VASCULAR DEVELOPMENTAL PROGRAMMING OF eNOS UNCOUPLING AND ENHANCED VASCULAR OXIDATIVE STRESS IN ADULT RATS AFTER TRANSIENT NEONATAL OXYGEN EXPOSURE

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Running Head: NOS uncoupling after newborn O₂ exposure

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ABSTRACT

We have previously shown that neonatal hyperoxic stress leads in adulthood to high blood pressure, impaired endothelium-mediated vasodilatation and increased vascular production of superoxide anion by NAD(P)H oxidase. However, it is unknown whether changes in nitric oxide (NO) production and/or bio-inactivation prevail, and whether NO synthase (NOS) is also a source of superoxide. The purpose of this study is to evaluate whether adult animals exposed to neonatal hyperoxic stress have impaired vascular NO production associated with NOS uncoupling participating to vascular superoxide production and vascular dysfunction.

In adult male rats exposed to 80 % oxygen from day 3 to 10 of life (H, n=6) vs. room air controls (CTRL, n=6), vascular (aorta) NO production is decreased at baseline (CTRL: 21±1 vs. H: 16±2 DAF-2 fluorescence intensity arbitrary units; p < 0.05) and after carbachol stimulation (acetylcholine analogue; CTRL: 26±2 vs. H: 18±2; p < 0.05). Pre-treatment with L-arginine (CTRL: 32±4 vs. H: 31±5) and L-sepiapterine (analogue of key NOS cofactor tetrahydrobiopterin (BH₄)) (CTRL: 30±3 vs. H: 29±3) normalizes NO production after carbachol. L-Sepiapterine also normalizes impaired vasodilatation to carbachol. Vascular eNOS immunostaining is reduced whereas total eNOS protein expression is increased in H (CTRL: 0.76±0.08 vs. H: 1.76± 0.21; p < 0.01). The significantly higher superoxide generation (CTRL: 20±2 vs. H: 28±3 hydroethidine fluorescence intensity arbitrary units; p < 0.05) is prevented by pre-treatment with the eNOS inhibitor L-NAME (CTRL: 21±4 vs. H: 22±4).

Taken together, the current data indicate a role for eNOS uncoupling in enhanced vascular superoxide, impaired endothelium mediated vasodilatation and decreased NO production in adult animals with programmed elevated blood pressure after a brief neonatal oxygen exposure.
Key Words:

Developmental programming, vascular dysfunction, premature newborn, oxygen, reactive oxygen species, endothelial nitric oxide synthase, superoxide anions.
INTRODUCTION

It is now recognized that conditions during early life can significantly impact adult health and disease, particularly the cardiovascular system; this concept is often termed “developmental programming”. Experimental and clinical studies have shown that, in addition to elevated blood pressure, endothelial-mediated vasodilatation can be impaired and that flow-mediated dilation is decreased in low birth weight and prematurely born children and young adults.\(^3\)\(^4\)

Among the many factors implicated in adverse perinatal conditions and developmental programming of hypertension, oxidative stress seems an important common denominator.\(^5\)\(^6\)\(^8\)\(^9\) Infants are exposed upon birth to relatively high concentrations of O\(_2\) compared to intrauterine life. Indeed, under physiologic conditions the foetus is hypoxic compared with the adult. In the fetal to neonatal transition blood O\(_2\) content and O\(_2\) availability abruptly increase in the first few minutes after birth to adult values eliciting the generation of a burst of O\(_2\) free radicals.\(^10\)\(^11\) Premature and small for gestational age infants have lower and less inducible antioxidant defences because it is only during the last trimester of a normal pregnancy that antioxidant enzyme levels increase.\(^12\)\(^13\)\(^16\)\(^17\) Therefore the combination of immature antioxidant system to face this surge in pO\(_2\) plus the need for therapy with O\(_2\) supplementation because of lung immaturity, unstable lung dynamics, sepsis etc. altogether can lead to significant oxidative stress in the immediate neonatal period.\(^16\)\(^18\) However, the possible long term consequences of neonatal peripheral vascular oxidative injury in susceptible individuals are only starting to be explored.

