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Biomarkers of Oxidative Stress and Antioxidant Status in Children Born Small for Gestational Age: Evidence of Lipid Peroxidation

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ABSTRACT: Children born small for gestational age are known to be at increased risk for adult diseases such as hypertension, diabetes, and coronary heart disease. Oxidative stress is a common feature of these pathogenic conditions and can be the key link between size at birth and increased morbidity later in life. The purpose of this study was to analyze the parameters of lipoperoxidation and changes in antioxidant defense system as well as assess their relationship to birth weight. Concentrations of thiobarbituric-acid-reactive-substances and F_2-isoprostanes, total antioxidant status, and the activity of both superoxide dismutase and glutathione peroxidase were measured in 65 children (33 boys, 32 girls; ages 8–13 y). Thiobarbituric-acid-reactive-substances and F_2-isoprostane levels were significantly elevated in children born small for gestational age. Nevertheless, superoxide dismutase activity was significantly elevated in these children and the levels of both glutathione peroxidase activity and total antioxidant status were unchanged. Moreover, we found that systolic blood pressure was positively associated with thiobarbituric-acid-reactive-substances levels in race- and gender-adjusted models but not in a multivariable regression model. In conclusion, the current study revealed that there is evidence of oxidative stress in children born small for gestational age as supported by increased lipid peroxidation. **(*Pediatr Res* 62: 204–208, 2007)**

It has been proposed that events occurring before birth may influence the risk in the development of many diseases, including type II diabetes, asthma, hypertension, and coronary heart disease (1–4). The fetal programming hypothesis proposes that these cardiovascular and related disorders derive from fetal adaptations, which increase the fetus's chance of survival in poor nutritional environments but result in permanent alterations of growth characteristics, postnatal metabolism, and physiology (1,2). Therefore, size at birth demands urgent attention not only because of the significantly increased risk it poses for infants and young children, but also for the risk of developing diseases in adulthood.

Multifactorial events are involved in the pathogenesis of disorders linked to fetal programming, and the identification of the mechanism is still a matter of discussion. It has been established that an excessive and/or sustained increase in free radical production associated with diminished efficacy of the antioxidant defense systems result in oxidative stress, which occurs in many pathologic processes and contributes significantly to disease mechanisms (5). It is reasonable to suggest that oxidative stress would be the key link between an adverse prenatal environment and increased morbidity later in life. In fact, adverse fetal growth is frequently associated with a number of oxidative insults. Longini *et al.* (6) demonstrated that elevated isoprostane concentrations could be detected in the amniotic fluid of fetal growth restriction pregnancies. In addition, Gupta *et al.* (7) demonstrated that malondialdehyde (MDA) concentration was increased whereas the activities of SOD, catalase, and glutathione were decreased in the cord blood of SGA infants. Urinary 8-hydroxydeoxyguanosine, a marker of oxidative stress, was increased in infants with very low birth weight (8). Inder *et al.* (9) reported a positive association between TBARS levels and adverse respiratory and ophthalmologic outcomes in very-low-birth-weight infants. Although evidence of an interaction between oxidative stress and size at birth was recently reported in neonates, little evidence of this correlation has been described in children.

To understand the long-term consequences of birth weight on oxidative or antioxidative pathways, we assessed parameters of lipoperoxidative degradation and antioxidant defenses in 8- to 13-y-old former SGA children. First, we investigated LPO *via* measurement of two biomarkers: TBARS and F_2-isoprostanes. The TBARS method measures levels of MDA, a decomposition product of peroxidized polyunsaturated fatty acids (10). Isoprostanes are noncyclooxygenase-derived prostaglandin isomers products of arachidonic acid that result from free radical-catalyzed lipid peroxidation. Measurement of these molecules has emerged as the most reliable method to

