Role of oxidative stress in multiparity-induced endothelial dysfunction

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Tawfik HE, Cena J, Schulz R, Kaufman S. Role of oxidative stress in multiparity-induced endothelial dysfunction. Am J Physiol Heart Circ Physiol 295: H1736–H1742, 2008. First published August 29, 2008; doi:10.1152/ajpheart.87.2008.—Multiparity is associated with increased risk of cardiovascular disease. We tested whether multiparity induces oxidative stress in rat vascular tissue. Coronary arteries and thoracic aorta were isolated from multiparous and age-matched virgin rats. Relaxation to ACh and sodium nitroprusside (SNP) was measured by wire myography. We also tested the effect of the superoxide dismutase mimetic MnTE2PyP (30 nM), the NADPH oxidase inhibitor apocynin (10 μM), and the peroxynitrite scavenger FeTPPs (10 μM) on ACh-mediated relaxation in coronary arteries. Vascular superoxide anion was measured using the luminol derivative L-012 and nitric oxide (NO) generation by the Griess reaction. Multiparity reduced maximal response and sensitivity to ACh in coronary arteries [maximal relaxation (Emax): multiparous 49 ± 3% vs. virgins 95 ± 3%; EC50: multiparous 135 ± 1 nM vs. virgins 60 ± 1 nM], and in aortic rings (Emax: multiparous 38 ± 3% vs. virgins 79 ± 4%; EC50: multiparous 160 ± 2 nM vs. virgins 90 ± 3 nM). Coronary arteries from the two groups relaxed similarly to SNP. Superoxide anions formation was significantly higher in both coronary arteries (2.8-fold increase) and aorta (4.1-fold increase) from multiparous rats compared with virgins. In multiparous rats, incubation with MnTE2PyP, apocynin, and FeTPPs improved endothelial dysfunction (33). Endothelial dysfunction has been shown to be at least partly dependent on the production of ROS and the subsequent decrease in vascular bioavailability of NO (10).

One important mechanism underlying endothelial dysfunction is inactivation of endothelium-derived NO by O2•−. NO reacts with O2•− to form peroxynitrite (ONOO−) (2). Unlike NO, which readily activates guanylyl cyclase and increases cGMP formation in vascular smooth muscle, ONOO− is a much weaker stimulus for guanylyl cyclase (31). Thus any reaction of NO with O2•− impairs NO-induced relaxation.

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formation. In addition, multiparity significantly reduced vascular eNOS expression. Our findings suggest that multiparity induces endothelial dysfunction through increased ROS and decreased eNOS expression, which leads to reduced NO bioavailability.

MATERIALS AND METHODS

Animals and housing. Female Long Evans multiparous rats (8–9 mo old) were obtained from Charles River (St. Foy, Quebec, Canada). These animals had undergone five pregnancies; their age at first pregnancy was 56 days. Control aged-matched virgin rats were raised in the same facility as the multiparous. A period of at least 1 mo from arrival was allowed to elapse before the experiments were started, during which time the rats were held in the University of Alberta animal facility on a 12:12-h light-dark cycle in a humidity- and temperature-controlled environment. To prevent obesity, they were restricted to just three pellets (20 g) of food per day (17). The experimental procedures were approved by the local Animal Welfare Committee in accordance with the guidelines issued by the Canada Council on Animal Care, which conforms to National Institute of Health guidelines.

Preparation of rat coronary arteries and aortic rings. Rats were anesthetized with pentobarbital sodium (60 mg/kg ip). A thoracotomy was performed; heart and thoracic aorta were quickly excised and placed in ice-cold oxygenated Krebs-Henseleit buffer. After the right ventricle and anterior wall of the left ventricle were removed under a stereomicroscope, intramyocardial second-order branches of the septal arteries were dissected from the septum facing the right ventricular cavity. Arterial segments (~2 mm long) were gently excised, transferred to the chamber of a small vessel myograph (Kent Scientific, Litchfield, CA) containing 5 ml buffer, and mounted on tungsten wires (diameter 40 μm). The arteries were allowed to equilibrate in oxygenated (95% O2-5% CO2) Krebs-Henseleit buffer. The composition of the buffer is (in mM): 118.3 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, 2.5 CaCl2, and 11.0 glucose. Vessels were allowed to stabilize for 30 min in the Krebs-Henseleit buffer under zero tension, during which time the buffer solution was changed at 10-min intervals. This was followed by a preconditioning stretch of 0.2 g tension, after which vessels were restretched at 0.2 g tension and allowed to stabilize in Krebs-Henseleit buffer for a further 30 min. The vessels were then radially stretched to their optimal lumen diameter for active tension development, i.e., to an internal circumference equal to 90% of that achieved in vessels exposed to a passive tension equivalent to a transmural pressure of 100 mmHg. Our preliminary experiments on rat coronary arteries indicated that the optimal active tension is 0.2 g.