Experimentally, we have shown that exposure of newborn rats to a hyperoxic stress is associated in adulthood with increased blood pressure and vascular dysfunction. Newborn rats display an immature stage of development of many organs (relative to term infants) and are a recognized model to study classical (lungs, eyes) O\(_2\)-related complications of prematurity.\(^19\)\(^20\)
The vascular dysfunction observed after transient neonatal hyperoxic exposure is characterized by impaired endothelium mediated vasodilatation and exaggerated vasoconstriction to angiotensin II, mediated at least in part by increased vascular production of superoxide anion by NAD(P)H oxidase. In addition, vascular dysfunction is normalized by superoxide dismutase analogue Tempol, suggesting that decreased bioavailability of NO by superoxide scavenging could prevail; however it remains undetermined whether this is associated with modifications in NO production, NOS expression and/or bioinactivation of NO, and whether NOS could also be a source of superoxide anion.

Endogenous NO is synthesized in vascular endothelial cells from conversion of L-arginine into L-citrulline via the enzymatic activation of constitutive homodimeric endothelial NO synthase (eNOS) with cofactors such as tetrahydro-L-biopterin (BH4). “Uncoupled” NOS may contribute to superoxide generation in conditions where L-arginine or more often BH4 are deficient. The purpose of this study was therefore to evaluate whether adult animals exposed to oxygen as newborn have impaired vascular NO production associated with programmed decreased eNOS expression and/or eNOS uncoupling which could result in enhanced vascular superoxide production. Unravelling mechanisms of adult vascular dysfunction after a transient neonatal exposure is important to understand pathways of developmental programming of adult cardiovascular diseases and target preventive/early therapies.
METHODS

Animals

Animals were used according to a protocol of the Animal Care Committee of the CHU Sainte-Justine-University of Montreal in accordance with the principles of the Guide for the Care and Use of Experimental Animals of the Canadian Council on Animal Care and with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) as previously reported. Briefly, Sprague-Dawley rat pups (Charles River, St-Constant, QC, Canada) were maintained either in a hyperoxic environment (80% O₂ by a mixture of medical grade 100% O₂ and room air (21% O₂; oxycycler ProOx model 110, Biosherix, Lacona, NY 13083) from day 3 to 10 of life (H group, n = 3 litters) or in room air (control (CTRL) group, n = 3 litters). To avoid maternal morbidity associated with high O₂ exposure, the dam of the O₂ exposed litter was alternated every 12 hours with a surrogate mother of a litter maintained in room air. We have previously shown that alternation of the dam does not impact later (adult) blood pressure and endothelium mediated vasodilatation. Pups were weaned at 4 weeks of age. Survival of the pups, their growth and food intake were similar between groups. Adult middle age (29 to 32 weeks) male rats were studied, n = 5-6 per group (max 2 animals per litter); blood pressure (tail cuff) in H vs. CTRL was significantly and similarly increased to previous study (systolic / diastolic: 159±8 / 120±5 vs. 141±4 / 105±2, p<0.05 using t-test). From each animal, carotid (vasoreactivity) and aorta (histochemistry) were sampled, as previously described. For the current study, H and CTRL males were studied considering that we previously had not observed difference in vascular (dys)function between genders nor between CTRL and NH animals.

Detection of vascular NO production

The measure of NO production was realized in aorta using the NO-specific fluorescent dye, namely 4,5-diaminofluorescein diacetate (DAF-2) according to a method adapted from
Franco et al. This molecule is taken up by the cells where the ester bonds are hydrolyzed by intracellular esterase in DAF-2; the reaction with NO leads to triazolofluorescein (DAF-2T) formation generating green fluorescence. Briefly, 8-μm sections of unfixed frozen aorta were loaded with DAF-2 (10 μM) and incubated in a light-protected humidified chamber at 37°C for 1 hr. Sections were then incubated for 30 min at 37°C in HEPES buffer (140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 10 mM glucose, 10 mM HEPES, pH 7.4) alone, with carbachol (100 μM), and with carbachol in the presence of L-arginine (100 μM, 1 hr pre-incubation) or of L-sepiapterin (100 μM, 30 min pre-incubation). Digital images were collected. CTRL and H samples were treated in parallel for each condition and analysed with identical conditions for image collection (exposure time, gain, light intensity). Fluorescence was evaluated with the Image software (http://rsbweb.nih.gov/ij) from at least four aortic sections per animal.

