NADPH oxidase and enhanced superoxide generation in intrauterine undernourished rats: involvement of the renin–angiotensin system

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Abstract

Objective: We previously reported that intrauterine undernutrition increased the oxidative stress by decreasing superoxide dismutase activity. In the present study, we tested whether NADPH oxidase, xanthine oxidase, cyclooxygenase or nitric oxide synthase are responsible for the increased O₂ generation observed in rats submitted to intrauterine undernutrition. In addition, we investigated the effect of angiotensin II (ANG II) on O₂ production via activation of NADPH oxidase.

Methods: Female pregnant Wistar rats were fed either normal or 50% of the normal intake diets, during the whole gestational period. At 16 weeks of age, the rats were used for the study of intravital fluorescence microscopy; microvascular reactivity, local ANG II concentration and AT₁, p22phox and gp91phox gene expression. In this study only the male offspring was used.

Results: Treatment of mesenteric arterioles with the xanthine oxidase inhibitor oxypurinol, the nitric oxide synthase inhibitor L-NAME or the cyclooxygenase inhibitor diclofenac did not significantly change superoxide production. Thus, these vascular sources of superoxide were not responsible for the increased superoxide concentration. In contrast, treatment with the NADPH oxidase inhibitor apocynin significantly decreased superoxide generation and improved vascular function. On the other hand, intrauterine undernutrition did not alter the gene expression for p22phox and gp91phox. The fact that the local ANG II concentration was increased and the attenuation of oxidative stress by blocking AT₁ receptor with losartan, led us to suggest that ANG II induces O₂ generation in intrauterine undernourished rats.

Conclusion: Our study shows that NADPH oxidase inhibition attenuates superoxide anion generation and ameliorates vascular function in rats submitted to intrauterine undernutrition. Although it is not clear which mechanisms are responsible for the increase in NADPH oxidase activity, a role for ANG II-mediated superoxide production via activation of NADPH oxidase is suggested.

Keywords: Angiotensin; Endothelial function; Gene expression; Hypertension; Nutrition

1. Introduction

Several experimental and clinical studies support the hypothesis that intrauterine undernutrition can contribute to the development of coronary heart disease, diabetes type II and hypertension in adult life [1,2]. In previous studies, we have shown that intrauterine undernutrition induces hypertension, impairs endothelial nitric oxide synthase (eNOS) gene expression/activity and decreases endothelium-dependent relaxation in adult offspring [3]. On the other hand, the response to sodium nitroprusside was not altered indicating a specific impairment of endothelium dependent dilation and excluding changes in the response of the smooth muscle cells. In fact, recent human studies have associated the intrauterine undernutrition with impairment of endothelium function in 9–11-year-old children [4] and young adult [5].

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The mechanisms whereby intrauterine undernutrition exerts its effects on the endothelium are not completely understood. In recent study, we demonstrated that the impairment of endothelium function after intrauterine undernutrition is partially due to an increased oxidative stress [6]. However, the source of reactive oxygen species (ROS) remained to be determined. Studies have identified a variety of intracellular sources of free radicals that include xanthine oxidase, nitric oxide synthase (NOS), cyclooxygenase (COX) and NADPH oxidase [7–9]. The xanthine oxidase reduces molecular oxygen leading to the production of both superoxide and hydrogen peroxide [10].

eNOS can produce considerable amounts of superoxide in the absence of sufficient tetrahydrobiopterin [11], whereas, iNOS-dependent superoxide generation has been shown only for the L-arginine-depleted enzyme [12]. The cyclooxygenase pathways generate superoxide by interaction of NADPH with intermediate radical form of the enzyme associated with the conversion of prostaglandin G₂ to prostaglandin H₂ [13]. Finally, the most important source of superoxide in vascular cells is NADPH oxidase [14,15]. This enzyme utilizing intracellular NADH or NADPH transfers electrons across the membrane to extracellular oxygen [8]. Previously, workers at our laboratory found that intrauterine undernutrition promotes increased superoxide anion (O₂⁻) concentration and that treatment with vitamins C and E normalizes O₂⁻ and hypertension in offspring [16]. In the present study, we tested whether NADPH oxidase, xanthine oxidase, COX or NOS are responsible for the increased O₂⁻ observed in rats submitted to intrauterine undernutrition. In addition, we investigated the effect of angiotensin II (ANG II) on O₂⁻ production via activation of NADPH oxidase.