The thoracic aorta was gently excised. Fat and connective tissue were removed, and the aorta was cut transversely into rings of 2–3 mm in width. The rings were mounted between two stainless steel wire hooks with extreme care being taken to avoid damaging the endothelium. They were then transferred to the chamber of a myograph (Kent Scientific) containing Krebs-Henseleit solution continuously gassed with 95% O2-5% CO2 (37°C, pH 7.4). Aortic rings were allowed to equilibrate for 60 min at an initial resting tension of 1 g, and the bathing solution was changed every 10 min according to the previously described protocol. Isometric force for all experiments was recorded online (Windaq data acquisition software).

Vascular reactivity. After the equilibration period, the responsive- ness of each individual coronary artery or aortic ring was confirmed by its vasoconstrictive response to KCl. The integrity of the vascular endothelium was confirmed pharmacologically by acetylcholine-induced relaxation of arteries that had been preconstricted with U-46619 (thromboxane A2 receptor agonist). Tissues that did not elicit a reproducible and stable contraction with U-46619 were discarded from the study. Preparations were then washed three times with Krebs-Henseleit buffer and allowed to relax freely for 30 min before the experimental protocol began.

The arteries were constricted with U-46619 at a submaximal dose of 1 μM (EC50). After reaching a plateau of contraction, cumulative concentration-response curves to ACh (0.1 nM–100 μM) and sodium nitroprusside (SNP, 0.1 nM–100 μM) were obtained to evaluate endothelium-dependent and -independent relaxations, respectively. In addition, we measured endothelium-dependent relaxation of rat aortic rings by performing cumulative concentration-response curves to ACh (0.1 nM–100 μM).

In a second series of experiments, ACh-induced vasodilation in rat coronary arteries was measured after 30 min incubation with one of the following: the cell-permeable superoxide dismutase mimetic MnTE2PYP (30 μM) (6); the NADPH oxidase inhibitor apocynin (10 μM) (32); the ONOO− scavenger FeTPPS (10 μM) (9); or the NOS inhibitor l-NAME (30 μM). In all cases, the responses were compared with those obtained using the same vessel before incubation with the test substance (control). The vasorelaxant responses are expressed as percent decrease from U-46619-induced contraction, i.e., the amount of contraction produced by 1 μM U-46619 in each vessel from its initial resting tension was considered to be 100%.

O2−/H2O2 generation in isolated coronary vessels and aorta was measured using a chemiluminescence assay. Vascular segments from each rat (virgin and multiparous) were incubated in Krebs-Henseleit buffer containing 500 μM of the highly sensitive luminol derivative L-012 (Wako Pure Chemical Industries, Osaka, Japan) (32). Chemiluminescence was measured in coronary arteries using the TD-2020 Luminometer (Turner Designs, Sunnyvale, CA) and in aortic tissue using the FB12 Luminometer (Berthold Detection System).

Tissues nitrate/nitrite level. For analysis of NO formation, we evaluated vascular homogenate NO by measuring total nitrate and nitrite levels using the Griess reaction method (Cayman Chemical). Although we measured vascular reactivity in both coronary arteries and aorta, tissue nitrate/nitrite levels were measured only in the aorta due to the difficulty of obtaining sufficient tissue from the coronary arteries.

briefly, aortic homogenate was incubated with nitrate reductase enzyme (10 μU) and NADPH (cofactor) for 3 h at 37°C. The total nitrate in each sample was then determined by adding sulphanilamide (Griess reagent 1), followed by N-(1-naphthyl)ethylenediamine (Griess reagent 2). The mixture was incubated at 37°C for 10 min. Absorbance at 540 nm was recorded, and the concentrations of NO2 were calculated from a standard curve constructed using NaNO2 and NaNO3 standards. Nitrate/nitrite levels were expressed as micromolar per milligram tissue protein.

