial dysfunction in O-DR precedes hypertension, suggesting that this mechanism could be involved in the genesis of hypertension in these animals. Furthermore, a greater impairment of the endothelium-dependent relaxation in arteries from 6- and 12-month-old O-DR was associated with a higher BP in these rats. The mechanism by which maternal diabetes impairs the endothelial function is not fully known. Insulin resistance, perhaps as a result of raised plasma cholesterol and triglycerides, is implicated in endothelial dysfunction (Johnstone *et al.*, 1993). Transient hyperglycaemia (Holemans *et al.*, 1997), oxidative stress (Siman and Eriksson, 1997) or the synthesis of advanced glycosylation end-products (Vlassara *et al.*, 1992) *in utero* have also been suggested as possible mechanisms involved in decreased endothelium-dependent vasodilatation in the adult offspring.

Insulin resistance is associated with a pro-inflammatory state of the vascular wall leading to vascular remodelling and endothelial dysfunction in systemic arteries (Chahwala and Arora, 2009). Various studies have shown that COX-2 is

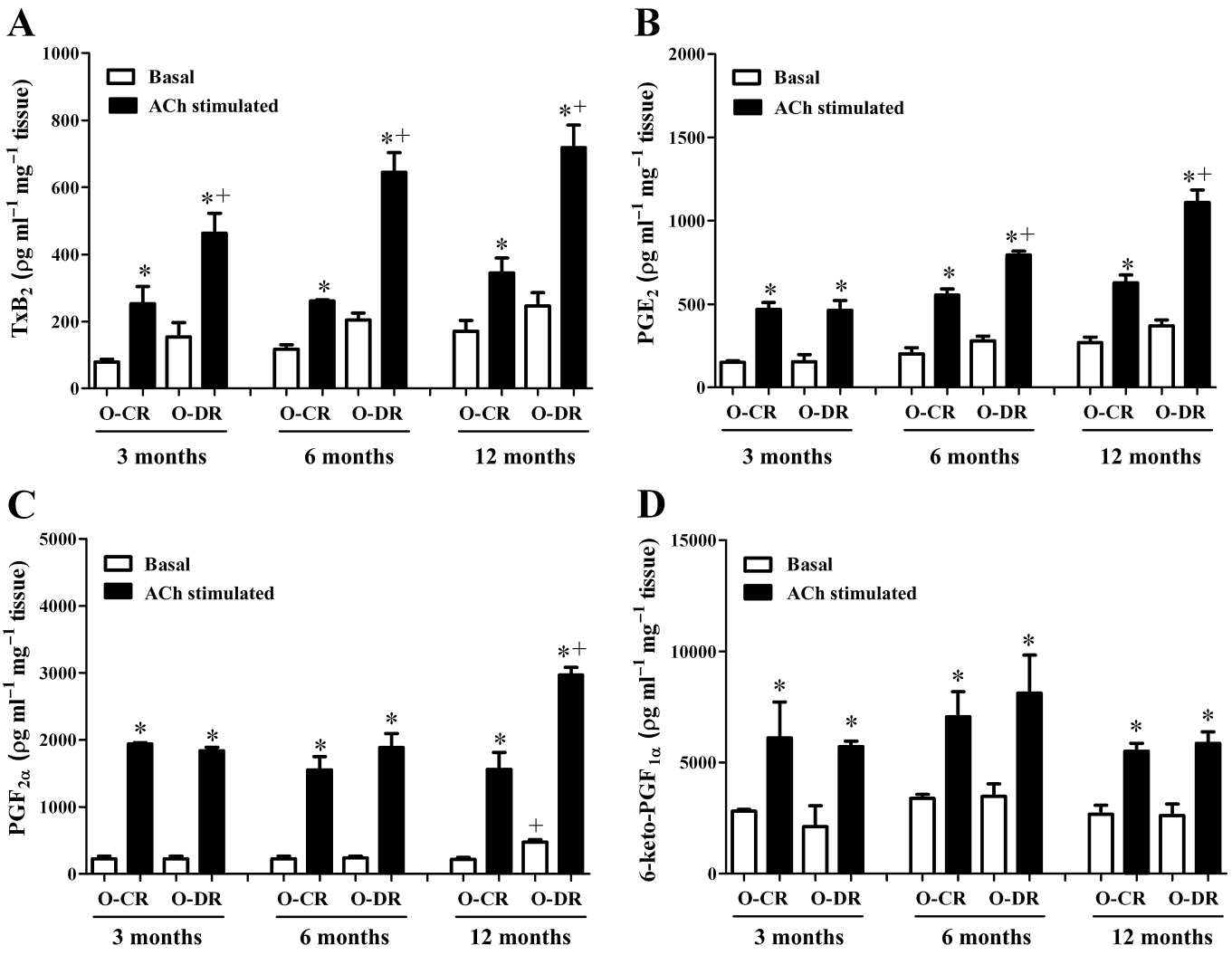

Figure 5

Basal and ACh-stimulated production of TxB_2 (A), PGE_2 (B), $PGF_{2\alpha}$ (C) and prostacyclin (6-keto-$PGF_{1\alpha}$, D) in mesenteric resistance segments from 3-, 6- and 12-month-old offspring of control (O-CR) or diabetic (O-DR) rats. n = 6–7 animals in each group. ANOVA: *P < 0.05 ACh-stimulated vs. basal; +P < 0.05 O-DR vs. age-matched O-CR.

induced during some inflammatory process and that prostanoids produced by this COX isoform are responsible for many inflammatory symptoms (Parente and Perretti, 2003). In this sense, COX-2-derived prostanoids have been shown to be associated with the development of vascular complications under conditions of insulin resistance and cardiovascular risk (Helmersson *et al.*, 2004; Bagi *et al.*, 2005; Virdis *et al.*, 2009; Elmarakby and Imig, 2010; Retailleau *et al.*, 2010, Félétou *et al.*, 2011), which in turn could contribute to elevation in BP (Tian *et al.*, 2011). The O-DRs used in the present study were insulin resistant and presented endothelial dysfunction in adulthood. To investigate the participation of COX-derived products in the ACh responses, we used the non-selective COX inhibitor indomethacin, the selective COX-1 inhibitor SC-560 and the selective COX-2 inhibitor NS-398. In arteries from 3-, 6- and 12-month-old O-DR, indomethacin or NS-398, but not SC-560, restored the ACh-induced relaxation to similar levels to that in O-CR, indicating that contractile prostanoids from COX-2, but not from COX-1, contribute to the endothelial dysfunction in these rats. In keeping with these functional results, Western blot analysis revealed an up-regulation of COX-2, but not COX-1, in arteries from all O-DR, reaching higher levels at 12 months of age. In O-CR arteries, inhibition of COX-1 or COX-2 did not affect the response to ACh, suggesting that in these rats COX isoenzymes do not have a significant effect on the ACh-induced relaxation, as previously reported (Xavier *et al.*, 2008).