2. Methods

All procedures used in this study were approved and performed in accordance with guidelines of the Ethics Committee of the Institute of Biomedical Sciences, University of São Paulo and conformed 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). Wistar rats from our colony (Laboratory of Hypertension, Institute of Biomedical Sciences, University of São Paulo) were maintained in a room at 22 ± 1 °C with a 12-h light cycle and 60% humidity.

2.1. Feeding protocol

Timed mating was performed in female Wistar rats (age range 9–11 weeks). To assess the stage of estrus of the females, vaginal smear was checked prior to introducing the males. Two females were mated to one male. A total of 12 adult virgin female Wistar rats were used to generate the 65 males used in the study. Day 1 of pregnancy was determined by the presence of spermatozoa in the vaginal smear. Following confirmation that mating had occurred, the females were housed individually in standard rat cages and randomly divided into two groups: control (n = 4), fed standard chow ad libitum (diet: protein 22%; carbohydrates 43.5%; fat 4.2%; cellulose 8%; minerals 10%; water 12.5%; plus salt and vitamin mixtures) and restricted group (n = 8), fed 50% of the ad libitum intake, determined by the amount of food consumed by the control group from day 1 of pregnancy until parturition (23 days). Following parturition, both groups of offspring received food ad libitum. In order to prevent any variation in neonatal growth through availability of milk intake during suckling, litter size was standardized to 8 pups at day 1. At 16 weeks of age, offspring of nutritionally restricted dams, named restricted, and offspring of control dams, named control groups were used for the study of intravital fluorescence microscopy; microvascular reactivity; local ANG II concentration and AT₁, p22phox and gp91phox mRNA expression. In this study only the male offspring was used.

2.2. Measurement of systolic blood pressure

Systolic blood pressure was determined in conscious rats by an indirect tail-cuff method (pneumatic transducer, PowerLab 4/S, AD Instruments). Rats were preheated at 37 °C for 5 min, and then three stable consecutive measurements of blood pressure were averaged. Care was taken in selecting an appropriate cuff size for each animal.

2.3. Intravital fluorescence microscopy

Reactive oxygen species generation in mesenteric arteriole wall was determined according to the modification of the method described by Suzuki et al. [17]. Rats were anesthetized with chloral hydrate (400–450 mg/kg, s.c.), and the mesentery was set for microscopic observation in situ [18]. The preparation was kept at 37 °C and continuously superfused (1.0 ml/min) with a Krebs–Henseleit bicarbonate-buffered solution saturated with 95% N₂ and 5% CO₂ gas mixture to minimize the production of oxygen free radicals by exposure of the tissue. Special precautions were taken to avoid interruption of the suffusion solution on the tissue because even superficial drying causes rapid cell injury. Blood vessels were classified according to their branching order beginning at the capillary level and reaching up to the arteriolar side. The smallest, precapillary arterioles were classified as A4, fed by the terminal arterioles (A3) branching from large arterioles (A2). Single unbranched A2 arterioles (15–25 µm) were selected for this study.

The mesenteric microcirculation was visualized through an intravital microscope (Axioscopic, Zeiss, Germany) with a 20× water immersion objective lens (Zeiss, Germany) by using a digital color charge-coupled device (CCD) camera (ZVS-47EC, Zeiss, Germany). To elicit fluorescent...
images, the preparation was illuminated with a 200-W mercury lamp. The light was passed through a fluorescence microscope attachment with quartz collector, excitation filter (490 nm, Zeiss, Germany) for epi-illumination, and a band-pass filter (590 nm, Zeiss, Germany). During the intervening periods, the shutter for the excitation light was kept closed. The fluorescent images were recorded by a computer system (KS-300, Kontron, USA) for later analysis. Transilluminated images were also recorded immediately before the fluorescence images.

After an initial 30-min stabilization period, when the mesenteric preparation was superfused with a standard buffer, a background auto-fluorescence image in the selected tissue area was recorded and stored in the memory of the computer. The preparation was then superfused with a buffer solution containing hydroethidine (HE: 5.0 µmol/l, Polysciences, USA) for 60 min. The number of nuclei labeled with ethidium bromide (EB-positive nuclei) along arterioles (NEB) was determined at each 15 min after the onset of HE superfusion. At the end of the experiments, the tissue was superfused with absolute ethanol for 5 min followed by EB superfusion to establish the total number of nuclei along the vessel wall (NT). The EB-positive number was counted as a double blind and expressed as a percentage of EB-positive nuclei = (NEB/NT) x 100 (%).

