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Journal of Nutritional Biochemistry xx (2015) xxx–xxx

Journal of Nutritional Biochemistry

Fetal undernutrition is associated with perinatal sex-dependent alterations in oxidative status

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Received 3 April 2015; received in revised form 30 July 2015; accepted 5 August 2015

Abstract

Intrauterine growth retardation predisposes to hypertension development, known as fetal programming. Females are less susceptible, which has been mainly attributed to estrogen influence. We hypothesize that perinatal differences in oxidative status might also contribute. We studied 21-day-old (prepuberal) and 6-month-old male and female offspring from rats fed *ad libitum* during gestation (Control) or with 50% of Control daily intake from day 10 to delivery (maternal undernutrition, MUN). We assessed *in vivo* blood pressure and the following plasma biomarkers of oxidative status: protein carbonyls, thiols, reduced glutathione (GSH), total antioxidant capacity, superoxide anion scavenging activity (SOSA) and catalase activities; we calculated a global score (oxy-score) from them. Estradiol and melatonin concentration was measured in young rats. Prepuberal MUN males were normotensive but already exhibited increased carbonyls and lower thiols, GSH, SOSA and melatonin; oxy-score was significantly lower compared to Control males. Prepuberal MUN females only exhibited reduced SOSA compared to Control females. Adult rats from all experimental groups showed a significant increase in carbonyls and a decrease in antioxidants compared to prepuberal rats; oxy-score was negative in adult rats suggesting the development of a prooxidative status as rat age. Adult MUN males were hypertensive and exhibited the highest increase in carbonyls despite similar or even higher antioxidant levels compared to Controls. Adult MUN females remained normotensive and did not exhibit differences in any of the biomarkers compared to Controls. The better global antioxidant status developed by MUN females during perinatal life could contribute to their protection against hypertension programming.

Keywords: Experimental models; Fetal programming; Hypertension; Melatonin; Plasma; Oxidative stress; Sex

1. Introduction

Noncommunicable diseases (NCDs) are the main contributors to the global disease burden in developed societies and are also increasing in developing countries [1,2]. Despite this fact, the causes of many NCDs are unknown and, therefore, ways to prevent them remain elusive. The conventional etiological model of NCDs based on a genetic predisposition and unhealthy lifestyle was challenged by Dr. Barker, who proposed that intrauterine life was an additional risk factor [3]. This hypothesis, known as fetal programming, is now well established through epidemiological and experimental studies evidencing the negative influence of intrauterine growth retardation (IUGR) in the development of NCDs, particularly cardiovascular diseases (CVD) and their risk factors obesity and hypertension [4,5].

It has been recently put forward that different NCDs might have common pathophysiological mechanisms, and oxidative stress might play a central role in their onset and development [6]. Therefore, it is plausible that exposure to stress conditions during intrauterine life could trigger an oxidative imbalance from early age, leading to long-term tissular damage and NCD pathology. In fact, oxidative stress has been implicated in fetal programming [7] and several studies in animal models of IUGR have evidenced that oxidative damage is present in key organs for cardiovascular control such as the heart [8], the kidney [9,10] and blood vessels [11]. However, the presence of oxidative damage in animals with already established pathology does not determine whether oxidative stress is the cause or consequence of the cellular alterations [12]. In addition, most studies have focused on individual biomarkers, and the complex and multifactorial nature of oxidative stress makes it difficult to assign a prevalent role to a particular biomarker and does not provide information on the global

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http://dx.doi.org/10.1016/j.jnutbio.2015.08.004
0955-2863/

oxidative status. We and others have previously used the calculation of a global score, based on individual plasma biomarkers of antioxidant defense and oxidative damage, to assess the overall oxidative balance in several CVD [13–15].

Fetal stress seems to have a lower impact on females, particularly in the development of hypertension [16–18]. This difference has been mainly attributed to the modulatory role of sex hormones, but other innate sex differences have also been proposed [19]. Despite the fact that "sex" is a critical variable to be taken into consideration [20], the sex-dependent phenotype of fetal programming has not been thoroughly investigated, since most studies have been performed on male animals. Based on the evidence that IUGR, induced by nutrient restriction to the fetus, is associated with oxidative stress, we hypothesize that females might counteract the deleterious effects of IUGR through the development of a better global oxidative balance during the critical developmental window of perinatal life. This early advantage, together with the cardiovascular protective effect of estrogens, could contribute to the milder consequences of IUGR on CVD programming on females.

To assess our hypothesis, we have studied a rat model of IUGR induced by maternal undernutrition during the second half of gestation (MUN), which has been previously demonstrated to program for hypertension and obesity in adult age [5,21–24]. In male and female offspring from MUN and Control dams, we have assessed individual plasma biomarkers of oxidative damage and antioxidant capacity and calculated a global score (oxy-score) from them. We have chosen to study two age points: weaning, as a critical developmental period prior to sexual maturity, and adult age, where hypertension is already established in males.

2. Materials and methods

Experiments were performed in Sprague–Dawley rats from the colony maintained at the animal house facility of the Universidad Autónoma de Madrid. All experimental procedures were approved by the Ethics Review Board of Universidad Autónoma de Madrid and conformed to the Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 85-23, revised in 1996), the Spanish legislation (RD 1201/2005) and the Directive 2010/63/EU on the protection of animals used for scientific purposes.

The rats were housed in buckets 36.5/21.5/18.5 cm (length/width/height) on aspen wood bedding, under controlled conditions of 22°C, 40% relative humidity and 12/12 light/dark photoperiod. The animal health monitoring indicated that they were free from pathogens that may interact with any of the parameters studied.