**Ex vivo vascular reactivity studies**

Freshly excised artery rings were studied as previously described, with a linear force transducer on a computerized data acquisition system (Kent Scientific, Litchfield, CT), using Dasylab 5.6 software (Data Acquisition System Laboratory, D-41189 Moenchengladbach, Germany). Four to eight rings from one rat were used for one experiment. The n presented with the Figure represents the number of animals studied. Vasorelaxation to carbachol (100 nM to 100 μM, see below) was measured after pre-contraction of the rings with thromboxane A2 analogue U46619 (0.3 μM; 15 min pre-incubation). For each experiment, half the rings were pre-incubated with L-sepiapterin (100 μM for 30 minutes prior to U46619 contraction; sepiapterin did not modify the contractile state of the vessel nor the response to U46619).

**eNOS expression**

eNOS protein expression was evaluated with immunohistochemistry and western blotting on H and CTRL aorta.
**Immunohistochemistry**

A primary antibody for eNOS (mouse IgG; diluted 1:50) was applied on fixed (ethanol/acetone) cryosections overnight at 4°C. To demonstrate endothelial integrity, a primary antibody for Von Willebrand factor (rabbit IgG; diluted 1:200) was applied on fixed (3.7% formaldehyde) cryosections overnight at 4°C. The sections were then washed with PBS and incubated for 1 hr with Alexa Fluor-488 (goat anti-mouse IgG; dilution 1:300 for eNOS and 1:500 for von Willebrand factor). After rinsing in PBS, sections were coverslipped. Digital images were collected and fluorescence evaluated as above on at least four aortic sections per animal.

**Immunoblotting**

Frozen aorta were disrupted with homogenizer and proteins extracted with RIPA (10% Na-deoxycholate, 100 mM EDTA, 10% SDS, 1 M Tris, 10% Igepal, 150 mM NaCl) supplemented with 0.05 X solution of cocktail protease inhibitors and 100 mM phenylmethanesulfonylfluoride (PMSF). Samples were centrifuged (10,000 x g, 10 min, 4°C) and the supernatant recovered for determination of protein content by Bradford assay using BSA as a standard.

Denatured (5min at 95°C) proteins (50 µg) were separated (SDS-polyacrylamide gel) and transferred overnight to polyvinylidene fluoride membranes. Membranes were blocked for 1 hr at room temperature with 10% BSA for eNOS; with 5% milk for iNOS and with StartingBlock (TBS) blocking buffer for β-actin. Membranes were then incubated with primary monoclonal antibodies: rabbit anti-eNOS (1/1000 in 5% BSA) overnight at 4°C; mouse anti-iNOS (1/500 in 5% milk), anti-nNOS (1/2500 in 5% BSA) overnight at 4°C; and mouse anti-β-actin (1/25 000), 1 hr at room temperature. Incubation with secondary antibodies was as follow: anti-rabbit eNOS (1/5000 in 5% BSA), 2 hrs at room temperature; anti-mouse iNOS (1/2500 in TBS-Tween 0.5%), anti-mouse nNOS (1/10 000 in TBS-Tween 0.5%) and anti-mouse β-actin
(1/25 000 in TBS-Tween 0.5%), 1 hr at room temperature.

Low-temperature SDS-PAGE (LT-PAGE) was performed for detection of eNOS dimers using reported procedures. As described by Marchesi et al., total proteins were incubated in 1 X non denaturing buffer (Tris-HCl 4 X, pH 6.8, 20% Glycerol and 0.02% bromophenol blue) without heating. The samples were then loaded onto a gradient gel (NuPAGE Novex, Invitrogen). Gels and buffers were equilibrated at 4°C before electrophoresis and the buffer tank placed in an ice bath during electrophoresis to maintain the temperature of the gel below 15°C. Subsequent to LT-PAGE, the gels were transferred overnight on nitrocellulose; the blots were then blocked and incubated with the antibodies as above.

The antibodies were visualized using an enhanced chemiluminescence method ECL plus (Perkin Elmer, MA, USA). Films (Bioflex Econo from InterSciences, Markham, ON, Canada) were scanned using a flatbed scanner and images analyzed with Image J (http://rsbweb.nih.gov/ij); results were normalized to β-actin for loading variations.

**Evaluation of vascular production of superoxide anion**

Aortic superoxide production was evaluated in samples of the H and CTRL groups using the oxidative fluorescent dye hydroethidine (2 µM) as described, in the presence or not of NOS blocker L-NAME (100 µM; 30 min pre-incubation at 37°C).