Activation of TP receptors allows vasoconstrictor prostanoids such as TxA_2 to contribute to endothelial dysfunction in different cardiovascular disorders (Alvarez *et al.*, 2005; Blanco-Rivero *et al.*, 2005; Xavier *et al.*, 2008). In the present study, blockade of the TP receptors (SQ29548) increased ACh-induced relaxation in mesenteric arteries from all O-DR. In the 3-month-old O-DR, this effect was similar to that produced by NS-398, suggesting that TxA_2 is the COX-2-derived

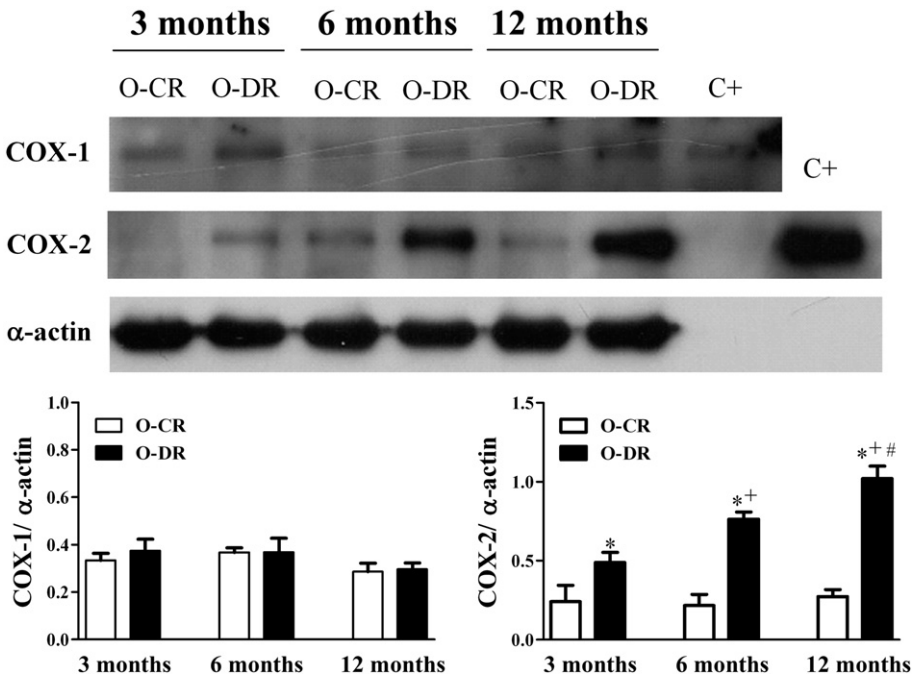

Figure 6

Representative Western blot for COX-1, COX-2, α-actin protein expression and the positive control (C+) for COX-1 and COX-2 in mesenteric resistance arteries from 3-, 6- and 12-month-old offspring of control (O-CR) or diabetic (O-DR) rats (upper panel). Graph shows densitometric analysis of the Western blot for COX-1 and COX-2. Results (means ± SEM) are expressed as the ratio between the signal for the COX-1 or COX-2 protein and the signal for α-actin. $n = 6$ animals in each group. ANOVA: *$P < 0.05$ O-DR versus age-matched O-CR; +$P < 0.05$ 6-month-old O-DR versus 3-month-old O-DR; #$P < 0.05$ 12-month-old O-DR versus 6-month-old O-DR.

prostanoid involved in the endothelial dysfunction in this group. Indeed, in arteries from these rats, ACh-stimulated release of TxB_2 (the TxA_2 metabolite) was increased. On the other hand, it should be noted that ACh-induced relaxation in arteries from 6- and 12-month-old O-DR was not entirely restored by SQ29548. In vessels from 6-month-old O-DR, relaxation to ACh was completely restored by additional application of the EP receptor blocker, AH6809, while the combined treatment of SQ29548 plus AH6809 and AL8810 (an FP receptor antagonist) was required to fully restore this response in arteries from 12-month-old O-DR. This implies that in arteries from 6-month-old O-DR, TxA_2 and PGE_2 contribute to the COX-2-dependent endothelial dysfunction while in 12-month-old O-DR, in addition to these two prostanoids, $PGF_{2\alpha}$ seems to play a role. In keeping with these functional findings, ACh-stimulated release of TxB_2 and PGE_2 was higher in arteries from 6-month-old O-DR. In addition to these two prostanoids, ACh-stimulated $PGF_{2\alpha}$ was also increased in arteries from 12-month-old O-DR. These findings are in accordance with previous results showing that COX-2 induces TxA_2, PGE_2 and $PGF_{2\alpha}$ production under inflammatory conditions (Kimura *et al.*, 1994; Alvarez *et al.*, 2005; Blanco-Rivero *et al.*, 2005; Retailleau *et al.*, 2010).

PGI_2 is another prostanoid that could contribute to impaired endothelial function in arteries from O-DR. This prostanoid also produces vasoconstriction that is mediated by activation of TP and EP receptors (Gluais *et al.*, 2005; Xavier *et al.*, 2008; 2009). Thus, the improvement of relaxation to ACh in arteries from O-DR, obtained in the presence of SQ29548 or AH6809, could be also due to blockade of the vasoconstrictor action of PGI_2. In a previous study, we reported that COX-2 overexpression is accompanied by increased PGI_2 release and blunted ACh-induced relaxation in rat resistance vessels (Xavier *et al.*, 2008). In the current study, the amount of ACh-stimulated 6-keto-$PGF_{1\alpha}$ in arteries from O-DR was comparable with that observed in arteries from age-matched O-CR. Nevertheless, the involvement of PGI_2 in the impaired ACh-induced relaxation in vessels from O-DR cannot be completely ruled out. Duong Van Huyen *et al.* (2010) reported that exposure to maternal diabetes is associated with decreased vascular expression of IP and impaired relaxation to a PGI_2 analogue, which in turn could contribute to the impaired endothelium-dependent relaxation.

Taken together our findings provide the first demonstration of the participation of an age-dependent increased COX-2-derived TxA_2, PGE_2 and $PGF_{2\alpha}$ in the changes in endothelial function in resistance vessels from adult offspring of diabetic rats, supporting the possible relevance of these prostanoids in cardiovascular changes induced by exposure *in utero* to hyperglycaemia. The fact that exogenously administered PGE_2, $PGF_{2\alpha}$ or the TP receptor agonist U46616 produced similar contractions in arteries from all groups studied eliminates the possibility that EP, FP or TP receptor initiated signalling mechanisms are altered in mesenteric resistance arteries from O-DR.

In summary, the present study demonstrates an age-dependent up-regulation of COX-2 coupled to an enhanced formation of vasoconstrictor prostanoids in resistance arteries

from adult offspring of diabetic rats. This increased formation of vasoconstrictor prostanoids plays a key role in the pathogenesis of endothelial dysfunction, which in turn could contribute to progression of vascular dysfunction in these rats.

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Conflict of interest

None.

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6. ARTIGO 2

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PLOS ONE

Increased Cyclooxygenase-2-Derived Prostanoids Contributes to the Hyperreactivity to Noradrenaline in Mesenteric Resistance Arteries from Offspring of Diabetic Rats

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Abstract

This study analyzed the effect of *in utero* exposure to maternal diabetes on contraction to noradrenaline in mesenteric resistance arteries (MRA) from adult offspring, focusing on the role of cyclooxygenase (COX)-derived prostanoids. Diabetes in the maternal rat was induced by a single injection of streptozotocin (50 mg/kg body weight) on day 7 of pregnancy. Contraction to noradrenaline was analyzed in isolated MRA from offspring of diabetic (O-DR) and non-diabetic (O-CR) rats at 3, 6 and 12 months of age. Release of thromboxane A_2 (TxA_2) and prostaglandins E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$), was measured by specific enzyme immunoassay kits. O-DR developed hypertension from 6 months of age compared with O-CR. Arteries from O-DR were hyperactive to noradrenaline only at 6 and 12 months of age. Endothelial removal abolished this hyperreactivity to noradrenaline between O-CR and O-DR. Preincubation with either the COX-1/2 (indomethacin) or COX-2 inhibitor (NS-398) decreased noradrenaline contraction only in 6- and 12-month-old O-DR, while it remained unmodified by COX-1 inhibitor SC-560. In vessels from 6-month-old O-DR, a similar reduction in the contraction to noradrenaline produced by NS-398 was observed when TP and EP receptors were blocked (SQ29548+AH6809). In 12-month-old O-DR, this effect was only achieved when TP, EP and FP were blocked (SQ29548+AH6809+AL8810). Noradrenaline-stimulated TxB_2 and PGE_2 release was higher in 6- and 12-month-old O-DR, whereas $PGF_{2\alpha}$ was increased only in 12-month-old O-DR. Our results demonstrated that *in utero* exposure to maternal hyperglycaemia in rats increases the participation of COX-2-derived prostanoids on contraction to noradrenaline, which might help to explain the greater response to this agonist in MRA from 6- and 12-month-old offspring. As increased contractile response in resistance vessels may contribute to hypertension, our results suggest a role for these COX-2-derived prostanoids in elevating vascular resistance and blood pressure in offspring of diabetic rats.