Western blot. For analysis of eNOS protein, frozen rat aortic arteries were crushed using a mortar and pestle that was cooled with dry ice. Again, we used aortic tissue homogenates to measure eNOS protein because of the small size of coronary arteries and the difficulty of obtaining sufficient tissue. The resulting powder was diluted 1:4 with 50 mM Tris-HCl (pH 7.4) buffer containing 31 mM sucrose, 1 mM dithiothreitol, 10 mg/ml leupeptin, 10 mg/ml soybean trypsin inhibitor, 2 mg/ml aprotenin, and 0.1% Triton X-100. This solution was then homogenized on ice using a microcentrifuge tube pestle. The homogenate was centrifuged at 10,000 g for 5 min at 4°C, and the supernatant was kept on ice.

Protein concentration was measured using the BCA protein assay (Pierce). Aortic homogenate samples (40 μg) were separated by 8% SDS-PAGE, transferred to nitrocellulose membrane, and probed with monoclonal antibody against eNOS (Cell Signaling, Boston, MA) followed by secondary antibody and enhanced chemiluminescence for detection (Amersham Pharma, San Francisco, CA). The membrane was stripped and reprobed for β-actin content using a species monoclonal antibody to verify equal loading, and results were analyzed using densitometry and Image J software.
Drugs. ACh, SNP, apocynin, and L-NAME were purchased from Sigma (St. Louis, MO), MnTE2PyP, U-46619, and FeTPPs were purchased from Calbiochem (San Diego, CA). L-012 was purchased from Wako Pure Chemical Industries (Richmond, VA). L-NAME, ACh, MnTE2PyP, SNP, and FeTPPs (stock 10 mM) solutions were made in deionized water. Apocynin was dissolved in dimethyl sulfoxide (DMSO). U-46619 was supplied as liquid in methylacetate and evaporated to dryness under a stream of nitrogen and dissolved in DMSO to stock solution (10 nM). Desired concentrations of SNP, apocynin, and U-46619 were made in deionized water. L-012 was dissolved in deionized water to a final concentration of 20 mM and then diluted into Krebs-Henseleit buffer to the assay concentration 500 µM.

Data analysis. All values are shown as means ± SE. Maximal relaxation (E_{max}) and half-maximal effective dose (EC_{50}) were calculated from individual dose-response curves. EC_{50} values were derived using Graph-Pad Prism. Statistical comparisons between these parameters for the virgin and multiparous groups were analyzed by one-way analysis of variance with Bonferroni’s test for comparison between all pairs of groups. In some experiments, statistical differences were determined by Student’s t-test. The results were considered significant when P < 0.05.

RESULTS

Effect of multiparity on ACh- and SNP-mediated coronary artery and aortic ring vasorelaxation. Concentration-response curves to U-46619 were constructed for coronary arteries and aortic rings from multiparous and virgin rats. U-46619 produced maximal contraction in both groups at a concentration of 10 µM (E_{max}) (n = 6, data not shown). A submaximal concentration (EC_{80}, 1 µM) was chosen for preconstriction in subsequent experiments; the constriction to this concentration of U-46619 was thus defined as 100% in the relaxation curves. There was no significant difference between the two groups (multiparous and virgins) with respect to the responses to KCl (40, 60, and 80 mM, n = 4, data not shown).

The reproducibility of the responses induced by ACh on the coronary arteries was assessed in a subset of ACh concentration-response experiments. Each vessel was exposed to the same ACh concentrations two times, separated by at 30 min of drug-free incubation. Our results produced qualitatively similar and reproducible effects on coronary arteries with repeated exposure (n = 5, data not shown). Thus it is evident that ACh-induced coronary vascular responses in our rat model do not exhibit tachyphylaxis with successive drug application.

ACh produced concentration-dependent relaxation in coronary arteries from both virgin and multiparous rats. E_{max} was higher in coronary arteries from virgin (95 ± 3%, n = 9) than from multiparous (49 ± 3%, n = 9, P < 0.05) (Fig. 1A) rats. There was also a rightward shift in the response of coronary arteries from multiparous rats (EC_{50} = 135 ± 1 nM) compared with the response from virgin rats (EC_{50} = 60 ± 1 nM, P < 0.05). L-NAME completely blocked ACh-mediated relaxation in vessels from both virgin and multiparous animals (Fig. 1A). Coronary arteries from multiparous and virgin rats responded similarly to the NO donor SNP (Fig. 1B). Aortic rings from multiparous rats exhibited decreased E_{max} to ACh (38 ± 3%, P < 0.05) compared with virgin rats (79 ± 4%) (n = 5 in both groups) and a rightward shift in the concentration-response curve (EC_{50} multiparous: 160 ± 2 nM vs. EC_{50} virgins: 100 ± 3 nM) (Fig. 2).