To evaluate the source of superoxide generation, the mesenteries from restricted group were treated with diclofenac (COX inhibitor, 10⁻⁵ M), l-NAME (NOS inhibitor, 10⁻³ M), oxyurinol (xanthine oxidase inhibitor, 10⁻⁶ M), apocynin (NADPH oxidase inhibitor, 10⁻⁴ M) or losartan (AT₁ receptor antagonist, 10⁻³ M). These concentrations were selected based on previously reported inhibitory activity.

2.4. Vascular reactivity in vivo in mesenteric microvessels

The mesentery was exteriorized and arranged for microscopic observation in situ according to the method of Zweifach [18]. The animals, under choral hydrate anesthesia (400–450 mg/kg s.c.) were kept on a special board, heated at 37 °C, which included a transparent plate on which the tissue to be transilluminated was placed. The mesentery was kept moist and warmed by irrigating the tissue with warmed (37 °C) Ringer–Locke solution (pH 7.2–7.4) containing 1% gelatin. The composition of the solution was (mM): NaCl 154.0, KCl 5.6, CaCl₂·2H₂O 2.0, NaHCO₃ 6.0, and glucose 5.0. The rate of outflow of the solution onto the exposed tissue was controlled to maintain the mesentery in continuous contact with a film of the liquid. Drugs, dissolved in Ringer–Locke solution, were topically added to the preparations in a standard volume of 0.01 ml.

In a series of experiments a 500-line television camera was incorporated to a tri-ocular microscope to facilitate observation of the enlarged image (3400×) on the video screen. An image-splitter micrometer was adjusted to the phototube of the microscope, as described by Baez [19]. The image-splitter sheared the optical image into two separate images and displaced one with respect to the other. By rotating the image-splitter in the phototube the shearing was maintained in a direction at right angles to the axis of the vessel. The displacement of one image from the other allowed measurement of the vessel diameter. The accuracy of the measurement is of the order of 0.1 μm [19]. Second order arterioles was distinguished on morphological grounds. Changes in vessels diameter were measured following the application of vasoactive drugs: acetylcholine (2x10⁻³ M), bradykinin (3x10⁻⁶ M) and apocynin (10⁻⁴ M). Drugs were removed by washing out with the warmed Ringer–Locke solution. For each animal, at least three different vessels were used for diameter estimation.

2.5. Reverse transcriptase-polymerase chain reaction (RT–PCR)

Rat mesenteries were dissected, frozen in liquid nitrogen, and stored at −70 °C. Total cellular RNA was isolated from the aortas using TRizol reagent (Gibco BRL, Life Technologies, Rockville, MD, USA). RNA (2 μg) was used for first-strand cDNA synthesis with AMV reverse transcriptase (RT) and oligo(dT) as primer. Polymerase chain reaction (PCR) amplifications were carried out on a portion of the cDNA produced using specific oligonucleotide primers for AT₁ receptor, p22phox, gp91phox and GAPDH (Table 1). GAPDH was used as an internal control for the co-amplification. In order to identify the optimal amplification conditions, a series of pilot studies were performed using a thermal cycler with temperature gradient (Eppendorf Mastercycler gradient; Eppendorf–Netheler–Hinz, Hamburg, Germany) at the annealing step, various amounts of RT products from 2 to 200 ng RNA, and 26–40 cycles of PCR amplification. PCR products were resolved on an 1% agarose gel containing ethidium bromide 0.5 µg/ml. The gel was subjected to ultraviolet light and photographed. The band intensities were measured using a software package (Kodak Digital Science, Eastman Kodak, New Haven, CT, USA) and the signals were expressed relatively to the intensity of the GAPDH amplicon in each co-amplified sample.

2.6. ANG II quantification by high performance liquid chromatography (HPLC–UV)

ANG II was measured using reverse phase chromatography coupled with ultraviolet detection (214 nm) as described by Monte et al. [20]. Fast isocratic separation was obtained using an Aquapore OD 300 column, 7 µm (4.6×250 mm) (Applied Biosciences, USA) equilibrated with 0.1% phosphoric acid in 5% acetonitrile. The peptides
Table 1  
Oligonucleotide primers used in study