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Introduction

Experimental and epidemiological studies have provided evidence that intrauterine exposure to maternal hyperglycemia produces several effects on offspring including insulin resistance, change in glucose metabolism and increased risk of hypertension in adult life [1–5]. This phenomenon termed "fetal programming" refers to the observation that an adverse environmental stimulus experienced *in utero* during the critical period of development correlates with a number of adult chronic diseases, including coronary heart disease, stroke and hypertension [6,7]. In the offspring of diabetic rats, the hyperglycemia-programmed hypertension is age-dependent [1,8,9] and has been partially attributed to baroreflex dysfunction [10] and nephron deficiency [4,8,11]. In addition, some studies have suggested that functional change in the vasculature could also contribute to increase blood pressure on offspring of diabetic rats [3,8]. These functional vascular changes are characterized by impaired endothelial function [8,9,12] and increased contractile responses to vasoconstrictor agents, such as noradrenaline and serotonin [2,12]. Recently, we have demonstrated that exposure to maternal hyperglycemia is associated with age-dependent vascular cyclooxygenase (COX)-2 up-regulation and enhanced formation of contracting prostanoids, which impairs the endothelium-dependent relaxation in resistance arteries from adult offspring [9].

COX catalyzes conversion of arachidonic acid to prostanoids, which is known as important inflammatory response mediators. In addition, endothelial prostanoids production contributes to vas-

cular tone regulation [13]. Two isoforms of COX have been identified in mammalian cells. COX-1 is constitutively expressed in most tissues, such as vascular endothelial cells, and is involved in homeostasis maintenance. Although COX-2 is expressed at low or undetectable level, it is readily upregulated by inflammatory, mitogenic and physical stimuli. Vascular COX-2 expression has been reported in several pathological conditions associated with cardiovascular risk, such as hypertension, diabetes, atherosclerosis, obesity and aging [13–20]. In addition to its contribution to impairment of the endothelium-dependent relaxations [9,17,19], COX-2-derived prostanoids have also been recognized as an important factor causing increased contraction in isolated arteries from hypertensive [15] and diabetic [18] rats. However, the impact of these endothelial factors on vascular contraction is specific to each experimental model or vascular bed studied. In this sense, we [19,21] and others [22] have demonstrated that in resistance arteries from spontaneously hypertensive rats the COX-mediated impairment of endothelium-dependent relaxation is accompanied by uncharged noradrenaline-induced contraction. In resistance arteries from offspring of diabetic rats the contribution of these prostanoids to noradrenaline-induced contraction is still unknown. Once sympathetic vasoconstriction in resistance arteries is an important peripheral blood pressure regulator, this study has important implications with regard to arterial diameter regulation and blood pressure in offspring of diabetic rats.

In view of that, the present experiments were designed to study whether *in utero* exposure to maternal hyperglycemia alters contractile responses to noradrenaline in resistance arteries from adult offspring as well as the possible role of COX-2-derived prostanoids in this effect. Considering that in offspring of diabetic rats the endothelial function change with age [9], in the current study we also investigated the influence of age on noradrenaline responses.

Methods

Animals

All experimental procedures were approved by the Animal Care and Use Committee of the *Universidade Federal de Pernambuco* (approval reference number: 23076.015755/2008-96) and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

Adult offspring of control (O-CR) and diabetic (O-DR) pregnant Wistar rats were studied. Diabetes mellitus in the maternal rat was induced experimentally by a single i.p. injection of streptozotocin (50 mg/kg body weight) on day 7 of pregnancy. The diabetes was confirmed by measuring plasma glucose concentrations (ACCU-CHEK®, Roche Diagnostics, Mannheim, Germany) on day 14 of pregnancy. Only pregnant females whose plasma glucose ranged between 250 and 500 mg/dL were included in the study. After birth, each litter was randomly reduced to six pups and restricted to male offspring only. The excess pups were sacrificed by CO_2 inhalation followed by cervical dislocation. When the male number was not enough to complete six, females were used but discarded at weaning. All rats were housed at a constant room temperature (21°C), humidity (55%) and light cycle (12 h light/dark) and had free access to tap water and standard rat chow *ad libitum*. In this study we used O-CR and O-DR with 3, 6 and 12 months of age.

Arterial Blood Pressure Measurement

Rats were anesthetized with ketamine, xylazine and acetopromazin mixture (64.9; 3.2 and 0.78 mg.Kg^{-1}, respectively, *i.p.*). The right carotid artery was cannulated with a polyethylene catheter (PE-50) that was exteriorized in the mid scapular region. Adequacy of anaesthesia was assessed by monitoring withdrawal reflexes. After 24 hours, arterial pressure was measured in conscious, freely moving rats. The arterial cannula was connected to a transducer and pressure signals were recorded for a 60-min period using a data acquisition system model PowerLab 4/35 (ADInstruments Pty Ltd, Castle Hill, Australia).

Vessel Preparation

Rats were anesthetized with ketamine, xylazine and acetopromazin mixture (64.9; 3.2 and 0.78 mg/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 mmol/L: 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 in 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 according to the method described by Mulvany and Halpern [23]. After a 15-min equilibration period in oxygenated KHS at 37°C and pH 7.4, segments were stretched to their optimal lumen diameter for active tension development. Optimal lumen diameter was determined based on the internal circumference/wall tension ratio of the segments by setting the internal circumference, L_0, to 90% of what the vessels would have if they were exposed to a passive tension equivalent to that produced by a transmural pressure of 100 mmHg [23]. Optimal lumen diameter was determined using specific software for normalization of resistance arteries (DMT Normalization Module; ADInstruments Pty Ltd, Castle Hill, Australia). Segments were washed with KHS and left to equilibrate for 30 min. Vessel contractility was then tested by an initial exposure to a high-K^+ (120 mmol/L) solution.

Experimental Protocols

After washout, segments were contracted with a concentration of noradrenaline that induced approximately 50% of the KCl contraction, and then acetylcholine (1 μmol/L) was added to assess the integrity of the endothelium. After 60 min, cumulative concentration–response curves for noradrenaline (10 nmol/L - 0.1 mmol/L) were generated. The role of the endothelium in the noradrenaline-induced contraction was evaluated in segments subjected to mechanical endothelium removal. The absence of endothelium was confirmed by the inability of acetylcholine (1 μmol/L) to induce relaxation and smooth muscle integrity was confirmed by the maintenance of the KCl- (120 mmol/L) induced contraction. The effect of the nonselective nitric oxide synthase inhibitor N-nitro-l-arginine methyl ester (L-NAME, 0.1 mmol/l) on concentration–response curves for noradrenaline was investigated.

The possible role of cyclooxygenase metabolites in noradrenaline-induced contraction was investigated in segments pre-incubated with either indomethacin (a COX-1 and COX-2 inhibitor, 10 μmol/L), [5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole] SC-560 (a COX-1 inhibitor, 1 μmol/L), N-(2-cyclohexyloxy-4-nitrophenyl) methansulfonamide (NS-398, a COX-2 inhibitor, 10 μmol/L), [1S-[1a,2a(Z),3a,4a]]-7-[3-[[2-(phenylamino) carbonyl]hydrazino]methyl]-7-oxabicyclo [2.2.1] hept-2-yl]-5-heptanoic acid (SQ29548, a TxA_2 receptor (TP) antagonist, 1 μmol/L), 6-Isopropoxy-9-oxoxanthene-2-carboxylic acid (AH6809, a PGE_2 receptor (EP_1, EP_2 and EP_3) antagonist, 30 μmol/L) or 9α,15R-dihydroxy-11β-fluoro-15-(2,3-

dihydro-1H-inden-2-yl)-16,17,18,19,20- pentanor-prosta-5Z,13E-dien-1-oic acid (AL8810, a $PGF_{2\alpha}$ receptor (FP) antagonist, 10 μmol/L). All drugs were added 30 min before the concentration-response curve to noradrenaline.

