Antenatal antioxidant prevents adult hypertension, vascular dysfunction, and microvascular rarefaction associated with in utero exposure to a low-protein diet

Gilles Cambonie,1,6 Blandine Comte,1,2* Catherine Yzydorczyk,1 Thierry Ntimbane,2 Nathalie Germain,1 Ngoc Loan Oanh Lê,1 Patrick Pladys,1 Cindy Gauthier,1 Isabelle Lahaie,1 Daniel Abran,1 Jean-Claude Lavoie,1 and Anne Monique Nuyt1

Departments of 1Pediatrics and 2Nutrition, Research Center, CHU Sainte-Justine, Université de Montréal, Montréal, Canada

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Ann J Physiol Regul Integr Comp Physiol 292: R1236–R1245, 2007. First published November 30, 2006; doi:10.1152/ajpregu.00227.2006.—Developmental programming of hypertension is associated with vascular dysfunction characterized by impaired vasodilatation to nitric oxide, exaggerated vasoconstriction to ANG II, and microvascular rarefaction appearing in the neonatal period. Hypertensive adults have indices of increased oxidative stress, and newborns that were nutrient depleted during fetal life have decreased antioxidant defenses and increased susceptibility to oxidant injury. To test the hypothesis that oxidative stress participates in early life programming of hypertension, vascular dysfunction, and microvascular rarefaction associated with maternal protein deprivation, pregnant rats were fed a normal, low protein (LP), or LP plus lazaroid (lipid peroxidation inhibitor) isocaloric diet from the day of conception until delivery. Lazaroid administered along with the LP diet prevented blood pressure elevation, enhanced vasomotor response to ANG II, impaired vasodilatation to sodium nitroprusside, and microvascular rarefaction in adult offspring. Livet total glutathione was significantly decreased in LP fetuses, and kidney eight-isoprostaglandin F2α (8-isoPGF2α) levels were significantly increased in adult LP offspring; these modifications were prevented by lazaroid. Renal nitrotyrosine abundance and blood levels of 1,4-dihydroxynone and 4-hydroxynonenal-protein adducts were not modified by antenatal diet exposure. This study shows in adult offspring of LP-fed dams prevented hypertension, vascular dysfunction, microvascular rarefaction, and of an increase in indices of oxidative stress by the administration of lazaroid during gestation. Lazaroid also prevented the increase in antioxidant glutathione levels in fetuses, suggesting an antenatal mild oxidative stress in offspring of LP-fed dams. These studies support the concept that perinatal oxidative insult can lead to permanent alterations in the cardiovascular system development.

hypertension; vascular dysfunction; developmental origin of adult disease; oxidative stress; antioxidants.

Cardiovascular diseases represent currently the leading cause of mortality in Western countries. The hypothesis of a developmental origin of these pathologies derives from epidemiological studies, indicating that, independent of genetic or lifestyle factors, the risks of hypertension (HT), stroke, and coronary heart disease in later life are inversely proportional to birth weight. Alteration of the vascular response to ACh, a decreased arterial compliance, and an increased incidence of atherosclerosis are all evidence further illustrating the vascular risk in children and adults born with low birth weight and/or intrauterine growth retardation (3, 4, 44, 47).

Experimentally, HT is observed in adults after malnutrition (proteins, calories, vitamins) or an excess of glucocorticoids during fetal life, which leads to, depending on the model, alteration of nephrogenesis, reprogramming of the hypothalomo-pituitary-adrenal axis and early activation of the renin-angiotensin system (RAS) (47). Studies have shown that programmed HT is associated with vascular dysfunction [characterized by impaired nitric oxide (NO)-mediated vasodilatation (9, 37) and enhanced vasomotor response to ANG II (60)], and with microvascular rarefaction appearing in the neonatal period (60).

In human adults, chronic HT and endothelial dysfunction are associated with oxidative stress. In this condition, the excessive production of superoxide anions, which participate in vascular dysfunction and remodeling seems secondary to the increased activation of the NADPH oxidase by ANG II (46). Considering the early role of the RAS in programmed HT (68), the impaired antioxidant defenses associated with fetal malnutrition (28, 45) and the fact that glucocorticoids have been shown to enhance susceptibility to oxidant stress (1, 56), especially in younger subjects (11, 57), one can postulate that infants born after intrauterine deprivation or who are relatively immature are at risk of sustaining early oxidative stress, which could lead to altered cardiovascular development and long-term consequences.

Oxidative stress can be evaluated using several markers. Eight-isoprostaglandin F2α (8-isoPGF2α), a prostaglandin-like family of compounds is a class of biologically active products of arachidonic acid with relevance to human vascular disease. Unlike prostaglandin production, isoprostane formation in vivo is produced from arachidonic acid through lipid peroxidation catalyzed by oxygen free radicals (58). Isoprostane production is recognized as a sensitive and reliable index of lipid peroxidation and oxidative damage (53). In addition, aldehydes that are produced from lipid peroxidation induced by radicals, such as peroxynitrite, are involved in specific responses and pathological events (83). Their much longer half-life than that of

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reactive oxygen species (ROS) make them good candidates to propagate and amplify effects elicited locally by free radicals to neighboring cells. The chemical modification of proteins by aldehydes is considered to be a key event in free radical-induced disturbances of metabolism and modulation of signal transduction. The most reactive species are considered to be 4-hydroxy-2-alkenals such as 4-hydroxynonenal (HNE). Accumulation of these modified proteins depends on the degree of the insult and can lead to the production of more ROS (6, 54, 66). HNE-modified proteins can be converted into less reactive compounds through reduction of HNE to 1,4-dihydroxynonenone (DHN) by aldose reductase and/or alcohol dehydrogenase.