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were initially separated by isocratic elution during 5 min followed by a linear gradient from 5 to 35% Acetonitrile in 0.1% phosphoric acid during 20 min under a flow rate of 1.5 ml/min. The tissues were previously homogenized in 8 ml 0.1 M sodium phosphate buffer containing 0.34 M sucrose and 0.3 M NaCl (pH 7.2), and ANG III (320 ng) was added to each sample as internal standard. The ANG II extraction was made using chromatography in column Sep-Pak-C_{18} (Millipore, USA). The column was activated with the following steps: 5 ml methanol, 5 ml tetrahydrofuran, 5 ml hexane, 5 ml methanol and 10 ml H_{2}O (MilliQ). After the activation, the samples were submitted to the column and eluted with the following steps: 10 ml H_{2}O, 5 ml 4% acetic acid and 5 ml ethanol/acetic acid/H_{2}O (90:4:6, v/v). The peptides were eluted in the last phase, which was evaporated to dryness in a Speed Vac SC 110 (Savant Instruments, Holbrook, NY, USA). Sample extracts were reconstituted with 500 μl 0.1% phosphoric acid in 5% acetonitrile, filtered and injected onto the analytical column of the HPLC system. Retention time was used to identify peaks of interest, previously determined by the elution of standard peptides. The calculations were based on peak area, and ANG II concentration was expressed as ng/g of tissue.

2.7. Drugs

The following drugs were used: hydroethidine (Polysciences, USA); ethidium bromide (Gibco, USA); losartan (MSD-Merck); diclofenac (Geigy); oxypurinol; chloral hydrate; apocynin, acetylcholine; bradykinin; L-NAME (Sigma/Aldrich, USA).

2.8. Statistical analysis

Data are presented as mean±S.E.M. Statistical analysis was performed using one-way ANOVA or t-test when appropriate (SigmaStat, version 2.0, Jandel Scientific Software). Values were considered statistically significant when P<0.05.

3. Results

At birth, the body weight in offspring exposed to intrauterine undernutrition was clearly lower (4.1±0.4 g; n=39; P<0.05) than in control group (7.9±0.3 g; n=16). Systolic blood pressure increased significantly in restricted (137±5.2 mmHg; n=39; P<0.05) compared with control group (115±2.3 mmHg; n=16).

3.1. Role of COX, NOS, xanthine oxidase and NADPH oxidase in superoxide generation

Superoxide anion concentration was higher in mesenteries from restricted than control group (Fig. 1). There were no changes in the number of EB-positive nuclei after

![Graph showing the time course of EB-positive nuclei in control and restricted groups.](image-url)
diclofenac (COX inhibitor), L-NAME (NOS inhibitor) or oxypurinol (xanthine oxidase inhibitor) treatments (Fig. 2A, B and C, respectively), demonstrating that there was no correlation between O$_2^*$ generation and these sources of oxidative stress in vascular wall. However, the enhanced number of EB-positive nuclei observed in restricted rats was significantly attenuated by treatment with the NAPDH oxidase inhibitor apocynin (Fig. 2D), suggesting that NADPH oxidase is involved in the overproduction of superoxide in restricted rats.

3.2. Role of NADPH oxidase in vascular reactivity

There were no differences in the baseline diameter between the control and restricted group (20.00±0.98 vs. 19.66±0.39 μm). The magnitude of acetylcholine (Fig. 3A) and bradykinin (Fig. 3B) responses was significantly less in restricted compared to control group. Topical application of apocynin did not alter the responses to acetylcholine and bradykinin in the control rats. In contrast, topical application of apocynin significantly im-
proved the response to both agents in the restricted group (Fig. 3A and B).

3.3. Role of AT1-receptor blockade in superoxide generation

Although we did not find an effect of AT1-receptor blockade on superoxide generation in control group (data not shown), the enhanced number of EB-positive nuclei observed in restricted rats was significantly attenuated by treatment with the AT1 receptor blockade losartan (Fig. 4). This finding suggests that ANG II induces O$_2^-$ generation in restricted group.

3.4. AT1, p22$^{phox}$ and gp91$^{phox}$ mRNA expression

RT-PCR analyses of mesenteric AT1, p22$^{phox}$ and gp91$^{phox}$ in restricted and control groups are shown in Fig. 5A, B and C, respectively. No changes in mRNA expression for these genes could be found in restricted compared with control group.

3.5. ANG II levels measurement

As illustrated in Fig. 6, ANG II concentration was augmented in mesenteric arteriolar beds from the restricted in comparison to the control group.
Fig. 5. Representative RT-PCR products of 2 μg cDNA extracted from mesenteries of control (open bars) and restricted (solid bars) groups. The bars graphs show the relative optical density values of AT₁ (A); p22^{phox} (B); and gp91^{phox} (C) bands obtained from the different groups. Values were normalized by the corresponding RT-PCR products for GAPDH, used as the internal control. Values are expressed as mean±S.E.M. and are representative of six experiments.