In another set of experiments, the vasoactive response to the TP receptor agonist (15)-hydroxy-11,9 -(epoxymethano)prosta-5,13-dienoic acid (U46619, 1 nmol/L - 10 μmol/L), PGE_2 (10 nmol/L –10 μmol/L) or $PGF_{2\alpha}$ (10 nmol/L to 10 μmol/L), was analyzed in endothelium-denuded arteries from 6- and 12-month old rats.

Prostanoids Production

To measure the release of TxA_2, PGE_2 and $PGF_{2\alpha}$, we used specific enzyme immunoassay kits (Cayman Chemical Company, Ann Arbor, MI, USA). The second, third and fourth branches of mesenteric artery were preincubated for 45 min in 200 μl of KHS at 37°C and continuously gassed with a 95% O_2 - 5% CO_2 mixture (stabilization period). Afterward, three washout periods of 7 min in a bath of 200 μl of KHS were run before incubation with noradrenaline (10 nmol/L - 0.1 mmol/L. The medium was collected 10 min after the last dose of noradrenaline. The different assays were performed following the manufacturer's instructions. Results were expressed as $pg.ml^{-1}$ mg wet $tissue^{-1}$.

Drugs

Drugs used were noradrenaline hydrochloride, acetylcholine chloride, indomethacin, L-NAME (Sigma; St. Louis, MO, U.S.A.), NS-398, SC-560, SQ29548, AH6809, AL8810, PGE_2, $PGF_{2\alpha}$ (Cayman Chemical Company; Ann Arbor, Michigan, U.S.A.) and U46619 (Calbiochem-Novabiochem GmbH). Stock solutions of acetylcholine was made in distilled water, noradrenaline was dissolved in a NaCl (0.9%)-ascorbic acid (0.01% wv^{-1}) solution, indomethacin, SQ29548, PGE_2 and $PGF_{2\alpha}$ were dissolved in ethanol and AH6809, AL8810 and U46619, which were dissolved in dimethyl sulfoxide. These solutions were kept at −20°C and appropriate dilutions were made on the day of the experiment.

Statistical Analysis

Noradrenaline, U46619, PGE_2 and $PGF_{2\alpha}$ contractile responses were expressed as a percentage of the maximum response produced by KCl.

All values are expressed as means±S.E.M. of the number of animals used in each experiment. Results were analyzed using two-way ANOVA for comparison between groups. When ANOVA showed a significant treatment effect, Bonferroni's post hoc test was used to compare individual means. Differences were considered statistically significant at $P<0.05$.

Results

O-DR presented higher blood pressure in adulthood. Although the mean arterial pressure of 3-month-old rats was similar in both groups (Figure 1) it was significantly increased in both 6 and 12 months (Figure 1) of age in O-DR compared to O-CR. The heart rate was similar in all O-DR groups compared to their respective age-matched O-CR (results not shown).

KCl (120 mmol/L) evoked similar contractions in vessels from all groups studied (3-month-old rats, O-CR: 3.15±0.04 *vs.* O-DR: 3.19±0.07 mN/mm; 6-month-old rats, O-CR: 3.28±0.12 *vs.* O-DR: 3.19±0.09 mN/mm; 12-month-old rats, O-CR: 3.21±0.02 *vs.* O-DR: 3.25±0.13 mN/mm; ANOVA (two-way), $P>0.05$). However, exposure to maternal diabetes promoted an increase in the contractile response to noradrenaline in a time-dependent manner in arteries from the offspring (Figure 2). While in arteries from 3-month-old O-DR the noradrenaline-induced contraction

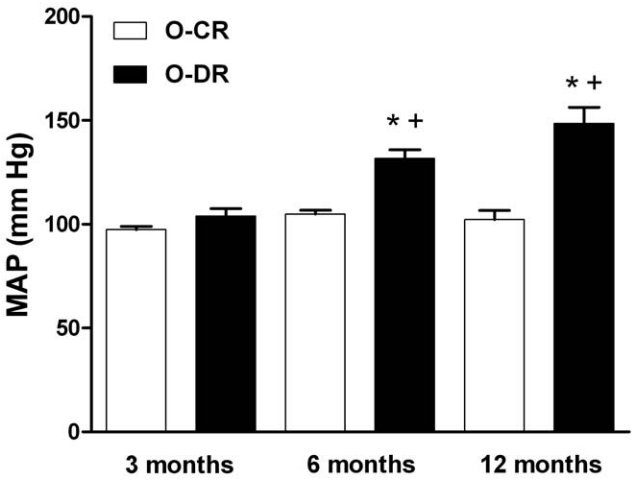

Figure 1. Effect of maternal diabetes on blood pressure levels in the offspring. Mean arterial pressure (MAP) significantly increased from 6 months in the offspring from diabetic mothers (O-DR) compared to control mother offspring group (O-CR). Results are expressed as means±SEM, N = 7–8 animals in each group. ANOVA (two-way): *$P<0.05$ O-DR *vs.* age-matched O-CR; +$P<0.05$ 6- or 12-month-old O-DR *vs.* 3-month-old O-DR.
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remained unmodified (Figure 2A), in both 6- and 12-month-old O-DR this response was increased when compared to age-matched O-CR (Figure 2B and C). In arteries without endothelium the contractile responses produced by noradrenaline were similar in all groups studied (Figure 2).

To assess the contribution of endothelium-derived nitric oxide to the noradrenaline responses, segments were incubated with the nitric oxide synthase inhibitor L-NAME. This drug similarly increased the response to noradrenaline in arteries from 3-, 6- and 12-month-old O-CR and 3-month old O-DR. (Figure 3). In arteries from 6- and 12-month-old O-DR L-NAME failed to produce significant increases of noradrenaline-induced contraction (Figure 3).

In mesenteric resistance arteries from 6- and 12-months old rats, incubation with either the unspecific COX inhibitor indomethacin or the specific COX-2 inhibitor NS-398 decreased the contractile response to noradrenaline only in O-DR (Figure 4C and D). In 3-month-old O-CR and O-DR, the contraction to noradrenaline did not change in presence of indomethacin or NS-398 (Figure 4A and B). Preincubation with the specific COX-1 inhibitor SC-560 did not alter the contraction to noradrenaline in any experimental group (results not shown).

To identify the contractile prostanoid involved in noradrenaline responses, arteries from 6- and 12-month-old O-DR were incubated with the TP receptor antagonist SQ29548, the EP_1, EP_2 and EP_3 antagonist AH6809 and the FP antagonist AL8810. In 6-month-old O-DR, SQ29548 decreased contraction to noradrenaline, but its effect was lower than to those obtained with NS-398 (Compare figure 4D with 5A). When co-incubated with SQ29548, AH6809 reduced the response to noradrenaline to similar values to those obtained with NS-398. AL8810 failed to decrease the effect of SQ29548 plus AH6809 (Figure 5A). In arteries from 12-month-old O-DR, SQ29548 partly decreased contraction to noradrenaline (Figure 5B). In arteries from this group, although co-incubation with AH6809 have increased the effect of SQ29548, an additional decreased of the noradrenaline response was observed when arteries were treated with SQ29548 in combination with AH6809 and AL8810 (Figure 5B).