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Abbreviations: AGA, appropriate birth weight for gestational age children; **BW,** birth weight; **GPx,** glutathione peroxidase; **LPO,** lipid peroxidation; **SGA,** small for gestational age children; **SOD,** superoxide dismutase; **TAS,** total antioxidant status; **TBARS,** thiobarbituric-acid-reactive-substances; **UA,** uric acid

assess oxidative stress status *in vivo* (11). In particular, F_2-isoprostane (8-Epi-$PGF_{2\ \alpha}$) has been implicated as a marker of antioxidant deficiency and oxidative stress and has the potential to assess human diseases (11). Secondly, we measured the activity of antioxidant enzymes such as SOD and GPx. These enzymes are the first line of defense against free radicals and are generally referred to as primary antioxidants. We also evaluated TAS, a measurement of the total antioxidant effect of the defense systems in circulation. These antioxidant systems include enzymatic (generally, if not exclusively, intracellular) and nonenzymatic (both intra- and extracellular). Lipid soluble and water-soluble antioxidants are involved in these processes. Thus, the overall antioxidant capacity may give more relevant biologic information compared with that obtained by measuring individual components because it considers the cumulative effect of all antioxidants present in circulation (12). Finally, we tried to find out whether there is a correlation between parameters of LPO and UA or lipid profiles, since these metabolic parameters could have pro-oxidant effects, which increase the rates of LPO reactions and hence can provide better insight into the implications of prenatal growth in SGA children.

METHODS

A total of 113 children aged 8–13 y were recruited and evaluated between November 2004 and July 2005. Participants were selected among 289 children screened in an anthropometric census performed in five shantytowns by the Nutritional Rehabilitation Center of the Federal University of São Paulo, Brazil. Initially, 18 shantytowns were identified within 15 km of the Federal University of São Paulo, which is located in the southern region of São Paulo. The region was then subdivided into three geographic areas and a secondary sample of five shantytowns was randomly selected taking into account this stratification by region. Personal and family medical histories were obtained from a questionnaire completed during an interview with the parents or guardians. This cohort was screened with the following characteristics: children born at term with birth weight (BW) $\leq$2.5 kg or $\geq$3.0 kg and gestational age between 38 and 40 wk; no family history or clinical signs of hypertension, cardiovascular disease, diabetes, or renal or chronic illness; no vitamin supplementation; and adequate amounts of blood or urine for evaluation of the biomarkers of oxidative stress and antioxidant status. A total of 65 children meeting these criteria were eligible to participate in this cross-sectional study. During enrollment, body weight, height, serum lipids, UA, and blood pressure levels were measured using methods whose details were described elsewhere (8). Children were divided into two groups according to their BW: 1) the AGA group, consisting of 27 children who had been born at term with appropriate BW (BW $\geq$3.0 kg) and 2) the SGA group, consisting of 38 children who had been born at term with small BW for gestational age (BW $\leq$2.5 kg). Cut-off points for classification of BW were recommended by the World Health Organization (13) and details of BW data validation have been previously published (14). The anthropometric indicators used to assess child nutritional status were height-for-age (HAZ), weight-for-age (WAZ), and BMI-for-age (BMIZ). The values were compared with the standards of the National Center for Health Statistics (NCHS, 2000) and were expressed as a Z-score (Epi Info Software Program, Version 3.3.2, Center for Disease Control). Hypertension was defined in accordance with the Fourth National Task Force on High Blood Pressure in Children and Adolescents (15). The study was approved by the Research Ethics Committee of the Federal University of São Paulo and informed consent was obtained from one of the parents of children enrolled in the study.

Blood and urine collection for oxidative and antioxidant biomarkers. Blood samples were centrifuged (1500× *g*, 5 min, 4°C) and the erythrocytes were washed three times with NaCl 0.9%. Aliquots of heparin plasma and lysate erythrocytes were stored at −80°C until assay. Urine samples (15 mL) were collected into polyethylene bottles; they were kept refrigerated during the collection period, after which they were immediately aliquoted and stored at −80°C until assay.

Plasma and erythrocyte assays of antioxidant enzymes. Plasma TAS was measured using the Calbiochem total antioxidant status assay kit (Novabiochem, San Diego, CA) according to the manufacturer's instructions. The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS+ by metmyoglobin. SOD activity was measured in erythrocytes using the Calbiochem assay kit (Novabiochem) based on the method developed by Nebot *et al.* (16). Erythrocyte GPx activity was measured by using a GPx-340 assay kit (Bioxytech, OXIS International, Portland, OR) as previously described by Paglia and Valentine (17). Hb concentration was measured to obtain erythrocyte SOD and glutathione concentration values in U/gHb.