The current study was undertaken to examine the presence of oxidative stress in early life (fetal and/or neonatal) and to verify the hypothesis that antioxidant in early development could prevent later elevation of blood pressure, vascular dysfunction, and microvessel rarefaction associated with in utero exposure to nutrient depletion. Using the animal model of developmental programming of HT associated with restriction of protein intake during pregnancy (37, 39, 60), our study shows an increase renal 8-iso-PGF2α in adult offspring and reduced liver antioxidant glutathione in fetuses. Antioxidant therapy administered during gestation in addition to the low-protein diet normalized glutathione in late fetal life and prevented HT, vascular dysfunction, microvascular rarefaction, and elevation of the marker of oxidative stress 8-iso-PGF2α of the adult offspring.

MATERIALS AND METHODS

Animals

Animals were used according to a protocol approved by the Animal Care Committee of the Research Centre of the Sainte-Justine Hospital, in accordance with the principles of the Guide for the Care and Use of Experimental Animals of the Canadian Council on Animal Care. Virgin Wistar rats (initial weight 225–250 g) were mated overnight and on the day of conception (determined by the presence of a vaginal plug), were allocated to feed ad libitum on a diet containing either 5% methionine to avoid sulfur deficiency and on the day of conception. All dams were weighed on the first day of gestation and weekly thereafter and had free access to food and water. A group of dams in each diet group (n = 7 for CTRL and LP and n = 3 for LP plus lazaroid groups) were anesthetized with intraperitoneal ketamine (65 mg/kg) and xylazine (7 mg/kg) at E21, and the fetal blood, kidneys, and liver were harvested. Within 12 h of delivery, the other dams were returned to regular rat chow. At P7, 10 pups from CTRL dams, 12 pups from LP and LP plus lazaroid dams were killed for tissue sampling (blood, kidneys, and liver; 2 pups/litter). Pups were weaned at 4 wk of age to regular chow. At 10–12 wk of age, in vivo blood pressure studies were performed in 6 male offspring per diet group (one/litter); a second group of adult offspring (n = 10 for CTRL, n = 12 for LP and LP plus lazaroid groups, one/litter/diet group) was killed for tissue sampling (blood, kidneys, liver, and tibialis anterior muscle).

Surgical Preparation

Male offspring (10–12 wk old) were anesthetized with intraperitoneal ketamine (65 mg/kg) and xylazine (7 mg/kg). Under sterile conditions, polyethylene catheters (PE50; Plastics One, Atlanta, GA) were inserted into a femoral artery and vein, tunneled subcutaneously to the back of the neck, threaded through a flexible metal spring, and connected to a dual-channel swivel mounted directly above the cage (Lomir Biochemical, Notre-Dame-de-L’Ile Perrot, Quebec, Canada). The spring was anchored onto a fabric jacket that was adjusted to the rat’s front legs and thorax. This setup allowed the rat freedom of movement within the cage. Each rat was given a dose of intravenous Cefazolin (25 mg/kg) and allowed to recover for 24 h before experiments were performed. We previously verified that the blood pressures obtained were not different whether animals were studied after a 24-h vs. a 4-day recovery period (59).

Experimental Procedures

In vivo studies. On the day of the experiment, the rats were brought in their cage to the laboratory and allowed to adapt for 1 h. During each experiment, mean arterial blood pressure (MABP) was monitored continuously using a pressure transducer (The Perceptor, Nemic, Glen Falls, NY) aligned to the level of the heart and a Grass recorder (Astro-Med, West Warwick, RI). The signal was displayed and simultaneously recorded on a computer via a Grass PVA-1A 8-channel analog-to-digital conversion board using the software Polyview (version 2.3, Astro-Med).

Resting MABP was recorded over 15 min before and 20 min after blockade of endogenous ANG II formation by angiotensin-converting enzyme inhibitor enalaprilat (150 μg/kg IV) (69). MABP responses to continuous intravenous infusions of ANG II (20 and 40 μg/kg−1 min−1) or of NO donor sodium nitroprusside (SNP; 10, 20, and 40 μg/kg−1 min−1) were then determined. Each infusion rate was maintained until MABP was stabilized for at least 1 min. A 30-min recovery period was allowed for MABP to return to resting values before the alternate drug was administered.