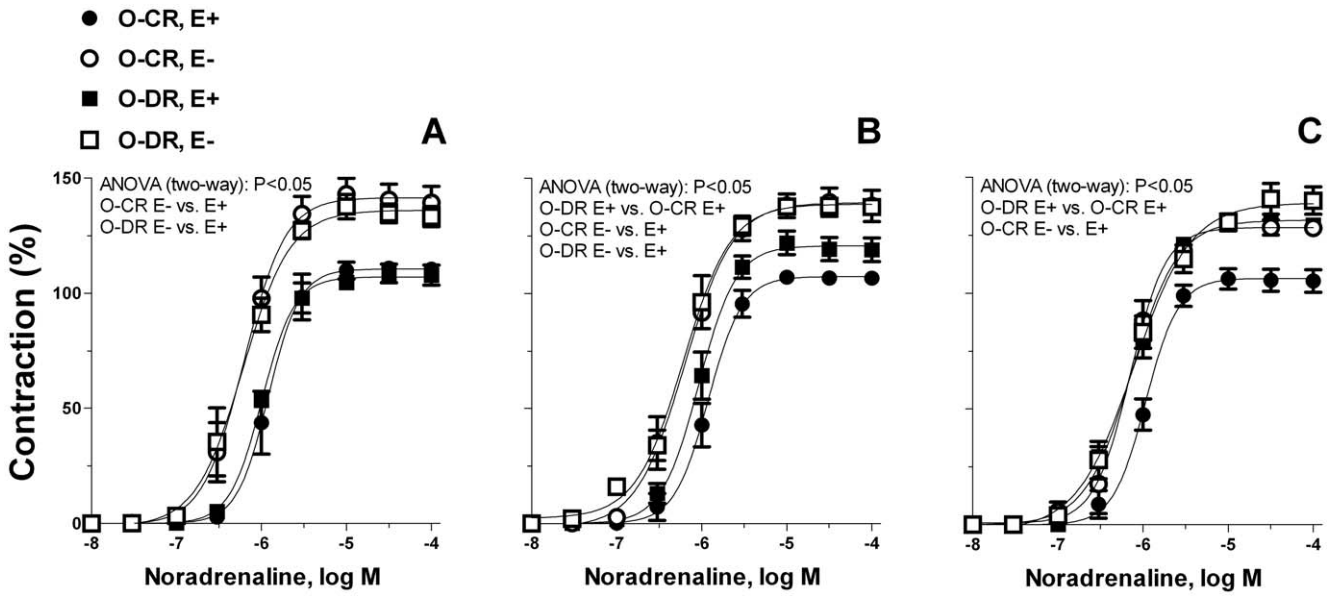

Figure 2. Concentration-dependent contraction to noradrenaline in endothelium-intact (E+) and denuded (E−) mesenteric resistance arteries from 3- (A), 6- (B) and 12-month-old offspring of diabetic (O-DR) and non-diabetic rats (O-CR). Results (mean±S.E.M.) were expressed as a percentage of the initial contraction elicited by KCl. N = 6−7 animals each curve.
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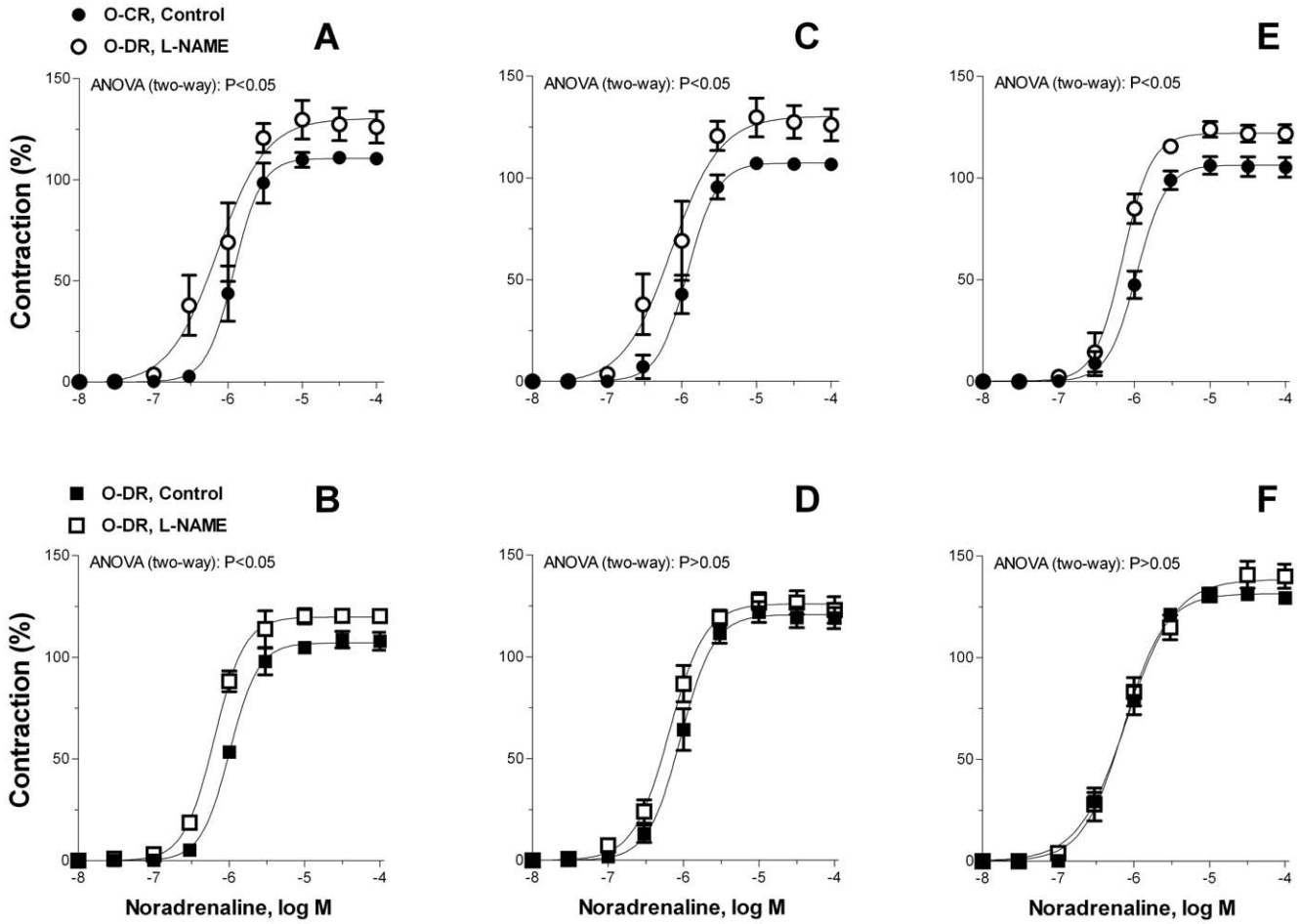