**Chemicals and reagents**

Carbachol, dihydroethidium, L-arginine, sodium deoxycholate; ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), phenylmethanesulfonylfluoride (PMSF), igepal, N-nitro-L-arginine methyl ester (L-NAME) and standard chemicals were purchased from Sigma Chemical (St Louis, MO, USA); Ketamine from Ayerst (Montreal, QC, Canada); Rompun (Xylazine) from Bayer HealthCare (Toronto, ON, Canada); 4,5-diaminofluorescein diacetate (DAF-2DA) from Calbiochem EMD Biosciences Inc (San Diego, CA, USA); L-
Antibodies: eNOS detection by immunohistochemistry: BD Transduction Laboratories (San Jose, CA, USA); Von Willebrand Factor: ab6994 from Abcam (Cambridge, MA, USA); eNOS detection by western blotting: Cell Signaling Technology (Danvers, MA, USA); secondary antibody Alexa Fluor-488 and gradient gel NuPAGE Novex were from Invitrogen (Burlington, ON, Canada); iNOS and nNOS: BD Transduction Laboratories (San Jose, CA, USA). BSA, Tris and milk were obtained from BioShop (Burlington, ON, Canada) and StartingBlock blocking buffer from Thermo Scientific (Rockford, IL, USA). Complete Mini, a cocktail of protease inhibitors, was obtained from Roche Applied Sciences (Laval, Quebec, Canada). Bradford reagent, polyvinylidene fluoride (PVDF) and nitrocellulose membranes were purchased from Bio-Rad (Mississauga, ON, Canada).

Statistical analyses

Values are expressed as means ± SEM. Vasomotor concentration-response curves to carbachol were analyzed by computer fitting to a four-parameter sigmoid curve using the Prism 3 program (GraphPad, San Diego, CA) to evaluate the EC$_{50}$ and E$_{max}$, the maximum asymptote of the curve. Analysis of differences within and between groups was performed using two-way repeated measure ANOVA followed by post-ANOVA comparison among means using the Bonferroni test, and by Student’s t test for paired or unpaired observations. Statistical significance was set at p < 0.05.
RESULTS

L-Arginine and L-sepiapterin restore vascular production of NO

To evaluate vascular NO production and possible NOS dysfunction in adults exposed to high oxygen as newborns, aortic sections from H and CTRL rats were studied with fluorescent DAF-2DA. Compared to CTRL, NO production was significantly decreased in H group in basal conditions (CTRL: 21 ± 1 vs. H: 16 ± 2 DAF-2 fluorescence intensity arbitrary units; p < 0.05) (Fig 1A) as well as after stimulation by carbachol (CTRL: 26 ± 2 vs. H: 18 ± 2; p < 0.05) (Fig 1B). However, pre-incubation with L-arginine (CTRL: 32 ± 4 vs. H: 31 ± 5) (Fig 1C) or with L-sepiapterin (CTRL: 30 ± 3 vs. H: 29 ± 3) (Fig 1D) restored NO production of H in response to carbachol to values similar to CTRL.

L-Sepiapterin restores impaired endothelial function of isolated vessels

As previously shown, isolated vessel rings from H (vs. CTRL) rats show impaired relaxation to acetylcholine analogue carbachol (Fig 2). Co-incubation with L-sepiapterine (precursor of the essential NOS cofactor BH₄) normalized cumulative concentration-response curves to carbachol of H vessels to CTRL values.

Effect of neonatal oxygen exposure on adult vascular NOS expression

We then questioned whether neonatal oxygen exposure is associated with changes in eNOS expression, vascular NO production and/or NOS function. eNOS immunostaining (Fig 3) is localized in the aorta endothelium and is significantly decreased in H vs. CTRL (CTRL: 16 ± 1 vs. H: 12 ± 1 fluorescence intensity arbitrary units; p < 0.05). However when evaluated by Western blots, total eNOS protein expression is significantly increased in H vs. CTRL (CTRL: 0.76 ± 0.08 vs. H: 1.76 ± 0.21; p < 0.01) (Fig 4A). Additionally, using the LT-PAGE method allowing the identification of the monomer and dimer forms, the results show that the abundance of the eNOS dimers are increased (CTRL: 7.0 ± 1.0 vs. H: 20.3 ± 4.3, ns) in
comparison to the monomer expression (CTRL: 37.7 ± 2.7 vs. H: 45.7 ± 8.4), resulting in a significant increase (CTRL: 0.19 ± 0.03 vs. H: 0.44 ± 0.03; p < 0.01) in the dimer:monomer ratio in H vs. CTRL (Fig 4B). We observed no significant difference between groups for iNOS and nNOS expression (data not shown).