Ex vivo vascular reactivity studies. Freshly excised carotid arteries from anesthetized offspring were placed in ice-cold modified Krebs bicarbonate solution of the following composition (in mM): 118 NaCl, 4.7 KCl, 25 NaHCO3, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, and 11 dextrose. They were cleaned of adherent connective tissue and precisely cut into rings of the same length (4 mm). Four to eight rings from one rat were used for one experiment. Rings were suspended horizontally between two stainless steel wires in organ chambers that contained 20 ml of Krebs bicarbonate solution maintained at 37°C and aerated continuously with a mixture 95% O2-5% CO2. The tension of the preparations was recorded with a linear force transducer on a computerized data acquisition system (Kent Scientific, Litchfield, CT). The rings were progressively stretched to a preload tension of 19.0 mN and allowed to equilibrate for 30 min with frequent washing and tension adjustments. After stabilization, rings were repeatedly exposed to KCl (80 mM) to test their viability and to determine a standard contractile response for each of them. When response to KCl...
was stable, endothelial integrity was then assessed in all experiments by a characteristic relaxation response to carbobal (100 μM) after contraction was evoked by phenylephrine (1 μM). Rings were then allowed to recover for 60 min, after which cumulative concentration-response curves were generated with ANG II (10⁻¹² to 10⁻⁷ M) or SNP (10⁻¹² to 10⁻⁵ M). For the latter, vasorelaxant responses were determined on rings precontracted with the thromboxane A₂ mimetic U46619 (3 × 10⁻⁷ M) added to the organ chamber 15 min before. We have previously shown that response to U46619 did not differ between groups (82).

**Biochemical Assays**

**Tissue collection.** Blood, muscle, kidneys, and liver were collected after decapitation (for E21 extracted from anesthetized dams and P7 offspring) or deep anesthesia (adult offspring). When tissues were collected for measurement of glutathione levels, samples were immediately processed, as described below. All other samples were rapidly frozen and stored at −80°C until further analyses.

**Microvasculature.** Morphological measurements of microvessel density were performed in tibialis anterior muscle of adult (10–12 wk old) LP, LP plus lazaroid, and CTRL offspring (method adapted from Ref. 15), as previously described (60). Briefly, muscle sections (transversal 12 μm) were cut on a cryostat (Microm Cryostat, Waldorf, Germany) at −25°C, thaw-mounted on microscope slides (Superfrost, VWR Scientific, West Chester, PA), and fixed in 50% ethanol (70%)–50% acetone for 10 min. Sections were incubated with 1/100 BS-1 lectin (Griffonia simplicifolia lectin; Sigma Chemicals, St. Louis, MO) or anti-rat CD31 (Serotec, Raleigh, NC) both for endothelial staining; the antibodies were TRITC or FITC-labeled for immunofluorescence. The images of the microvasculature obtained from an epifluorescence microscope (Nikon E800) were analyzed using a computerized image-analysis system (Image-Pro plus, Media Cybernetics, Silver Spring, MD). The density of the capillaries was studied on sections obtained from the midbelly of each tibialis anterior muscle. A square counting frame (area 0.3 mm²) was placed over the image (total magnification ×100). Within each muscle section, analyses were performed on four specific areas (two in the cortex and two in the core region of the muscle). The capillary density was expressed in number of capillaries/mm².

**Glutathione.** Freshly isolated liver were immediately homogenized in 5% metaphosphoric acid and centrifuged for 1 min at 5,000 g. Oxidized (GSSG) and reduced (GSH) glutathione in diluted supernatants (in 1% metaphosphoric acid), were separated by zonal capillary electrophoresis (PACE™ MDQ Capillary Electrophoresis System from Beckman Coulter). GSSG and GSH were detected at 200 nm and quantified by using standard curves. The retention time for each species was confirmed by adding in some samples a spike of GSSG and/or GSH. Thresholds were 2 and 45 pmol for GSSG and GSH, respectively, and coefficients of variation were 7% and 12%, respectively, for the intra-assay and interassay (Core Laboratory, Research Centre, Sainte-Justine Hospital). Total glutathione (μmol/g tissue; GSH+2GSSG) and redox ratio [%; 2GSSG/GSH+2GSSG] were determined as indices of antioxidant capacity of LP and LP plus lazaroid compared with CTRL offspring (50).

**8-isoprostaglandin-F₂α.** Eight-isopGF₂α, were assayed in samples of frozen kidney as described (26, 36), using the enzyme immunoassay technique (Cayman Chemical, Ann Arbor, MI).

**HNE and DHN-protein adducts.** To avoid any bias in the measurements, samples were coded before being processed. After complete analyses of the results, the codes for the sample identification were revealed. Assessment of HNE and DHN-protein adducts was done with the sensitive isotope dilution gas chromatography-mass spectrometry method, as described (75) which quantifies HNE-protein adducts formed via Michael-type reactions with the following modifications. Briefly, samples of whole blood (400 μl) were added in a buffer containing 2,6-tert-buty1-4-methylphenol to minimize lipid peroxidation during sample processing. After addition of sodium borohydride (200 μl, 1 M NaBH₃) to reduce HNE to the chemically stable [³H]DHN and sulfosalicylic acid (200 μl) to precipitate the proteins, the pellet was extracted twice with mixed-solvent methanol:chloroform (3 ml; 2:1, vol:vol) to remove contaminants and rinsed 3 times with water (1 ml). The protein pellet was then resuspended into 0.5 ml of guanidine buffer (pH 7.2) and spiked with 0.1 nmol of internal standard [³H]DHN. Two grams of Raney Nickel and 1.5 ml of water were added, and the sample was incubated overnight at 55°C, which converts these alcohols to their corresponding saturated hydroxynonane derivatives. Then, after acidification (pH < 2, 12N HCl), the supernatant was extracted twice with anhydrous ethyl acetate (5 ml) and evaporated under N₂ to dryness, while the residue was treated with 75 μl N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide for derivatization. Samples were heated at 90°C for 3 h and then left overnight at 70°C.