Figure 3. Effect of L-NAME (0.1 mmol/L) on the concentration dependent contraction to noradrenaline in mesenteric resistance segments (A) 3-month-old O-CR, (B) 3-month-old O-DR, (C) 6-month-old O-CR, (D) 6-month-old ODR, (E) 12-month-old O-CR and (F) 12-month-old ODR. Results (mean±S.E.M.) are expressed as a percentage of the initial contraction elicited by KCl. N = 7−8 animals each curve.
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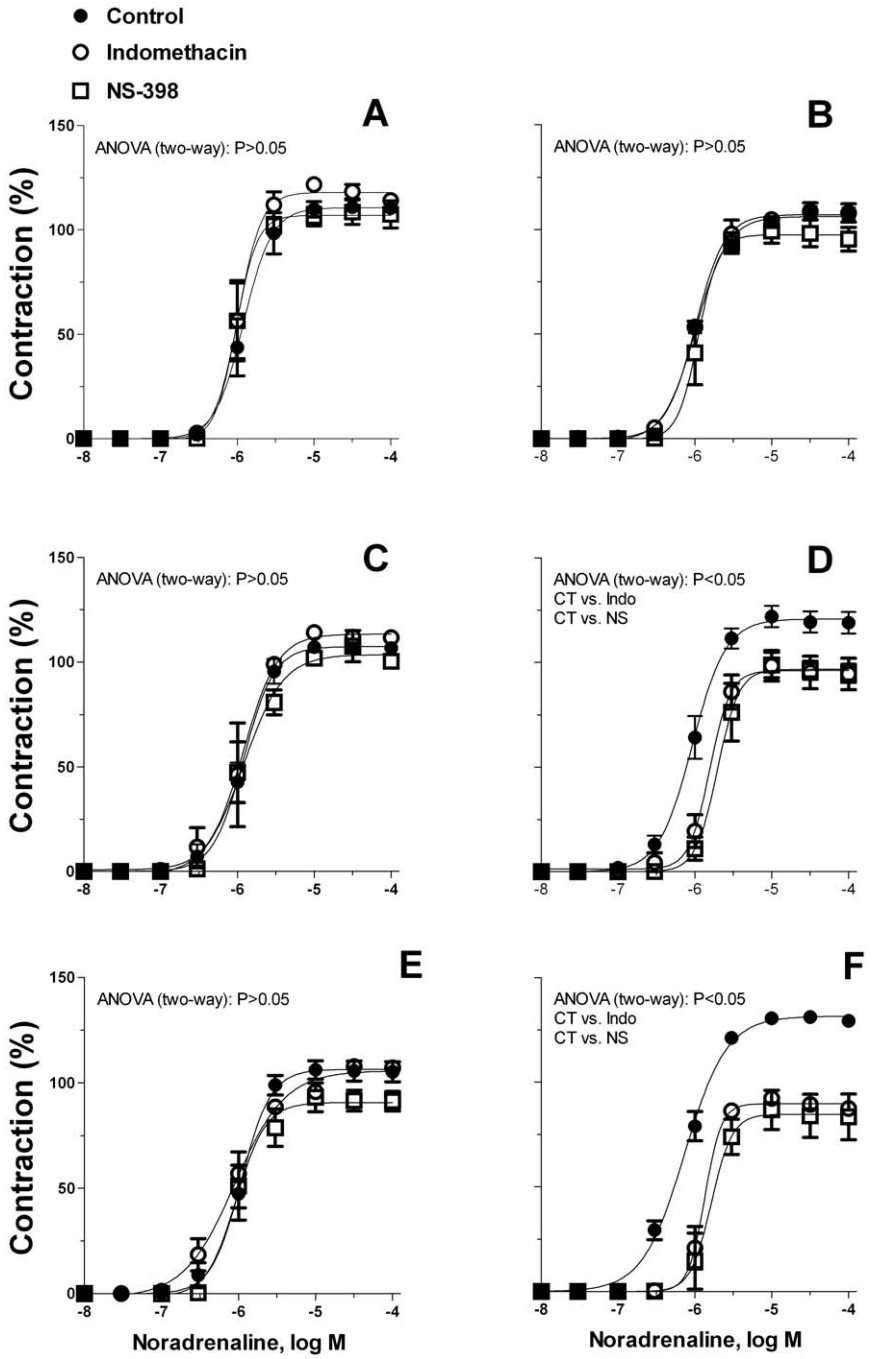

Figure 4. Effect of indomethacin (Indo, 10 μmol/L) or NS-398 (NS, 10 μmol/L) on the concentration dependent contraction to noradrenaline in mesenteric resistance segments (A) 3-month-old O-CR, (B) 3-month-old O-DR, (C) 6-month-old O-CR, (D) 6-month-old ODR, (E) 12-month-old O-CR and (F) 12-month-old ODR. Results (mean±S.E.M.) are expressed as a percentage of the initial contraction elicited by KCl. N = 6−7 animals each curve.
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U46619, PGE_2 and $PGF_{2\alpha}$ caused cumulative concentration-dependent contractions in quiescent resistance arteries. These contractions were not significantly different between arteries of O-CR and O-DR (Figure 6). Contraction to U46619, PGE_2 and $PGF_{2\alpha}$ were inhibited by SQ29548, AH6809 and AL8810, respectively (Figure 6).

In mesenteric resistance arteries from all groups, noradrenaline increased the release of TxB_2, PGE_2 and $PGF_{2\alpha}$ (Figure 7). The noradrenaline-stimulated TxB_2 levels were higher in arteries from both 6- and 12-month-old O-DR compared to their respective age-matched O-CR (Figure 7A). In arteries from 6- and 12-month-old O-DR, the levels of noradrenaline-stimulated PGE_2 were higher compared to O-CR (Figure 7B and C). The noradrenaline-stimulated $PGF_{2\alpha}$ levels were higher only in arteries from 12-month-old O-DR (Figure 7C).

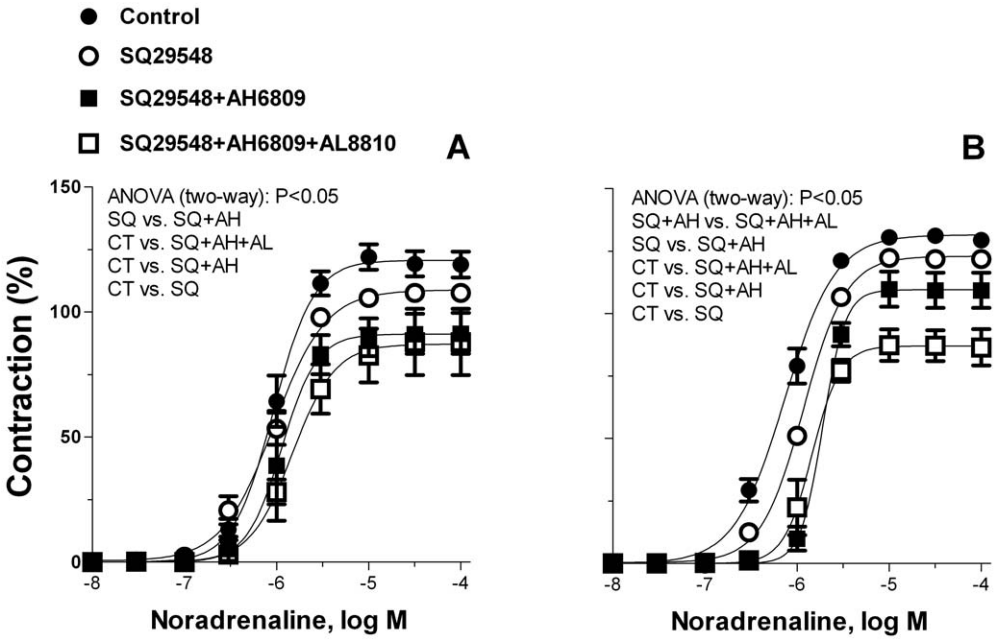

Figure 5. Effect of SQ29548 (SQ, 1 μmol/L) alone or in association with AH6809 (AH, 30 μmol/L) or AH6809 plus AL8810 (AL, 10 μmol/L) on the concentration-dependent contraction to noradrenaline in mesenteric resistance segments from 6- (B) and 12-month (mo)-old (C) diabetic (O-DR) offspring rats. Results (mean±S.E.M.) are expressed as a percentage of the initial contraction elicited by KCl. N = 6−7 animals in each group.
doi:10.1371/journal.pone.0050593.g005

Discussion

Results presented here demonstrate that *in utero* exposure to maternal hyperglycaemia in rats increases the participation of COX-2-derived TxA_2, PGE_2 and $PGF_{2\alpha}$ on contraction to noradrenaline. This participation, associated to decrease in nitric oxide modulation, might help to explain the greater noradrenaline responses observed in mesenteric resistance arteries from 6- and 12-month-old offspring of diabetic rats, which could partially contribute to the development of hypertension and vascular disease in these rats.