**Effect of NOS blockade on vascular production of superoxide anion**

To evaluate whether eNOS could participate in the enhanced vascular reactive oxygen species production we previously reported in adult H rats, we measured the aortic production of superoxide anion using the oxidative fluorescent dye hydroethidine in the presence or not of eNOS blocker L-NAME (Fig 5). Pre-treatment of the aortic sections with L-NAME significantly reduced the enhanced superoxide generation in H aortic sections to values similar to CTRL and was without effect in CTRL (without L-NAME: CRTL: 20 ± 2 vs. H: 28 ± 3 fluorescence intensity arbitrary units; p < 0.05, Fig 5A; with L-NAME: CTRL: 21± 4 vs. H: 22 ± 4, Fig 5B).
DISCUSSION

The current study shows that rats exposed to high oxygen during a short period in neonatal life present as adults impaired vascular (aorta) production of NO at baseline as well as in response to acetylcholine analogue carbachol. In these adults, NOS cofactors improve vascular NO production and endothelium mediated vasodilatation, suggesting impaired eNOS function. Compared to CTRL, H adult rats showed a decreased expression of vascular eNOS evaluated by immunohistostaining, whereas when evaluated by Western blots eNOS total protein expression and dimer:monomer ratio were increased. Our data also show that eNOS blocker L-NAME inhibits exaggerated vascular superoxide production of adult H rats. Taken together, these results suggest a role for eNOS uncoupling in increased vascular superoxide anion production in adult rats with developmental programming of high blood pressure and vascular dysfunction after neonatal hyperoxic stress.

In endothelial dysfunction, vascular NO bioavailability is affected by the expression of functional NOS, the rate of NO synthesis (influenced by substrate/cofactor availability) and NO bioinactivation by superoxide anion. In the current study, decreased vascular NO production in H adult rats is normalized by L-arginine and L-sepiapterine, both essential substrates for proper NOS function. BH$_4$ has a role in stabilization of the dimeric eNOS, facilitates binding of L-arginine and donates electrons to the ferrous–dioxygen complex in the oxygenase domain as the initiating step of L-arginine oxidation and NO synthesis. Reduced levels of BH$_4$ can lead to eNOS uncoupling as BH$_4$ promotes coupling of NAD(P)H oxidation to NO synthesis and inhibits superoxide formation. Therefore under shortage of BH$_4$, eNOS catalyzes an uncoupled NAD(P)H oxidation (uncoupled from L-arginine oxidation and NO formation), leading to generation of superoxide. Current results suggest that BH$_4$ availability is impaired in H adult rats. In another model of developmental programming of hypertension associated with intrauterine growth restriction, BH$_4$ also reversed impaired endothelium NO production and
normalized excess vascular superoxide production. BH₄ bioavailability can be reduced through reduced production, increased oxidation or in impaired recycling from the oxidized form (BH₂). The latter mechanism is particularly important in determining BH₄ homeostasis in cells and eNOS coupling. Dihydrofolate reductase (DHFR) which converts back BH₂ to BH₄, can be down-regulated by angiotensin II and its activity decreased in aorta of other animal models of cardiovascular diseases. Whether DHFR expression/activity can be (permanently) altered by deleterious perinatal conditions or exposure to hyperoxic stress remains to be explored.

L-arginine, the substrate of eNOS, stabilizes the enzyme in the dimerization state. In the current study, normalization of NO production after carbachol stimulation with L-arginine pre-incubation in adult H rats suggested that its availability could play a role in impaired NO production. Plasma concentrations of L-arginine usually far exceed the K_M for eNOS but bioavailability for eNOS can be reduced by highly expressed cellular arginases, as demonstrated in animal models of cardiovascular diseases associated endothelial dysfunction, or by local competition with the endogenous eNOS inhibitor asymmetric dimethyl-L-arginine (ADMA). Interestingly, ADMA, proposed as an early marker of endothelial dysfunction, is increased in premature vs. term infants, more so in male vs. female preterm, and in infants born to preeclamptic mother or with neonatal infections, which are all pro-oxidant clinical perinatal situations. Whether ADMA is increased or the ratio L-arginine to ADMA decreased after neonatal oxygen exposure and perpetuated to adulthood is still currently not known.