A 2-μl aliquot is injected in duplicates into an Hewlett Packard 6890 Series GC System version A.02.14 (Hewlett Packard, Palo Alto, CA) equipped with a HP-5 capillary column (50 m × 0.2 mm × 0.33 μm) coupled to a mass spectrometer (Agilent Technologies Mass Selective Detector 5973 Network) operated in the positive chemical ionization mode with ammonia. The sample was injected in the pulsed splitless mode with helium as the gas carrier. The injection port and transfer line were set at 280°C and 320°C, respectively. The detection in the single ion monitoring detection mode with a dwell time of 75 ms enables the monitoring of m/z 389, 390, 400 and 257, 258, and 268 corresponding to the molecular ions and one of its fragments for the analysis of DHN, [³H]DHN and the internal standard [³H]DHN, respectively. The temperature program of the chromatograph was as follows: 170°C for 1 min, increased by 10°C/min up to 210°C, increased by 5°C/min up to 280°C and then by 20°C/min up to 320°C for 10 min. The MS source and quadrupole were set at 180 and 126; and 300 and 176°C for the two sets of ions, respectively. Levels of HNE and DHN-protein adducts were calculated from the average of duplicate or triplicate injections for each set of ions.

**Western Blot Analysis of Nitrotyrosine Abundance in Kidney**

Frozen kidney tissues were disrupted using mortar and pestle in liquid nitrogen. Proteins were extracted with RIPA buffer containing 50 nm Tris, pH 7.5, 150 mM NaCl, 1% (vol/vol) Igepal, 0.5% (wt/vol) sodium deoxycholate, 1 mM EDTA, and 0.1% (wt/vol) SDS, supplemented with 1 mM PMSF and 1× solution of cocktail protease inhibitors (Roche Applied Science, GE). Samples were centrifuged at 10,000 g for 10 min, and supernatant was recovered for determination of protein content by BCA assay (Pierce, Rockford, IL) using BSA as standard. Samples containing 50 μg of total crude extract of protein in 2× Laemmlı buffe (200 mM DTT, 20% (vol/vol) glycerol, 100 mM Tris, pH 6.8, 0.2% (wt/vol) bromophenol blue, and 4% (wt/vol) SDS) were boiled for 5 min and applied to a 12% SDS gel, with plates treated with Surfasil siliconizing fluids (Pierce). Proteins were separated by SDS-PAGE and electrophotoblated to a PVDF membrane (Bio-Rad, Mississauga, ON, Canada). The membrane was stained by Ponceau solution to verify that the protein transfer to the membrane was complete. The PVDF membrane was incubated with StartingBlock blocking buffer (Pierce) for 1 h, followed by incubation with a monoclonal antibody for nitrotyrosine (Upstate Technology, Temecula, CA) in a 1:250 solution for 1 h at room temperature or monoclonal antibody for β-actin (Novus Biologicals, Littleton, CO) in a 1:5,000 solution, as internal control. After washing with TBS-T (10 mM Tris, pH 7.6, 15 mM NaCl, and 0.1% (vol/vol) Tween), membranes were incubated for 1 h with a horseradish peroxidase (HRP)-conjugated secondary antibody against mouse IgG (Amersham Life Science; Baie d’Urfé, CA) at a 1:7,500 dilution. After washing with TBS-T, HRP labeling was detected using enhanced chemiluminescent plus (ECL +) reagent (Amersham Life Science) and exposure to X-ray film (X-Omat, Kodak). The autoradiograms were scanned with a
FluoChem 8800 apparatus (Alpha Innotech, San Leandro, CA) to determine the integrated density values of each band. The nitrotyrosine density values were normalized according to the related β-actin values.

Chemicals

The following agents were purchased: ketamine (Ayerst, Montreal, QC, Canada); xylazine (Bayer, Montreal, QC, Canada); enalaprilat (Vasotec, Merck Frosst, Montreal, QC, Canada); ANG II, sodium nitroprusside, carbamylcholine chloride (carbachol) and U46619 (Sigma Chemical, St. Louis, MO); Lazaroid (des-methyl-tirilazad, U-74389G; BioMol Research Lab, Plymouth Meeting, PA).

For the assay of glutathione and protein adducts, chemicals and organic solvents were supplied by the Mat Laboratory (Montreal, QC, Canada), Sigma (St. Louis, MO), and J. T. Baker (Phillipsburg, NJ). Unlabeled HNE was obtained from BioMol Research Lab, while the derivatization agent N-methyl-N-(t-butylmethylsilyl)-trifluoroacetamide was supplied by Regis Chemical (Morton Grove, IL). NaB\(^{3}H_{4}\) and trans-4-hydroxy-2-nonenal-[5,5,6,6,7,7,8,8,9,9,9-\(^{2}H_{11}\)]diethyl acetalate were supplied by Cambridge Isotope Laboratories (Andover, MA) and CMD Isotopes (Pointe-Claire, QC, Canada). Solution of internal standard of \[^{2}H_{11}\]DHN was prepared as previously described (75). All solutions were kept frozen at \(-80^\circ\text{C}\).