Determination of lipid peroxidation parameters. Erythrocyte TBARS content was measured according to Ohkawa *et al.* (18). TBARS concentrations were expressed as nmol/gHb using 1,1,3,3-tetraethoxypropane as a standard. Urine concentrations of F_2-isoprostane (8-Epi-$PGF_{2\alpha}$) were determined using an enzyme immunoassay kit based on the method developed by Roberts and Morrow (19) (Bioxytech, OXIS International). Urinary content of F_2-isoprostane was indexed to creatinine and expressed as ng/mg of creatinine.

Statistical analysis. Categorical variables were compared using the χ^2 test. Comparisons of continuous variables were performed using the *F* statistic. Pearson's coefficients and stepwise forward multiple linear regression analyses of the overall cohort were used to investigate associations between antioxidant or pro-oxidative parameters and other variables. Values of continuous variables were expressed as mean ± SEM. Statistical tests were two-tailed and the significance level was set at $p < 0.05$.

RESULTS

Baseline characteristics. Clinical, anthropometric, and metabolic characteristics of the children are shown in Table 1. No difference was observed in the anthropometric evaluation, and, with exception of the serum UA level, the groups did not differ significantly with respect to the laboratory parameters. The mean UA value was significantly greater in SGA compared with AGA children [means difference: 1.2 mg/dL, 95% confidence interval (CI): 0.8 to 1.6 mg/dL], and no race ($p = 0.207$) or gender ($p = 0.907$) differences in UA levels were observed in the entire cohort. Mean SBP was higher in SGA than in AGA children (means difference: 8.9 mm Hg, 95% CI: 2.7 to 15.0 mm Hg), and mean diastolic BP levels were similar in both groups. There were no significant race ($p = 0.291$) or

Table 1. *General and biochemical characteristics of the population*

Variables	AGA $n = 27$	SGA $n = 38$	p
Gender (%)			0.573
Boys (%)	59	50	
Girls (%)	41	50	
Race			0.495
White (%)	44	34	
Black (%)	56	66	
Birth weight (g)	3171.9 ± 32.8	2373.3 ± 27.4	0.001
Gestational age (wk)	39.2 ± 0.2	39.1 ± 0.1	0.422
Age (y)	9.38 ± 0.4	9.61 ± 0.3	0.476
Weight (kg)	28.2 ± 1.5	28.1 ± 1.2	0.661
Height (cm)	133.6 ± 2.4	133.9 ± 1.8	0.672
WAZ	-0.98 ± 0.20	-0.99 ± 0.15	0.926
HAZ	-0.74 ± 0.19	-0.76 ± 0.15	0.862
BMIZ	-0.97 ± 0.23	-0.89 ± 0.15	0.740
Cholesterol (mg/dL)	156.2 ± 5.4	152.2. ± 4.9	0.938
LDL-c (mg/dL)	93.7 ± 5.0	94.7 ± 4.0	0.702
HDL-c (mg/dL)	51.0 ± 2.5	53.3 ± 2.3	0.486
Uric acid (mg/dL)	3.0 ± 0.1	4.2 ± 0.2	0.001
Mean systolic BP (mm Hg)*	98.5 ± 2.6	107.4 ± 2.0	0.001
Mean diastolic BP (mm Hg)*	63.6 ± 2.4	68.2 ± 2.0	0.122

Values expressed as means ± SEM or percentage. LDL-c, low-density-lipoprotein cholesterol; HDL-c, high-density-lipoprotein cholesterol.

* Average of two measurements made at 2-min intervals.

Table 2. *Oxidative stress markers in AGA and SGA children*

	AGA $n = 27$	SGA $n = 38$	p
Antioxidant defenses			
Erythrocyte superoxide dismutase activity (U/g Hb)	1737.2 ± 121.6	2749.8 ± 181.5	0.001
Erythrocyte glutathione peroxidase activity (U/g Hb)	64.1 ± 4.0	73.6 ± 3.9	0.356
Plasma total antioxidant status (mmol/L)	1.3 ± 0.1	1.1 ± 0.1	0.628
Pro-oxidants			
Erythrocyte TBARS (nmol/g Hb)	215.8 ± 10.5	286.7 ± 16.9	0.002
Urinary F_2-isoprostanes (ng/mg creatinine)	29.1 ± 3.1	45.9 ± 4.6	0.006

Values expressed as mean ± SEM.

gender ($p = 0.096$) differences in the mean SBP levels in the whole study group.