The intrauterine exposure to maternal hyperglycaemia is a significant risk factor for development of metabolic syndrome components, including glucose intolerance, insulin resistance and hypertension [5,24]. The mechanisms of this hyperglycemia-programmed hypertension are complex and involve renal, neural and vascular factors [1,2,4,8,10]. One of the most remarkable vascular changes in rats born from diabetic mothers is the reduction of endothelium-dependent relaxation [2,8,12]. Ingram *et al.* [25] reported that *in utero* exposure to diabetic environment severely results in general decline in proliferative capacity of fetal circulating endothelial progenitor cells, producing a decrease in the number of endothelial cells and their premature senescence. These alterations may predispose infants born from diabetic mothers to develop endothelial dysfunction and ultimately cardiovascular disease.

In a recent study we reported that offspring of diabetic rats manifest glucose intolerance, insulin resistance and hypertension at 6 and 12 months of age [9]. In addition, resistance arteries from these rats also displayed impaired endothelial function, associated with increased COX-2-derived vasoconstrictor prostanoids formation [9]. Several studies have demonstrated a correlation between endothelial dysfunction and the hyperreactivity to the vasoconstrictor agents [15,26–28]. Herein, we have provided clear evidence that compared to O-CR, mesenteric resistance arteries isolated from O-DR is indeed hyperreactive to noradrenaline. This effect is endothelium-dependent, since in endothelium-denuded arteries noradrenaline contraction was similar between O-CR and O-DR. The unmodified K^+-induced contraction in resistance arteries from O-DR suggests no alteration in contractile ability of the smooth muscle. Our results also show that the increased response to noradrenaline in arteries from O-DR correlates with changes of blood pressure in these rats, suggesting that this mechanism could be responsible for the enhanced arteriolar tone and consequently elevated systemic blood pressure in these rats.

The next objective was to determine possible quantitative or qualitative differences in the participation of endothelial factors in this contractile response in both O-CR and O-DR. Nitric oxide synthesis inhibition (L-NAME) increased the vasoconstriction to noradrenaline in arteries from all O-CR groups, while in 6- and 12-month-old O-DR it failed to produce significant increases of noradrenaline-induced contraction. This suggests a reduction on nitric oxide release/bioavailability in arteries from 6- and 12-month-old O-DR, which in turn could contribute to the augmented noradrenaline response in these rats.

COX-1 and COX-2 activity may contribute to underlying vascular hyperreactivity in some cardiovascular disease models [15,18,29–32]. Many studies have shown that COX-2 is induced during some inflammatory process and prostanoids produced by this isoform are responsible for many inflammatory signs [13,33]. In this sense, COX-2-derived prostanoids have been shown to be associated with the development of vascular complications under insulin resistance conditions and cardiovascular risk [13,14,16,20,34,35]. Results presented here and previously [9] provide several lines of evidence suggesting that COX-2 contributes to the hyperreactivity to noradrenaline in mesenteric resistance arteries from O-DR. First, the immunoblot analysis clearly shows that COX-2, but not COX-1, is up-regulated in

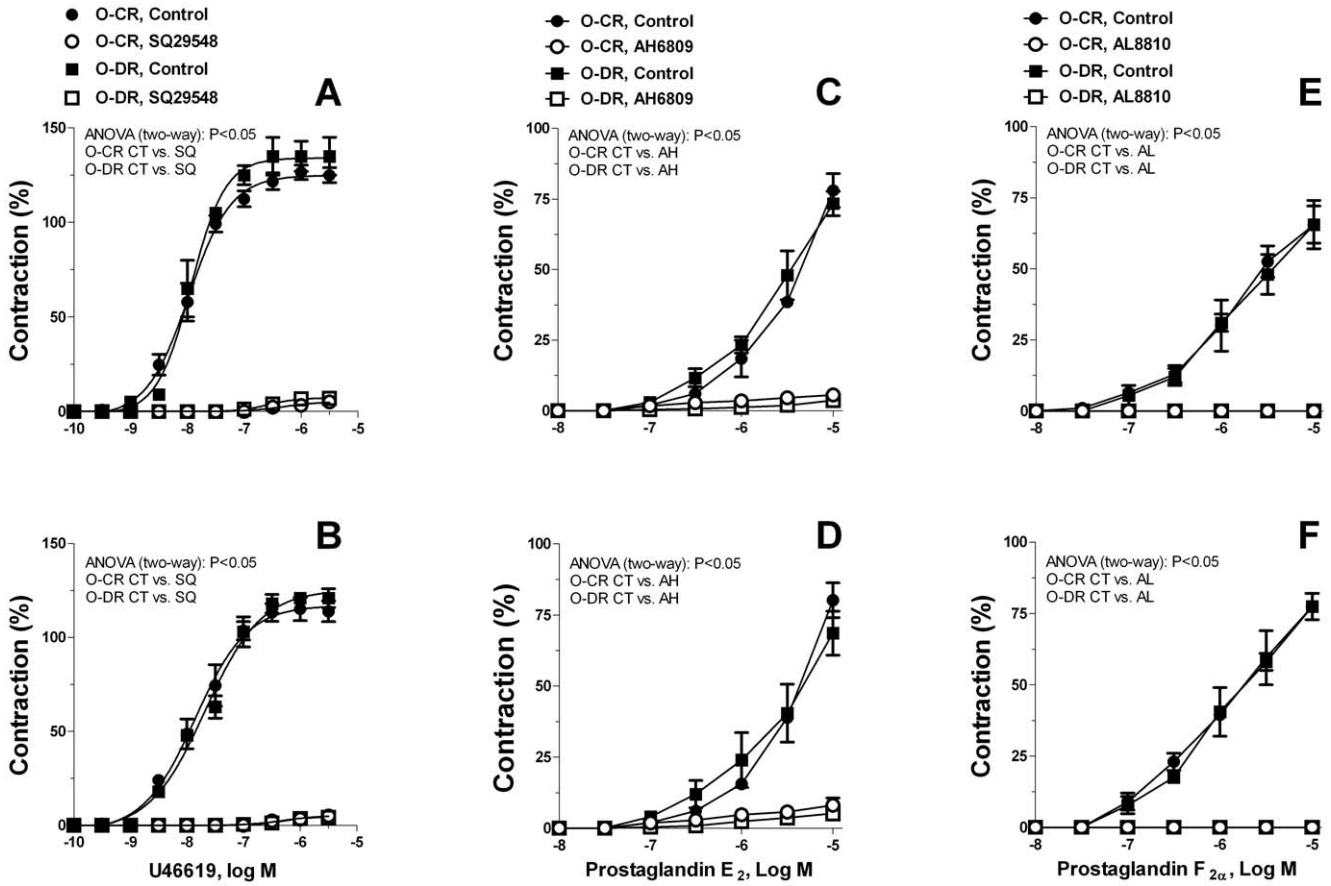

Figure 6. Concentration-dependent contraction to U46619 (A and B), PGE_2 (C and D) and $PGF_{2\alpha}$ (E and F) in endothelium-denuded mesenteric resistance arteries from 6- (A, C and E) and 12-month-old (B, D and F) offspring of diabetic (O-DR) and non-diabetic rats (O-CR). Curves to U46619, PGE_2 and $PGF_{2\alpha}$ were performed in absence (Control, CT) and in presence of SQ29548 (SQ), AH6809 (AH) or AL8810 (AL), respectively. Results (mean±S.E.M.) are expressed as a percentage of the initial contraction elicited by KCl. N = 4−6 animals in each group.
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arteries from O-DR [9]. Second, the time course of COX-2 up-regulation [9] coincides with the appearances of hyperreactivity to noradrenaline in O-DR (6 and 12 months of age). Third, selective inhibition of COX-2 activity with indomethacin or NS-398, but not the inhibition of COX-1 with SC-560, significantly reduced the noradrenaline-induced contractile responses in arteries isolated from 6- and 12-month-old O-DR, whereas these drugs did not affect the contractions in arteries from O-CR. Taken together, these results suggest that the selective COX-2 up-regulation in mesenteric resistance arteries is responsible for the contractile hyperreactivity in offspring of diabetic rats.