Statistical Analysis

Values are expressed as means ± SE. In vivo results were analyzed with Student’s \(t\)-test or factorial ANOVA for concentrations and treatments followed by post-ANOVA comparison among means using the Tukey-Kramer test. Ex vivo concentration-response curves 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 \(E_{\text{max}}\), the maximum asymptote of the curve. Analysis of differences within and between groups were performed using two-way repeated-measure ANOVA and by Student’s \(t\)-test for paired or unpaired observations. Levels of protein adducts, glutathione, and isoprostanes were analyzed using factorial ANOVA. Statistical significance was set at \(P<0.05\).

RESULTS

Net weight gain during pregnancy was similar for all diet groups: 142 ± 5 g (\(n = 19\)), 144 ± 7 g (\(n = 18\)), and 132 ± 7 g (\(n = 10\)) for CTRL, LP, and LP plus lazaroid-fed dams, respectively. Diet group allocation neither significantly affected the litter size at birth (CTRL: 15 ± 1, \(n = 8\); LP: 15 ± 1, \(n = 7\); LP plus lazaroid: 12 ± 1, \(n = 8\)) nor the survival rate of the offspring during the 14 wk of the study period. Birth weight of the LP offspring (5.3 ± 0.1 g, \(n = 14\)) was not significantly lower than that of the CTRL offspring (5.6 ± 0.2 g, \(n = 14\)), but pups born to LP plus lazaroid-fed dams weighed significantly more (5.7 ± 0.1 g, \(n = 29\)) than LP pups and were comparable to the CTRL pups.

Effects of Antenatal Diet and of Lazaroid on Adult Blood Pressure at Baseline and in Response to Vasoactive Agents

Figure 1 shows that resting MABP of LP adults was significantly higher than that of CTRL offspring. MABP of LP plus lazaroid offspring was significantly decreased compared with LP and similar to CTRL offspring. Systemic administration of enalaprilat significantly reduced MABP of LP without altering MABP of CTRL and of LP plus lazaroid offspring. After blockade of endogenous ANG II formation, blood pressure responses to ANG II and SNP infusions were significantly shifted to higher values in LP-exposed rats relative to CTRL. Administration of lazaroid throughout gestation to LP-fed dams prevented the exaggerated pressor response to ANG II and the altered hypotensive response to NO donor SNP of the adult offspring.

Effects of Antenatal Diet and of Lazaroid on Ex Vivo Vascular Reactivity

Vasoconstrictor response of carotid arteries segments to ANG II was increased, and vasodilator response to SNP was decreased in LP compared with CTRL offspring, as presented in Fig. 2. Antenatal administration of lazaroid to the LP-fed dams prevented both exaggerated vasoconstriction to ANG II and impaired vasorelaxant response to SNP of their adult offspring.

Effects of Antenatal Diet and of Lazaroid on Microvascular Rarefaction

Capillary density in the tibialis anterior muscle of male adult offspring exposed during gestation to the different diets was measured. Our data showed prevention of significant decrease in capillary density in LP plus lazaroid offspring compared with LP (60); capillary density of LP plus lazaroid offspring was found similar to CTRL: 1.107 ± 0.09, 791 ± 52, and 1.045 ± 24 for CTRL, LP, and LP plus lazaroid diets, respectively; for 6 animals/group and on average 15 to 20 sections/animal analyzed. \(* P<0.05\) vs. CTRL and LP plus lazaroid using unpaired \(t\)-test.

Effects of Antenatal Diet and of Lazaroid on Markers of Antioxidant Defense and Oxidative Stress

To investigate the mechanisms involved in the previous observation in adulthood, markers of antioxidant defense and

Fig. 1. Mean arterial blood pressure (MABP) response to blockade of endogenous ANG II formation with angiotensin-converting enzyme inhibitor enalaprilat (150 µg/kg iv), followed by continuous intravenous infusions of ANG II (20 and 40 µg/kg \(^{-1}\text{min}^{-1}\); left) or of nitric oxide donor sodium nitroprusside (SNP; 10, 20, and 40 µg/kg \(^{-1}\text{min}^{-1}\); right) of 10- to 12-wk-old rats exposed during gestation to a control (CTRL), low-protein (LP), or LP plus lazaroid diet. Data are expressed as means ± SE of five experiments in each group; \(* P<0.05\) vs. baseline of the same study group; \(* P<0.05\) vs. LP under the same experimental condition and \#P < 0.05 vs. CTRL under the same experimental condition using two-way ANOVA.
of oxidative stress were measured in different tissues of E21,
P7, and adult offspring exposed to CTRL, LP, and LP plus
lazaroid antenatal diet. Antioxidant total glutathione levels
were measured in liver, the major site of glutathione synthesis.
The total glutathione level (GSH \( \div \text{H}_{2}\Gamma \text{GSSG} \)) was significantly
decreased by 30% in E21 LP offspring compared with the
animals with the control diet (Fig. 3A), and this effect of the LP
diet was prevented by the treatment with lazaroid. Total glu-
taxhione levels in livers of P7 offspring displayed a similar
pattern with a decrease in LP prevented in LP plus lazaroid
offspring; however, these later results did not reach statistical
significance. The redox ratio of glutathione (%) was unaltered
by antenatal diet exposure.