The rats were fed with breeding diet (SAFE A03) containing 51.7% carbohydrates, 21.4% protein, 5.1% lipids, 3.9% fiber, 5.7% minerals and 12.2% humidity (Safe Augy, France). Drinking water was provided *ad libitum* in all cases.

2.1. Experimental model (MUN)

Maternal undernutrition (MUN) was induced as previously described [25]. Day 1 of gestation was determined by observation of sperm in the vaginal smear. Dams were then divided in two groups, one with *ad libitum* diet throughout pregnancy (Control group, C, $n=6$) and the other one with *ad libitum* diet during the first 10 days of gestation and 50% of the daily intake from day 11 to the end of gestation (MUN, $n=6$). The maximum daily intake of rat chow was previously determined in a group of pregnant rats as 24 g/day. Therefore, during the second half of gestation, the MUN group received 12 g of rat chow per day. After delivery, both C and MUN rats were offered food *ad libitum* during the suckling period.

At 24 h after birth (postnatal day 1, P1), the pups were sexed and weighed individually, and the litter was standardized to 12 individuals, 6 males and 6 females, if possible (smaller litters were not used). At weaning (21 days, postnatal day 21, P21), 1–2 males and 1–2 females from every litter were used to test blood pressure, plasma parameters of oxidative status and estrogen levels.

One male and one female rat from each litter were kept for another 2 weeks under changed light/dark cycle to measure peak plasma levels of the antioxidant hormone melatonin. At the end of the 2-week period, sufficient for a change in melatonin pattern of secretion [26], the rats were anesthetized by CO_2 and the blood was collected by cardiac puncture at 11.00 h, peak time of melatonin secretion under the changed cycle. The rest of the litter was maintained until the age of 6 months to assess blood pressure, plasma biomarkers of oxidative status and for other studies.

2.2. Blood pressure measurements

Systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) were measured in rats under anesthesia (80 mg/kg ketamine and 10 mg/kg xylazine, ip) as previously described [27]. Briefly, a cannula connected to a pressure transducer (Statham; Harvard Apparatus) was inserted in the right iliac artery and pressure wave was recorded in a PowerLab system (ADInstruments) during 60 min. SBP, DBP and HR were measured in the trace at the end of the 60 min, averaging the data from approximately 1 min recording period.

2.3. Biomarkers of oxidative status in plasma

After blood pressure measurements, blood was collected from the cannula inserted in the iliac artery in eppendorf tubes containing 5% heparin. The blood was centrifuged for 10 min (900*g* at 4°C) and the plasma was aliquoted and stored at −70°C until use.

2.3.1. Total protein carbonyls

Plasma protein carbonyls were assessed with a 2,4-dinitrophenylhydrazine-based assay [28], as previously described [27]. The protein carbonyl concentration was determined using extinction coefficient of 2,4-dinitrophenylhydrazine ($\varepsilon=22{,}000$ M/cm) and expressed as nanomoles per milligram (nmol/mg) of protein. Protein content was assessed by Coomassie-blue-based microtiter plate assay, according to manufacturer's instructions (Bio-Rad). The absorbance was measured at 595 nm in a microplate reader (Synergy HT Multimode; BioTek).

2.3.2. Total antioxidant capacity (TAC)

TAC assay is based on enhanced horseradish-peroxidase-catalyzed luminol chemiluminescence [29,29]. The relative luminescence (RL) was calculated by the following formula: RL=[1−luminescence (t)]/[luminescence ($t0$)]. RL was used to calculate the area under the curve using GraphPad Prism software (GraphPad, San Diego, CA).

2.3.3. Reduced glutathione (GSH) content

Plasma GSH was assessed by a fluorimetric micromethod based on the reaction with *o*-phthalaldehyde as previously described [27]. Fluorescence was measured in the microplate reader at 360±40 nm excitation and 460±40 nm emission wavelengths. GSH concentration of the samples was expressed as micromoles per milligram (μmol/mg) of protein.

2.3.4. Total thiols

Plasma thiols were assessed by a 5,5′-dithiobis(2-nitrobenzoic acid) assay [28], adapted to a microplate reader, as previously described [27]. The absorbance was measured at 412 nm and thiol content was expressed as nanomolar of GSH per milligram of protein.

2.3.5. Superoxide anion scavenging activity (SOSA)

SOSA was determined using a luminescence assay with coelenterazine as detection probe [29,29], adapted to microplate reader [14]. SOSA values were quantified by comparing the luminescence inhibition of each sample with that observed from superoxide dismutase (SOD) activity standard curve (0–4 U/ml) and expressed as milliunits of SOD per milligram of protein.

2.3.6. Catalase activity

Catalase activity was assessed by Amplex Red catalase assay (EnzChek Myeloperoxidase Assay Kit with Amplex Ultra Red reagent; Invitrogen). Catalase activity was expressed as units per milligram of protein.

2.4. Calculation of a global score of oxidative status (oxy-score)

The biomarkers of oxidative status mentioned above were used to calculate a global index (oxy-score) for each experimental group, using the statistical methodology previously described [13,14], using the following steps:

(1) Analysis of the normality of the different biomarkers through the Kolmogorov–Smirnov test and Q–Q graphs.
(2) Normalization of those parameters that did not show a normal distribution through a logarithmic transformation.
(3) Parameter standardization.
(4) Calculation of oxy-score based on the partial indexes (OXY and ANTIOX), according to the following equations:

$$\text{oxy-score} = (\text{ANTIOX}-\text{OXY})$$

$$\text{OXY} = \text{standarized values of protein carbonyl, as biomarker of oxidative damage}$$

$$\text{ANTIOX} = \text{sum (standardized antioxidant biomarkers)}$$

Based on this calculation, a positive oxy-score indicates prevalence of antioxidant capacity and a negative oxy-score indicates predominance of oxidative damage.