Data from our group [9,19] and others [15,35,36,37] have showed that COX-2 up-regulation is associated with increased production of the vasoconstrictor prostanoids, such as TxA_2. This

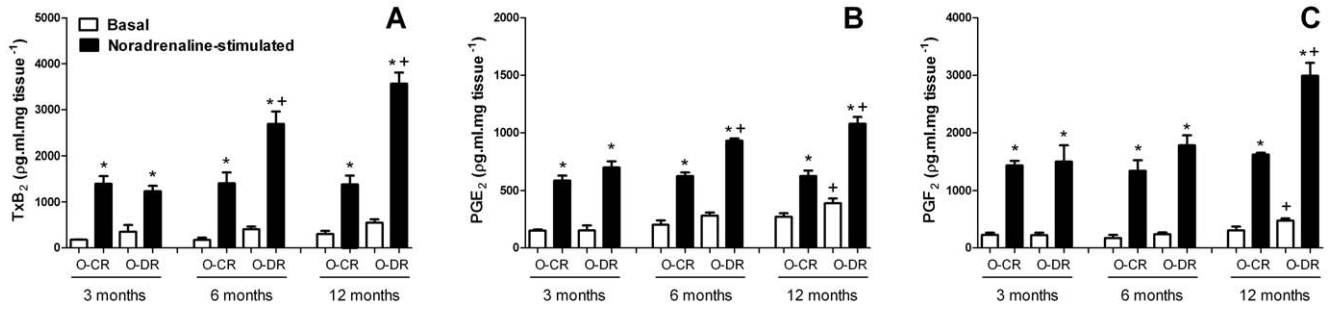

Figure 7. Basal and noradrenaline-stimulated production of thromboxane B_2 (TxB_2, A), prostaglandin E_2 (PGE_2, B) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, C) in mesenteric resistance segments from 3-, 6- and 12-month-old control (O-CR) and diabetic (O-DR) offspring rats. N = 6−7 animals in each group. ANOVA (two-way): *P<0.05 noradrenaline-stimulated vs. basal; +P<0.05 O-DR vs. age-matched O-CR.
doi:10.1371/journal.pone.0050593.g007

denotes that the increased production of TxA_2 is one potential mechanism mediating COX-2-induced vascular hyperreactivity. Indeed, TP receptor blockade by SQ29548 diminished the vascular smooth muscle hyperreactivity in both 6- and 12-month-old O-DR. In addition, noradrenaline-stimulated release of TxB_2 (the TxA_2 metabolite) was increased in arteries from those rats. However, it should be noted that in arteries from 6 and 12-month-old O-DR the decrease in noradrenaline-induced contraction produced by SQ29548 was lower than that observed in the presence of indomethacin or NS-398. In mesenteric arteries from 6-month-old O-DR, reduction in the noradrenaline response to the level produced by COX-2 inhibition occurred when these vessels were preincubated with SQ29548 plus EP receptor blocker, AH6809. Among 12-month-old O-DR the combined treatment of SQ29548 plus AH6809 and AL8810 (a FP receptor antagonist) was required to reduce noradrenaline response to the level produced by NS-398 or indomethacin. This indicates that in resistance arteries from 6-month-old O-DR the increase in noradrenaline-induced contraction was produced by an increase in both TxA_2 and PGE_2 while in arteries from 12-month-old O-DR the hyperactivity to noradrenaline was produced by an increase in PGE_2, TxA_2, and $PGF_{2\alpha}$ release. Consistent with these functional findings, in addition to TxA_2, noradrenaline-stimulated PGE_2 release was also higher in arteries from 6- and 12-month-old O-DR. Besides these two prostanoids, noradrenaline-stimulated $PGF_{2\alpha}$ was also increased in arteries from 12-month-old O-DR. The fact that exogenous PGE_2, $PGF_{2\alpha}$ or TP receptor agonist U46616 administration have produced similar contraction in endothelium-denuded arteries in all groups studied discard the possibility that EP, FP or TP receptor initiated signaling mechanisms are altered in mesenteric resistance arteries from O-DR.

As reported by several studies, increased activation of TP, EP and/or FP receptors represents a key mechanism to increased vascular tone and blood pressure in several models of cardiovascular disease, including hypertension and diabetes [38–46]. Rutkai *et al.* [38] demonstrated that EP receptor activation in resistance arteries may contribute to the development of high blood pressure in type-2 diabetic mice. In these animals the augmented pressure- and angiotensin II-induced arteriolar tone, as well as the elevated systolic blood pressure were normalized by the EP antagonist, AH6809. Similarly, Guan *et al.* [39] demonstrated that EP receptor activation contributes to hypertension in two well-established experimental models: SHR and chronic angiotensin II-infused mice. Respect to TxA_2, various studies have reported that TP receptor activation contributes to the severity of blood pressure elevation as well as cardiac hypertrophy and increased vascular responsiveness in hypertensive rats [40,41,42,43]. $PGF_{2\alpha}$ has also been involved in the pathogenesis of hypertension. Tian *et al.* [44] demonstrated that COX-2-derived $PGF_{2\alpha}$ contributes to endothelial dysfunction and elevation of blood pressure in renovascular hypertensive rats. In addition, Yu *et al.* [45] have also reported that FP receptor deletion decreased blood pressure in mice, indicating that $PGF_{2\alpha}$ may act via FP receptor to modulate blood pressure regulation.

Results presented here provide evidences of participation of COX-2-derived TxA_2, PGE_2 and $PGF_{2\alpha}$ in the altered regulation of mesenteric arterial responsiveness in offspring of diabetic rats. As abnormal vascular reactivity may contribute to the etiology of hypertension [47–49], our results suggest a crucial role for these COX-2-derived prostanoids in elevating vascular resistance and systemic blood pressure in offspring of diabetic rats. However, these results should be careful interpreted once blood pressure was not analyzed after chronic COX-2 inhibition or TP, EP and FP receptors blockade. Further studies have yet to be performed to demonstrate this effect in offspring of diabetic rats.

Collectively, our results suggest that a) age-dependent hyperreactivity to noradrenaline is present in mesenteric resistance arteries of adult offspring of diabetic rats, b) it may result from a reduction on nitric oxide release/bioavailability and increased TxA_2, PGE_2 and $PGF_{2\alpha}$ vasoconstrictor prostanoid releasing, and c) the mechanisms underlying this hyperreactivity may involve up-regulation of COX-2 [9]. However, further studies need to be carry out to determine whether and for what extent changes in mesenteric prostaglandin synthesis might contribute to blood pressure alterations in these rats. Nevertheless, changes in COX-dependent modulation of contractile response should be taken into consideration in future studies with offspring born from diabetic mothers.

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Author Contributions

Conceived and designed the experiments: FEX GPD. Performed the experiments: FERA DBQ JSR. Analyzed the data: FERA DBQ JSR. Contributed reagents/materials/analysis tools: FEX GPD. Wrote the paper: FERA DBQ FEX.

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7. CONCLUSÕES

Os resultados obtidos neste estudo demonstram que a exposição intrauterina e perinatal à hiperglicemia materna induz hipertensão arterial, associada à disfunção endotelial e hiper-reatividade vascular à noradrenalina em artérias de resistência da prole adulta. Este efeito é influenciado pela idade e é mediado por um aumento, nestas artérias, da expressão protéica da COX-2 e conseqüente liberação de prostanóides vasoconstritores, como o TxA_2, PGE_2 e a $PGF_{2\alpha}$.