Figure 3C shows the levels of 8-isoPGF\(_{2\alpha}\) measured in
kidney tissue, the major site of vasoconstrictive action of
8-isoPGF\(_{2\alpha}\). Levels were significantly increased in LP vs.
CTRL adult offspring (but not at E21 or P7). Levels in LP plus
lazaroid-exposed adult animals were lower than for LP ani-
mals, but this did not reach statistical significance. For the
CTRL and LP diets, we observed a significant age effect: the
levels in P7 offspring were significantly lower than those
measured in E21 (62%) and adults (74% and 248% for CTRL
and LP, respectively; \( F(1,29) = 22.58, P < 0.01 \) for E21-
adults vs. P7); lazaroid had no effect on this observation.

Another marker of oxidative stress has been measured in
whole blood of these animals, namely HNE- and DHN-protein
adducts. Total (HNE- and DHN) -protein adducts of adult LP
offspring were higher (by 65%) than CTRL, but this did not
reach statistical significance (\( P < 0.2 \)). Overall, total adducts
were not significantly modified by antenatal diet exposure at
the three ages studied. However, and interestingly, contrary to
what was observed in kidney 8-isoPGF\(_{2\alpha}\) levels, the blood
concentrations of protein adducts were relatively increased in
P7 compared with E21 and to adult offspring of the CTRL and
LP diet groups; this reached statistical significance for the
CTRL group. LP diet abolished this significant modulation
with age. No difference in nitrotyrosine abundance in kidney
tissue was observed between groups (Fig. 4).
DISCUSSION

There is convincing epidemiological evidence that altered environment during development is associated with vascular dysfunction and HT in adult life (4, 23, 43, 44). Considering that oxidative stress is an important pathophysiological element underlying endothelial dysfunction associated with chronic HT and that intrauterine nutrient depletion can decrease antioxidant capacity of the newborn, we tested the hypothesis that perinatal oxidative stress may play a key role in developmental programming of HT. Our results reveal that antioxidant glutathione is decreased in E21 LP offspring, that oxidative stress marker 8-iso-PGF2α is significantly increased in adult LP offspring, and these changes are prevented by administration of lazaroid to LP fed dams. More importantly, we show that the elevated blood pressure, vascular dysfunction, and microvascular rarefaction of the adult offspring can be prevented by antioxidant therapy administered during gestation to the LP fed dam.

Hypertension in LP offspring is seen equally in male and female in most (40, 79) but not all studies (80). However, the mechanisms underlying the increased blood pressure are probably sex-specific. HT in LP offspring at 4 wk of age is glucocorticoid mediated in males but not females (40, 47, 49, 79). Renal expression of ANG II receptors in LP offspring differ according to gender (48, 49, 63, 64). Gender-specific mechanisms are also reported for other animal models of developmental programming of elevated blood pressure and vascular dysfunction, such as in offspring of lard-fed pregnant rats (33). We have restricted these studies to male offspring in the adults; therefore the results of that age group cannot be extended to female offspring. However, sex could not be determined at E21 and P7, and one can therefore suppose that the significant differences observed then apply to both genders or alternatively that combination of both genders could have masked more significant differences between groups.

In human adults, chronic HT and endothelial dysfunction are associated with oxidative stress and a major cause of ROS generation (mostly superoxide) is ANG II (17, 52, 71). Increased vascular production of superoxide in developmental programming of HT has previously been shown. Adult offspring of LP-fed dams and of dams subjected to a 50% caloric restriction during gestation display increased vascular superoxide anion concentration at baseline and after simulation with ANG II (19, 82). Reversal of HT and altered vasodilatation has been reported in LP offspring treated as adults with antioxidant vitamins C and E (18). These effects were associated with decreased vascular superoxide anion concentration. However, it is unknown whether oxidative stress was involved in early life and could have initiated vascular dysfunction and HT. Lazaroid is a potent inhibitor of free radical formation and of superoxide-mediated lipid peroxidation. Previous studies with U-74389G have shown beneficial effects of this lazaroid on blood pressure in three different models of experimental hypertension; namely, the lead-induced (72), the 5/6 nephrectomy (14), and the spontaneously hypertensive rat (SHR) (73) models. In all of these models, U-74389G administration rapidly normalized arterial pressure, plasma malondialdehyde concentration (which is an aldehyde produced by plasma lipid peroxidation, as HNE), urinary excretion of nitric oxide metabolites, and accumulation of nitrotyrosine in the brain of the animals with chronic renal failure. Particularly in the SHR model, lazaroid treatment increased NO bioavailability leading to NO/NOS feedback restoration and modulation of NO synthase expression in different tissues. Such a mechanism could be involved in the present study, as NO-mediated vasodilatation was impaired in vivo (Fig. 1) and ex vivo (Fig. 2) in the LP offspring and normalized by the coconcomitant administration of U-74389G.