Evaluation of antioxidant activity. Our results showed that erythrocyte GPx activity and plasma concentration of the TAS did not differ between AGA and SGA children (Table 2). For the entire cohort, no correlation was found between BW and TAS ($r = 0.03$, $p = 0.810$) or GPx ($r = 0.04$, $p = 0.720$). On the other hand, activity of the SOD enzyme was significantly higher in the SGA than in the AGA group (means difference: 1012.6 U/g Hb, 95% CI: 584.3 to 1440.9 U/g Hb) (Table 2). No significant race ($p = 0.968$) or gender ($p = 0.481$) differences in the mean SOD levels in the whole study group were observed. For the entire cohort, erythrocyte SOD activity correlated significantly with BW ($r = -0.453$, $p < 0.001$), whereas adjustment for race, gender, age and BMIZ did not significantly modify the strength of this association ($r = -0.375$; $p = 0.003$).

Evaluation of oxidative stress indices. Erythrocyte TBARS and urinary F_2-isoprostane levels were higher in SGA than in AGA children (Table 2) (means difference: 70.9 nmol/g Hb, 95% CI: 29.1 to 112.7 nmol/g Hb; and 16.8 ng/mg creatinine, 95% CI: 6.3 to 27.3 ng/mg creatinine, respectively). No statistical differences were found in gender ($p = 0.666$) or race ($p = 0.790$) in the TBARS levels. However, we observed significantly greater levels of F_2-isoprostanes in black children compared with white children (44.4 ± 4.3 ng/mg creatinine *versus* 27.8 ± 2.9 ng/mg creatinine, respectively, $p = 0.009$). No difference was noted between males and females ($p = 0.177$) for this marker of lipid peroxidation. In addition, we investigated the correlation between LPO and BW and observed that levels of both TBARS ($r = -0.342$, $p = 0.003$) and F_2-isoprostanes ($r = -0.269$, $p < 0.03$) were inversely correlated with BW. After controlling for gender and race, these associations remained significant [TBARS ($r = -0.302$, $p = 0.004$) and F_2-isoprostanes ($r = -0.251$, $p = 0.050$)].

Relationship between tbars, F_2-isoprostanes, lipid profile, and uric acid. A correlation was found between TBARS and F_2-isoprostanes ($r = 0.401$, $p = 0.003$). This was present after adjusting for race, gender, and BW ($r = 0.376$, $p = 0.006$). We also observed that serum cholesterol levels were positively associated with levels of both TBARS ($r = 0.658$, $p < 0.001$) and F_2-isoprostanes ($r = 0.265$, $p = 0.038$). Adjustment for race, gender, BW, age, and BMIZ did not modify these associations [TBARS ($r = 0.797$, $p < 0.001$) and F_2-isoprostanes ($r = 0.304$, $p = 0.024$)]. Erythrocyte TBARS and LDL-c levels were significantly correlated ($r = 0.438$, $p < 0.001$), whereas UA concentration was positively associated only with F_2-isoprostane levels ($r = 0.295$, $p = 0.022$). Similar relationships were seen when we adjusted for race, gender, BW, age, and BMIZ [TBARS ($r = 0.464$, $p < 0.001$) and F_2-isoprostanes ($r = 0.315$, $p = 0.018$)]. Subsequently, in a multiple regression analysis testing BW, age, BMIZ and blood lipids as independent variables, only cholesterol ($\beta = 2.533$; SE = 0.226; $p < 0.001$) and BW ($\beta = -0.075$; SE = 0.015; $p < 0.001$) showed significant associations with levels of TBARS in our population. On the other hand, when the dependent variable was F_2-isoprostanes, only BW was a significant predictor ($\beta = -0.0168$; SE = 0.006; $p = 0.010$) even after including age, BMIZ, UA, and blood lipids in the model.