Effect of superoxide dismutase mimetic (MnTE2PyP) on endothelium-dependent relaxation of rat coronary arteries. Incubation of coronary vessels from multiparous rats with MnTE2PyP significantly improved maximal relaxation (E_{max} MnTE2PyP = 74 ± 5%, n = 6 vs. vehicle = 41 ± 5%, n = 6, P < 0.05) and sensitivity (EC_{50} MnTE2PyP = 61 ± 0.5 nM vs. vehicle = 91 ± 6 nM) to ACh (Fig. 3A). The submaximal contraction obtained at 1 µM of U-46619 was not altered by MnTE2PyP, nor did MnTE2PyP alter ACh-mediated relaxation in coronary arteries from virgin rats (data not shown).

Effect of the ONOO\(^-\) scavenger (FeTPPs) on endothelium-dependent relaxation of rat coronary arteries. Incubation of coronary vessels from multiparous rats with FeTPPs significantly improved maximal relaxation (E_{max} FeTPPs = 72 ± 3%, n = 7 vs. vehicle = 46 ± 3%, n = 7) and sensitivity (EC_{50} FeTPPs = 61 ± 0.5 nM vs. vehicle = 91 ± 6 nM) to ACh (Fig. 3B). The submaximal contraction obtained at 1 µM of U-46619 was not altered by FeTPPs, nor did FeTPPs alter ACh-mediated relaxation in coronary arteries from virgin rats (data not shown).

Effect of the NAPDH oxidase inhibitor apocynin on endothelium-dependent relaxation of rat coronary arteries. Incubation of coronary vessels from multiparous rats with apocynin significantly improved maximal relaxation (apocynin = 73 ± 3% vs. vehicle = 46 ± 3%, n = 7) and sensitivity (EC_{50} apocynin = 61 ± 0.5 nM vs. vehicle = 91 ± 6 nM) to ACh (Fig. 3C).
3% vs. vehicle = 41 ± 3%) and sensitivity (EC_{50} apocynin: 45 ± 3 nM; EC_{50} vehicle: 91 ± 6 nM) to ACh (Fig. 3C). The submaximal contraction obtained at 1 μM of U-46619 was not altered by apocynin, nor did apocynin alter ACh-mediated relaxation in coronary arteries from virgin rats (data not shown).

**Effect of multiparity on ROS levels in rat coronary arteries.** To evaluate the contribution of O_{2•} in multiparity-reduced ACh-mediated relaxation, we assessed the effect of multiparity on O_{2•} formation using chemiluminescence. O_{2•} production was significantly higher in both coronary vessels and aortic tissue from multiparous compared with virgin rats (Fig. 4, A and B).

**Effect of multiparity on NO production in rat aorta.** To test whether the increased O_{2•} formation in vascular tissues from multiparous rats reduces NO availability, we measured nitrate/nitrite using the Griess reaction. Nitrate/nitrite levels were significantly lower in aortic tissues from multiparous rats compared with those from virgins (Fig. 5).

**Effect of multiparity on eNOS levels in rat aorta.** To establish whether there are other, non-ROS-mediated, mechanisms by which multiparity might reduce endothelial-dependent relaxation, we measured eNOS protein level. eNOS protein was significantly lower in aortic tissue from multiparous rats compared with that in tissue from virgins (Fig. 6, A and B).

**DISCUSSION**

Circulating markers of oxidative stress increase during pregnancy (23, 26, 34). However, the question as to whether this returns to normal after parturition and whether this might contribute to the increased risk for cardiovascular disease observed in older multiparous women (7, 8, 13, 18, 22, 27, 28) had not previously been addressed. Our data demonstrate that, indeed, there is increased oxidative stress in both coronary arteries and aorta from multiparous rats and that this does contribute to compromised vasodilation in both the coronary and aortic vasculature.

An imbalance between the production of ROS and NO in the vessel wall is considered to play an important role in the development of endothelial dysfunction. O_{2•} reacts with NO to form ONOO^{-} (1). ONOO^{-} can oxidize the NOS cofactor tetrahydrobiopterin (24) and also reduces cellular transport of L-arginine, the eNOS substrate for NO production (29). It is believed by many that these events contribute to increasing •OH production over NO production (24). Ultimately, endothelial dysfunction reflects the inability of the endothelium to generate adequate amounts of NO (or reduced bioavailability of NO) and to produce NO-mediated vasorelaxation.