ANG II can increase blood pressure through central and peripheral mechanisms. Brain ANG II blunts arterial baroreflex modulation and activates efferent sympathetic activity to the vessels and the kidneys (increasing renin secretion), thereby increasing blood pressure. Interestingly, increased intracellular superoxide production in the brain cardiovascular regulating areas seems critical in the development of ANG II-induced HT (85, 86). We have previously reported increased expression of AT1 receptors in brain cardiovascular regulating areas of adult LP offspring (59). Whether oxidative stress indices are detectable in LP brain and whether the increase in AT1 expression (and perhaps of oxidative stress markers) was abrogated by the current experiments was not examined. Nonetheless, to parallel what was reported with 5/6 nephrectomized rats where lazaroid reduced blood pressure and tyrosine nitration in the brain (14), we could hypothesize that lazaroid prevented RAS upregulation in LP brain. This, in turn, could participate in the prevention of elevation of blood pressure by lazaroid in LP offspring.

Whether lazaroid crosses the placenta is unknown, but the latter cited study among others indicates it crosses the blood-brain barrier (14, 25). Our results suggest that either low-level...
oxidative stress is ongoing during gestation which could cause or be concomitant with reduced antioxidant defenses and is partially prevented by lazaroid (see below), or alternatively, that lazaroid is still present in the newborn tissues and helps prevent oxidative insult associated with increase in $P_{O2}$ at birth.

The current data support a role for early oxidative stress as a key element in the pathogenesis of programmed cardiovascular dysfunction. The mechanism by which oxidant injury in early life could have long-term cardiovascular consequences remains to be demonstrated, but one has to consider a potential role for RAS. Blockade of ANG II formation during the first week of life of LP offspring (68) prevents later elevation of blood pressure. Generation of ROS by early activation of RAS could permanently alter cardiovascular development and function. Within the vasculature, excess ROS is involved in several signal pathways resulting in inflammation, generation of vasoconstrictors, such as isoprostanes, and reduction of NO bioavailability (10, 29); all of these elements contribute to vascular dysfunction associated with chronic HT. In addition, ROS can further increase the local expression of RAS, including ANG II, as well as its receptor AT$_1$ (16, 17, 27), which amplifies oxidative stress. Indeed, there is increased AT$_1$ expression in adult LP offspring in the cardiovascular-regulating areas of the brain (59), carotids (82), and in 4-week-old male LP kidneys (64, 74). Taken together, these effects could lead to early and permanent (vascular) damage.

We have previously reported microvascular rarefaction in striated muscle of P7 and adult but not of late fetal (E21) offspring of LP-fed dams, suggesting postnatal disruption of capillary development. As we show prevention of microvascular rarefaction with early antioxidant administration, this indeed supports a role for oxidative stress in disrupting normal capillary development. Immature animals exhibit increased vulnerability to microvascular oxidant stress-induced injury (especially the endothelial cells) (8, 35). Vasoconstriction and endothelial cell damage lead to degenerative processes in terminal arterioles and capillaries inducing anatomical rarefaction (24, 76). Decrease in NO bioavailability by ROS scavenging could also interfere with angiogenesis (34).

Hypertension results from vascular dysfunction and vascular structural anomalies. In programmed HT, whether vascular dysfunction appears along with microvascular rarefaction or is a later and/or secondary event is unknown. In this model, vascular dysfunction results from enhanced constriction to ANG II, impaired NO-mediated vasodilatation along with increased vascular generation of superoxide. These dysfunctions could be induced in the neonatal period by an oxidative insult and perpetuated throughout adulthood. Indeed, in addition to increasing RAS components expression, ROS can decrease NO production by NO synthase reduced expression or uncoupling (51), it can decrease the effect of vascular NO by decreasing expression of soluble guanylate cyclase (5, 12), and it can increase NADPH oxidase expression (41); all of these elements will, in turn, further increase ROS production. In addition, glutathione depletion has been shown to decrease endothelial NO synthase activity (42). One has also to evoke the possibility of epigenetic changes such as DNA methylation patterns and histone modifications of genes, which have been suggested to mediate the effect of altered intrauterine environment on adult onset diseases (47) and which can be caused by oxidative stress; such changes involving genes implicated in vascular development and function remain to be demonstrated.

Prevention of adult cardiovascular manifestations was observed after supplementation of the LP-fed pregnant dam with glycine (31). Glycine is a nonessential amino acid, which has been found to protect against oxidative stress in several pathological situations; however, antioxidant capacity or indices of oxidative stress were not evaluated in this study. More recently, Stewart et al. (70) reported enhanced oxidative stress determined by quantitative immunoblotting for nitrotyrosine in the kidneys of 4-week-old LP offspring; in this study, administration of superoxide dismutase analog before 4 wk of age prevented the increase in nitrotyrosine abundance in the kidney as well as the development of high blood pressure. In spontaneously hypertensive rats, HT is prevented by perinatal administration of L-arginine (essential for NO synthesis) and antioxidant vitamins C and E (61, 62).