Association between lipid peroxidation and systolic blood pressure. We investigated the relationship between LPO and systolic blood pressure. Controlling for gender and race we found that TBARS correlated positively with systolic blood pressure levels ($r = 0.243$, $p = 0.040$) for the entire cohort. In contrast, F_2-isoprostanes were not significantly correlated with this clinical parameter ($r = 0.593$, $p = 0.658$). In a multiple regression analysis, systolic blood pressure was significantly associated with BW ($\beta = -0.0120$; SE = 0.003; $p = 0.001$) and gender ($\beta = 8.609$; SE = 2.949; $p = 0.005$), whereas race, age, BMIZ, UA, blood lipids, and TBARS did not reach statistical significance.

DISCUSSION

It has been proposed that events occurring before birth may influence the risk of developing chronic cardiometabolic diseases in later life, notably hypertension and diabetes (1–4). Oxidative stress is a common feature of these pathogenic conditions and can be the key link between prenatal growth and increased morbidity later in life. This study was undertaken to test the hypothesis that oxidative imbalances are associated with birth weight in 8- to 13-y-old SGA children. Our results revealed elevated concentrations of LPO end products in blood and urine samples of SGA children, indicating that oxidative stress is identified a decade after insults during fetal life have occurred.

The body has developed a complex defense strategy to minimize the damaging effects of various oxidants. Central to this defense are the antioxidant enzymes of the blood, which include SOD, GPx, and catalase. Previous studies demonstrated that the activity of SOD, catalase, and glutathione were lower in cord blood of the SGA newborn, indicating the presence of a deficient antioxidant defense mechanism (7). This does not appear to occur in children with SGA since we observed that both GPx activity and TAS levels were unchanged. This discrepancy might be due to differences in efficiency of the antioxidant mechanisms existing in newborns and children. The Po_2 *in utero* has been found to range between 11 and 60 mm Hg. These values correspond to approximately 1–9% oxygen (compared with atmospheric conditions of 21% oxygen), indicating that the earliest stages

of development take place under a low oxygen concentration (20). Therefore, immediately after birth humans are exposed to a higher oxygen pressure, promoting increased activation of the antioxidative pathways. In fact, Asikainen *et al.* (21) reported that expression of SOD mRNA in the lung and liver are very low in fetuses and increases progressively toward adulthood. Neonates have low antioxidant defenses in both their extracellular and intracellular pathways (20). Thus, it is possible that defense systems against free radicals in newborns are insufficient. On the other hand, we also found that erythrocyte SOD activity is significantly elevated in SGA children. Whatever the explanation for this current finding, it suggests an important compensatory mechanism that protects these children against the excess production of superoxide radicals. In fact, this protective phenomenon can be observed in some disorders characterized by the presence of oxidative stress like type I and type II diabetes (22,23). However, longitudinal studies are needed to confirm whether this adaptive mechanism observed in the current study really occurs in SGA children.

LPO is a part of normal metabolism and its increased levels are thought to be a consequence of oxidative stress. This is a process whereby unsaturated fatty acids, phospholipids, glycolipids, cholesterol esters, and cholesterol itself are peroxidized by free radical-mediated reactions and results in cell damage (24). TBARS concentrations in erythrocytes represent an index of free radical attack with potentially cytotoxic consequences because oxidized lipids have wide-ranging effects on membrane function and cell metabolism (12). On the other hand, isoprostanes are formed in the phospholipid domain of cell membranes; they reflect lipid peroxidation and are excreted in the urine (11). In the current study, TBARS and F_2-isoprostanes showed an evident increase in SGA children. For the entire cohort, we observed a positive association between these oxidative biomarkers, suggesting that they are responding to a common stimulus or are otherwise physiologically linked. Moreover, we also found a significant inverse correlation between birth weight and both TBARS and F_2-isoprostanes in race- and sex-adjusted models. Other investigators have noted the presence of LPO only among SGA neonates or during adverse prenatal life (6–9) but not in SGA children. It is unclear how fetal programming augments LPO in children a decade later. From the present study, it is difficult to infer the possible mechanisms involved in the oxidative stress induced by fetal growth restriction. The "fetal programming" hypothesis proposes that the fetus adapts to an adverse intrauterine life to ensure survival, favoring the development of some organs over that of others, thus promoting long-range effects through childhood and into adult life (1,2). Moreover, reprogramming of the redox state has been proposed as a key adaptation enabling the fetus to survive in a limited nutrient environment (25). Further studies are warranted to assess the importance of this hypothesis and its subsequent correlation with morbidity in later life and to elucidate the mechanisms involved in this oxidative damage promoted by fetal programming.