Our data show that coronary arteries and aortic rings from multiparous rats exhibit significantly decreased NO-dependent relaxation in response to ACh. That is, repeated pregnancy causes a long-term reduction in endothelium-dependent va-
sorelaxation. This finding is consistent with our previous studies showing that the enhanced vasoconstriction to PE of mesenteric arteries from multiparous rats is also NO dependent (7). We have also shown that the decreased NO-dependent relaxation in arteries from multiparous rats was associated with increased O$_2^\cdot$/H$_2$O$_2$ levels as shown by chemiluminescence. These data are in agreement with reports of decreased NO-dependent relaxation in association with other oxidative stress conditions such as diabetes (32), smoking (15) and hypertension (20).

We evaluated endothelium-independent relaxation in rat coronary arteries by performing concentration-response curves to the NO donor SNP. Our results showed that coronary arteries from both multiparous and virgin rats relaxed similarly to SNP. These data confirm that, in our model, multiparity caused impairment only of endothelium-dependent relaxation, i.e., that the vascular smooth muscle response to NO was normal. Furthermore, L-NAME blocked ACh-mediated relaxation in the coronary arteries. These data confirm that, under the conditions used in these experiments, ACh-mediated relaxation was mediated through endothelium-derived NO and that the reduction in ACh-mediated relaxation in coronary arteries from multiparous rats may indeed be attributed to endothelial dysfunction.

Our finding that multiparity reduced endothelium-dependent relaxation and increased O$_2^\cdot$ levels prompted us to assess the effect of the superoxide dismutase mimetic MnTE2PyP on ACh-mediated relaxation. Incubation of coronary vessels from multiparous rats with MnTE2PyP significantly improved ACh-mediated relaxation. To evaluate the specific contribution of O$_2^\cdot$ in the multiparity-induced decrease in ACh-mediated relaxation, we assessed the effect of apocynin (NADPH oxidase inhibitor) on ACh-mediated relaxation. Apocynin improved ACh-mediated relaxation in coronary arteries from multiparous rats. These data suggest that multiparity decreases vascular relaxation by a mechanism involving O$_2^\cdot$ formation and subsequent reduction in NO availability. This concept was supported by our finding that multiparity was also associated with reduced nitrate/nitrite level in multiparous rat aortic homogenates. Our results are thus in agreement with previous reports that multiparity attenuates the production of NO in the kidney (30).

An important characteristic of endothelial dysfunction is inactivation of NO by its reaction with O$_2^\cdot$ to form ONOO$^-$.
FeTTPs is a drug that catalyzes the conversion of ONOO− into nitrate, thus preventing the oxidizing and nitrat- ing reactivity (25). In accordance with this concept, we showed that incubation with FeTTPs improved ACh-mediated relax- ation in coronary vessels from multiparous rats. This finding suggests that multiparity increases O2•− formation in vascular tissues, which then scavenges NO to form ONOO−. We thus speculated that the impaired relaxation to ACh seen in coro- nary arteries from multiparous rats is the result of increased "OH/ONOO− and decreased l-arginine availability to eNOS. Consequently, there is a reduction in NO bioavailability. We tested the NO level and eNOS protein in aortic tissue homoge- nates. We have shown that multiparity decreases endothelium- dependent relaxation in two functionally distinct vascular tis- sues, namely coronary arteries and the aorta. Our data suggest that this is due to ROS-mediated endothelial damage.

Impaired endothelium-dependent reactivity can also be caused by changes in eNOS. We therefore measured eNOS protein in rat aortic homogenates. Multiparity significantly reduced vascular eNOS protein levels. Although we recogn- ize that eNOS expression does not necessarily reflect eNOS activity, it is likely that a reduced eNOS protein would be associated with impaired endothelium-dependent relaxation. A possible mechanism by which oxidative stress could reduce eNOS is via ONOO− activation of RhoA and its downstream target Rho kinase; there is evidence that Rho kinase decreases eNOS mRNA expression (19). Another possible mechanism could be through activation of P21 Ras. ONOO− has been shown to increase the activity and ex- pression of P21Ras which, in turn, could inhibit eNOS protein expression (4).

In summary, our data show that multiparity (repeated pregnancy) reduces endothelium-dependent relaxation in rat coronary arteries and aortic rings. This effect was associated with increased oxidative stress. Increased O2•− would en- hance ONOO− formation, thus reducing eNOS expression and NO biosynthesis. We conclude that multiparity induces vascular endothelial dysfunction by facilitating the formation of ROS, which then decrease NO production and bioavailability.

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