Our results also showed a decreased expression of vascular eNOS evaluated by immunohistostaining, whereas when evaluated by Western blots eNOS total protein expression and dimer:monomer ratio are found increased. The following elements could reconcile the apparently conflicting results. Detection of the total eNOS protein expression was first performed by immunoblotting in denaturating conditions whereas the LT-PAGE conditions allow detection of proteins as dimers as well as monomers. Indeed, SDS breaks only non-disulfide bonds of proteins and cold temperature maintained during electrophoresis made the
dimers identified. It has long been considered that homodimerization of eNOS is essential for NO production\textsuperscript{38} and that, under certain conditions, eNOS can remain monomeric and lead to production of superoxide anion rather than NO.\textsuperscript{39} However it is now recognized that changes in eNOS dimer:monomer ratio are not directly related to eNOS (un)coupling (\textit{i.e.} uncoupled from NO production), because the oxidase activity of the monomers is limited and the dimeric form is more active and able to generate superoxide as well as NO.\textsuperscript{40} Therefore increased expression of eNOS as dimer can lead to increased formation of superoxide anion when cofactors are unavailable. In addition to conformational changes and post-translational phosphorylation, activation of eNOS is dependent on its specific subcellular localization. Most activable eNOS are located in membrane plasmalema caveolae.\textsuperscript{40} In conditions such as diabetes (kidney), aging (vascular) and hypertension, eNOS can be differentially expressed in cytosol vs. membranes, resulting in a decreased NO production\textsuperscript{41, 42} and uncoupling when cytosolic expression is increased.\textsuperscript{43} Taken together, these elements suggest that total vascular eNOS protein expression and eNOS dimer:monomer ratio are increased in adult rats exposed to oxygen as newborn along with decreased expression of active coupled eNOS at the endothelial cell membranes, resulting in decreased NO and increased superoxide productions.

In other experimental models of hypertension and endothelial dysfunction, a decreased eNOS dimer:monomer ratio was reported with the total amount of eNOS protein unchanged or similarly to current data, increased.\textsuperscript{26, 44, 45} In a different model of developmental programming of vascular dysfunction associated with intrauterine undernourishment, impaired endothelium mediated vasodilatation was associated with decreased aortic eNOS expression (evaluated by RT-PCR).\textsuperscript{46} By immunostaining, eNOS is decreased (similar to current data) in luminal endothelial cells of atherosclerotic plaques, associated with impaired NO release.\textsuperscript{47} In summary, these studies and current data underline the fact that different pathways can ultimately result in decreased NO bioavailability and vascular dysfunction.

In the current study, we also examined the expression of two other NOS isoforms,
neuronal (nNOS) and inducible (iNOS). Although iNOS is constitutively present in tissues such as lung epithelium and kidney distal tubules, it can become the predominant isoform in vascular smooth muscle in response to cytokines and other inflammatory mediators. Our results do not show any significant change in aorta iNOS expression in the H group and the inhibitor we used has a poor affinity for iNOS. This is in agreement with a study by Franco et al. showing similar iNOS expression between hypertensive offspring of undernourished dams and control. The involvement and role of NO produced by nNOS in cardiovascular control remains unclear but data suggest nNOS could be involved in the modulation of myogenic tone and systemic arterial pressure. We observed no difference in nNOS expression between groups. Taken together, these elements suggest that iNOS and nNOS do not significantly participate to the enhanced vascular superoxide production in adult rats subjected to neonatal hyperoxic stress.

In the same model, we have previously shown an impaired endothelium-dependent relaxation, an increased in vascular superoxide anions production and a normalization of the endothelial dysfunction by superoxide dismutase analogue Tempol. These data suggested a role for superoxide anion production in impaired endothelium-mediated vasorelaxation through reduced NO bioavailability. Apocynine decreased these exaggerated production of superoxide anions observed in H adult rats, suggesting an increased production by NADPH oxidase. The current study shows that eNOS inhibitor L-NAME also decreased aorta superoxide anion production of H, as observed in other models of hypertension and vascular diseases, both in aorta and resistance vessels. Contribution of both NADPH oxidase activity and eNOS dysfunction to increased total vascular superoxide production and reduced NO bioactivity was also shown in experimental diabetes in rats and in atherosclerotic apoE-/- mice. Superoxide can in turn further contribute to NO bio-inactivation and maintain eNOS uncoupled through oxidation of key cofactor such as BH₄, as well as decrease the caveolae number in endothelial cells. The current studies were realized in aorta; we therefore cannot conclude that the observed eNOS dysfunction plays a role in resulting hypertension and vascular dysfunction.
observed in this model.\textsuperscript{21}