Noteworthy were the data regarding the race-specific differences in urinary isoprostanes concentration. The present study revealed that isoprostane levels were significantly higher in black children than in white children. The reason for race-specific differences in urinary isoprostanes could not be identified in this study, but some reports have demonstrated that in acute hyperlipidemia, higher F_2-isoprostane levels are found in African Americans than in Caucasians (26). In addition, higher levels of the polyunsaturated fat eicosapentaenoic acid are detected in patients of African origin than in Caucasians, contributing to increases in lipid hydroperoxide concentration (27). It was also reported that cultured endothelial cells derived from African American patients release greater quantities of superoxide and peroxynitrite radicals compared with Caucasians, suggesting an inherent basis for the racial differences in redox metabolism (28). Environmental factors, race differences in the distribution of genetic polymorphisms, or both could contribute to differences observed in isoprostane levels.

Lipid abnormalities and oxidative modification of LDL-c may contribute to increased rates of LPO reactions (29). The findings of our study support a direct relationship between these variables since we found that total cholesterol levels were positively correlated with both TBARS and F_2-isoprostanes. Moreover, we also observed significant association between TBARS and LDL-c concentrations; adjustment for BW, race, gender, age, and BMIZ did not modify the strength of this association. The results of multiple regression analysis indicate that BW and cholesterol were significantly and independently related to TBARS in the combined study population. These findings suggest that oxidative damage that can be attributed to lipid peroxidation occurs to a greater degree in the context of low birth weight.

Another surprising feature of this study was the positive correlation between F_2-isoprostanes and UA levels that was observed in the entire cohort. An important consideration is how the increase in UA levels should be converged with lipid peroxidation. UA is known to act as an antioxidant and may be one of the strongest determinants of plasma antioxidative capacity (30). On the other hand, under determined pathologic circumstances this antioxidant paradoxically becomes prooxidant. This is demonstrated through its direct oxidation by oxygen radicals, chemical oxidation with concomitant formation of allantoin, oxonic/oxaluric acid, and parabanic acid, and urate anion free radical formation through a one-electron redox process (30).

There is evidence that increased UA levels could have pro-oxidant effects and hence promote lipid oxidation in vascular tissue. It has been suggested that urate anion reacts with a polyunsaturated fatty acid leading to the formation of a lipid radical that, in the presence of molecular oxygen, would generate a lipid peroxyl radical, thus propagating LPO within the LDL-c particle (31). Although it is unknown whether circulating LDL-c might represent a source of augmented urinary F_2-isoprostanes, studies have demonstrated that F_2-isoprostanes is formed in LDL-c when it is oxidized *in vitro* (31).

Finally, we found that systolic blood pressure was marginally and positively associated with TBARS levels in race- and gender-adjusted models, but not in a multivariable stepwise model where BW as well as age emerged as significant predictors of blood pressure. A number of experimental stud-

ies have found strong positive association between a marker of oxidative stress and hypertension. However, these relationships may only apply to certain hypertensive states such as those associated with high renin levels or salt sensitivity (32,33). On the other hand, in human beings the determinants of blood pressure are likely multifactorial, thus the specific proportion of cases with oxidative stress–mediated hypertension may be too small to drive a significant association between these variables.

The community-based sample and adjustment for socioeconomic status and other confounders strengthen the findings of our investigation. Potential limitations of our study include the relatively small sample size of children studied and the predominant black race among SGA children that limit the generalizability of our results to other populations.

In summary, the current study revealed that there is evidence of oxidative stress in SGA children as evidenced by increased lipid peroxidation. In contrast, increases in erythrocyte SOD activity was also seen in these children. Although our study did not allow us to identify a mechanism for the increased oxidative status found in children a decade after the insult caused by adverse fetal life, these observations contribute to a better understanding of the link between birth weight and later development of disease and has implications in determining health risks in children.

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