Immature subjects (such as prematurely born infants) have lower and less inducible antioxidant defenses, such as superoxide dismutase, catalase, and glutathione peroxidase (22, 32, 65). Intrauterine gestational retardation and premature infants also display indices of increased oxidative stress (32, 67). Glutathione is a central component of the antioxidant array because of its high intracellular concentrations and its ubiquity (81), and its metabolism is highly sensitive to dietary regulation. Antioxidant defenses can be reduced by protein malnutrition (28). Intrauterine oxidative stress could be attributed to exposure to excess maternal glucocorticoids (21, 30) and to nutrient restriction, which can, in itself, induce oxidative stress in the pregnant dam with placental and potentially fetal repercussion (13). In addition, elevated glucocorticoids can increase the susceptibility to oxidative stress, especially in young animals, in part through impairment of antioxidant status (1, 11, 56, 57). Therefore, the combination of increased production of ROS by ANG II and decreased antioxidant capacity (through nutrient restriction and exposure to excess glucocorticoids) can increase the susceptibility of the “programmed” fetus to oxidative stress at birth when tissue $P_{O2}$ increases dramatically. At E21, our results show total glutathione levels in the liver that are significantly decreased by LP exposure, but the redox ratio was similar between groups. These results, along with the other markers of oxidative stress in which no difference was detected at E21, could suggest that oxidative stress is absent in fetal life but that antioxidant capacity is decreased. However, the fact that administration of lazaroid along with the LP diet normalized glutathione levels suggests the presence of a mild oxidative imbalance and that glutathione levels are sufficient to buffer the excessive oxidative reactions, which are not detectable by the oxidative stress markers used in this study.

Eight-isoPGF$_{2\alpha}$ is a reliable and specific indicator of lipid peroxidation and oxidative damage (53) produced independently of the cyclooxygenase pathway; its formation results from oxidation by free radicals of the ubiquitous arachidonic acid in plasma membranes. Increased levels of 8-isoPGF$_{2\alpha}$ in kidneys from LP adults is in agreement with findings in hypertensive adults and in other high blood pressure animal models (78). The significant age effect in the kidney levels of 8-isoPGF$_{2\alpha}$ (Fig. 3C) that we observed is supported by the recent study of Ward et al. (77), who showed, in rats, that isoprostane levels in kidney increase with age but also of Friel
et al. (20), who reported that plasma levels of isoprostanes decline in infants from 1 to 12 mo of age. Although caloric restriction can decrease kidney isoprostanes concentrations (77), our results show that LP antenatal diet did not significantly modify them. Even though we were not able to measure isoprostanes levels in E21 offspring of LP plus lazaroid fed dams, our results suggest that the effect of age (P7 vs. adults) is not modified by the addition of an antioxidant in the prenatal diet.

Hydroxynonenal is among the most reactive aldehydes and reacts spontaneously with specific amino acid residues of proteins (cysteine, lysine, and histidine). These chemical modifications of proteins are considered to be a key event in free radical-induced disturbances of metabolism and modulation of signal transduction (6, 54, 66). The accumulation of HNE-modified proteins depends on the intrinsic capacity of the cells to metabolize HNE into nontoxic products through reduction to DHN or conjugation with glutathione. Because of this efficient metabolism, it is important to measure HNE, as well as DHN not to underestimate the true magnitude of lipid peroxidation under specific pathological conditions (75). Increased HNE-modified proteins have been reported in blood, heart, and brain in pathological conditions associated with oxidative stress (2, 7, 55, 75, 83). Interestingly, in the CTRL condition, the pattern of protein-adduct concentrations in the blood is the reverse to what is observed with kidney 8-isoPGF2α levels with significantly higher levels in P7 vs. E21 and vs. adult animals (Fig. 3D). Even though there is a trend of increased protein-adduct concentrations in the blood of adults LP offspring, this difference did not reach statistical significance, but the kinetics observed with age in the CTRL diet is dampened. It is interesting to note that two markers of oxidative stress assayed in different tissues show different patterns, justifying the use of several indices to evaluate the importance and the role of that phenomenon in the development of the complex pathology. Therefore, our results suggest that a mild antenatal oxidative stress prevails and that glutathione levels are sufficient to buffer the excessive oxidative reactions. Some important considerations should, however, be kept in mind: 1) even though statistical significance is not reached, it is not possible to exclude that metabolic consequences of the mild oxidative stress are present; 2) the significance could be potentially reached with more sensitive methods for the assessment of oxidative stress, particularly for isoprostane determination (i.e., using mass spectrometry); and 3) finally, oxidative stress can occur very locally at the tissue level, such as the (developing) endothelium and was not assessed by the methodology used.

In summary, the current study shows in adult offspring of LP-fed dams, prevention of elevated blood pressure, of microvascular rarefaction, and of vascular dysfunction both in vivo and ex vivo by the administration of peroxidation inhibitor lazaroid concomitantly with the LP diet during gestation. Moreover, there are indirect indications that an antenatal mild oxidative stress occurs in late fetal life and in the adult offspring of dams fed a LP diet during gestation, processes that are, in part suppressed by the addition of lazaroid concomitantly with the LP diet during gestation. These findings support the concept that perinatal oxidative insult can lead to permanent alterations in the cardiovascular system development.

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Present addresses: G. Cambonie, Service de réanimation pédiatrique et Néonatale, Hôpital Arnaud de Villeneuve, 34295 Montpellier, France. P. Pladys, Unité de médecine néonatale, Hôpital Sud, CHU Rennes, 35203 Rennes Cedex 2, France.

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