CONCLUSION

The pathways by which a transient neonatal hyperoxic exposure results in adulthood to perpetuated changes resulting in eNOS uncoupling are yet to be fully unravelled. Epigenetic changes can be triggered by a neonatal exposure, and could involve a number of genes along the proteins involved in eNOS function and substrates availability. Accelerated vascular aging could also underlie these observations. Vascular aging is characterized by functional and structural impairment, increased vascular stiffness (as we have recently shown in H adult rats\textsuperscript{55}) and endothelial dysfunction with increased superoxide production by NAD(P)H oxidase and eNOS\textsuperscript{51}, eNOS uncoupling with reduced BH\textsubscript{4} production\textsuperscript{50, 56, 57}, and with decrease in the proportion of active plasma membrane-bound eNOS relative to cytosol subcellular localization.\textsuperscript{58} Whether deleterious neonatal conditions result in epigenetic changes and/or premature aging of the vascular system remains to be fully explored and is important for the growing proportion of young adults who survived premature birth.
ABBREVIATIONS

ADMA: asymmetric dimethyl-L-arginine
BH4: tetrahydro-L-biopterin
CTRL: control group
DAF-2: 4,5-diaminofluorescein diacetate
DHFR: dihydrofolate reductase
H: hyperoxic group
L-NAME: N(G)-nitro arginine methyl ester
NADPH: nicotinamide-adenine-dinucleotide-phosphate
NO: nitric oxide
NOS: NO synthase
eNOS: endothelial NO synthase
nNOS: neuronal NO synthase
iNOS: inducible NO synthase
FIGURE LEGENDS

FIGURE 1: Evaluation of NO production using DAF-2DA (10 µM) in aorta of adult rats exposed to high oxygen as newborns (H) vs. control (CTRL) in baseline conditions (A), after stimulation with the acetylcholine analogue carbachol (100 µM) (B), and with carbachol after pre-incubation with L-arginine (100 µM) (C) and with L-sepiapterine (100 µM) (D). The microphotographs are representative of experiments performed in n = 6 animals per group, compiled in the bar graphs. Bar scale = 100 µm. *: p < 0.05 H vs. CTRL using unpaired t-test.

FIGURE 2: Vasomotor response of carotid artery rings to carbachol from adult rats exposed to oxygen as newborns (H), H in the presence of L-sepiapterin (S; 100 μM; red line) and control (CTRL). Vasodilatation is expressed as percent reversal of U46619 (0.3 μM)-induced vasoconstriction. Data are mean ± SEM of n = 5-6 rats per group. *: p<0.05 compared with CTRL and with H plus sepiapterin using two-way ANOVA.

FIGURE 3: eNOS (A, B and C) and von Willebrand factor (D, E and F) immunostaining in aorta of adult rats exposed to high oxygen as newborns (H; B and E) and control (remained in room air) (CTRL) (A and D). The microphotographs are representative of experiments performed in n = 6 animals per group, compiled in the bar graphs (C and F). eNOS immunostaining is localized to the endothelium and is significantly decreased in H vs. CTRL. Von Willebrand factor immunostaining shows endothelial integrity of the studied aorta segments. Bar scale = 100 µm; e: endothelium. *: p < 0.05 H vs. CTRL using unpaired t-test.

FIGURE 4: Expression of total eNOS (A: histogram and immunoblots) relative to β-actin proteins (n = 6 per group). Expression of eNOS dimer:monomer ratio (B: histogram and immunoblots) in aorta from adult rats exposed to oxygen as newborns (H) and control (CTRL) (n = 3 per group) as determined by immunoblotting quantified by with Image J (http://rsbweb.nih.gov/ij). *: p < 0.05 H vs. CTRL using unpaired t-test.
**FIGURE 5**: Representative sections of aorta from H and CTRL adult rats, after treatment with hydroethidine (2 µM) without L-NAME (A) or with L-NAME (100 µM) (B) pre-treatment (see Methods). Images were obtained with a laser scanning confocal microscope (LSM 510 laser scanning microscope, Zeiss) equipped with an argon laser. Fluorescence was detected with a 514-nm long-pass filter. The microphotographs are representative of six performed experiments (compiled in the bar graph). Bar scale = 100 µm. *: p < 0.05 H vs. CTRL using unpaired t-test.

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