4. Discussion

In the present study, we provide strong evidence that NADPH oxidase is involved in the superoxide generation in mesenteric arterioles of intrauterine undernourished rats. Furthermore, we found that inhibition of NADPH oxidase with apocynin improves endothelium-dependent vasodilatation observed in these rats. The restorative effect of this agent may be due in part to an increased NO bioavailability.

The methodology used to evaluate the sources of vascular oxidative stress is based on the oxidation of hydroethidine, a cell permeable non-fluorescent probe, to the fluorescent ethidium bromide (EB) by oxyradicals. Intracellularly, in the presence of oxyradicals, hydroethidine is rapidly converted to EB, which binds to DNA and is detected by its red fluorescent light. The extend of nuclei labeled with EB along arteriolar wall shows a quantitative estimate of oxidative stress in each vessel. In this methodology the hydroethidine oxidation to EB is caused more rapidly by superoxide anions than by others oxyradicals [21]. It has been demonstrated that superoxide is the most likely oxyradical involved in hydroethidine oxidation [22].

In the present study, inhibition of xanthine oxidase, NOS or COX did not significantly affect superoxide production in microvessels from intrauterine undernourished rats, suggesting that these pro-oxidative enzymes are not involved in the enhanced oxidative stress observed in these animals. On the other hand, inhibition of NADPH oxidase with apocynin attenuated the superoxide generation in microvessels from intrauterine undernourished rats. Corroborating these data, treatment of mesenteric arterioles with apocynin corrected the decreased endothelium-dependent relaxation induced by intrauterine undernutrition, demonstrating that NADPH oxidase plays an important role in the vascular superoxide generation that led to reduced vasodilatory response in these animals.

To better understand how intrauterine undernutrition...
interfered with NADPH oxidase, we investigated the gene expression of p22phox and gp91phox, two subunits of this enzyme. Although it has been demonstrated that gp91phox is not an essential compound of the vascular NADPH oxidase [23] the existence of gp91phox in blood vessels has been well documented in endothelial cells in culture [24–26]. In addition, in the gp91phox knockout mouse aorta an enhanced endothelium-dependent relaxation was observed [25], suggesting that the subunit participates in superoxide anion generation that limits NO bioactivity in vascular cells in situ. An overexpression of the p22phox has been linked to the increased superoxide generation in some pathological conditions, such as hypertension. In fact, Zalba et al. [27] demonstrated that p22phox is essential for superoxide anion generation in spontaneously hypertensive rats. However, in microvessels of intrauterine undernourished rats no alterations in expression of these two subunits were found. Therefore, activity and not lack of NADPH oxidase might be altered and contribute to the alterations observed.

Intrauterine undernutrition is associated with markedly alteration in renin–angiotensin system. In fact, increased angiotensin-converting enzyme activity is observed in intrauterine undernourished rats [28]. Sherman and Langley-Evans [29] demonstrated that the treatment with captopril decreased blood pressure levels in these rats. On the other hand, an association between NADPH oxidase activity and ANG II has been suggested. Rajagopalan et al. [30] demonstrated that this peptide induces superoxide generation in aortic rings of SHR by direct stimulation of NADPH oxidase. In addition, exposure of cultured vascular human endothelial cells to ANG II increased the activity of NADPH oxidase and the subsequent formation of reactive oxygen species [31]. Our data showing that superoxide generation was significantly attenuated after losartan treatment, led us to suggest that ANG II, via AT1 receptors, can stimulate superoxide production through activation of NADPH oxidase. To test this hypothesis gene expression of AT1 receptors was investigated. Since it was not altered in rats submitted to intrauterine undernutrition, we may exclude overexpression of this receptor as responsible for the increase in oxidative stress. However, intrauterine undernutrition markedly enhanced the local ANG II concentration. This allows us to suggest that ANG II is the main responsible for the increased activity of NADPH-oxidase that led to the increased generation of superoxide. The cause of the excess ANG II in intrauterine undernourished rats remained to be determined. However, studies in our laboratory are in progress to clarify this issue.

In summary, our study shows that NADPH oxidase inhibition attenuated superoxide anion generation and ameliorated vascular function in rats submitted to intrauterine undernutrition. Although it is not clear which mechanisms are responsible for the increase in NADPH oxidase activity, a decrease in superoxide anion generation after losartan treatment associated with an increased production of ANG II was observed, suggesting a role of ANG II-mediated superoxide production via activation of NADPH oxidase.

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