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Original Contribution

Intrinsic disturbance of cellular redox balance enhances blood lymphocyte apoptosis in the spontaneously hypertensive rat

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Abstract

The spontaneously hypertensive rat (SHR) shows an altered cell apoptosis rate compared to normotensive controls by a mechanism that may involve redox imbalance. This study was designed to determine whether cellular oxidative stress and apoptosis in blood lymphocytes are enhanced in the SHR by intrinsic cellular abnormalities and/or by factors derived from selected organs (kidney, adrenals). We identified apoptosis and free radical production in isolated peripheral blood lymphocytes with flow cytometry before and after serum withdrawal, which exposes the cells to a pro-oxidative condition. Freshly drawn SHR lymphocytes showed higher levels of apoptosis and intracellular oxygen radicals than lymphocytes from normotensive Wistar–Kyoto rats ($p < 0.05$). Apoptosis and intracellular oxygen radicals were profoundly elevated after serum-free incubation in SHR cells and to a lesser extent in normotensive controls suspended in exactly the same medium ($p < 0.01$, SHR vs normotensives). Cell-permeable antioxidants, L-cysteine and Tempol, attenuated serum deprivation-induced apoptosis as well as cellular oxidative stress. Blood plasma and organ extracts from the SHR exerted pro- or antiapoptotic activity to the same degree as those from normotensives. The results suggest that blood lymphocyte apoptosis is enhanced in the SHR due to intrinsically disturbed cellular redox balance rather than altered activity in extracellular humoral factors.

Keywords: Apoptosis; Hypertension; Lymphocyte; Oxidative stress; Free radicals

Increasing evidence suggests that enhancement and a possible imbalance of cell replication and apoptosis are the basis for the pathogenesis of the hypertensive disease. Proliferation of vascular smooth muscle cells results in medial thickening of small arteries [1]. Meanwhile, enhanced cell apoptosis in microvascular endothelial cells seems to promote the rarefaction of microvessels in hypertension [2]. These pathologic changes may enhance tissue hypoperfusion, leading to end-organ damage in hypertensives.

In hypertensive humans [3] and animal models, including spontaneously hypertensive rats (SHR) [1,4], genetically hypertensive mice [5], and Sprague–Dawley rats fed a high-salt diet [6], cell apoptosis is reported to be both increased and decreased in different cell types and at different ages compared to their normotensive controls, but the mechanism for an altered cell apoptosis rate is incompletely understood. The fate of cells, death or survival, can be determined by: (1) pro- or antiapoptotic extracellular stimuli such as exposure to tumor necrosis factor (TNF) super family members and cell-type-specific growth factors and (2) apoptosis-prone or -resistant intracellular milieu, e.g., expression of bcl-2 family proteins and cellular redox status.

We specifically explore the role of reactive oxygen species (ROS) as mediators of an apoptosis/proliferation imbalance in hypertension. Oxygen radicals modify redox-sensitive intracellular elements, such as mitogen-activated protein kinase [7], nuclear factor κB [8], mitochondria [9], and Bcl-2 family proteins [10], and may regulate mitogenesis and apoptosis. In the SHR, blockade of oxidative stress attenuates the endothelial cell apoptosis in microvessels [2].

Abbreviations: FSC, forward scatter; HE, hydroethidine; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PBMC, peripheral blood mononuclear cells; ROS, reactive oxygen species; SHR, the spontaneously hypertensive rat; SSC, side scatter; TNF, tissue necrosis factor; WKY, Wistar-Kyoto.

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Abnormalities both in extracellular humoral factors (raised catecholamine [11], angiotensin [12], and glucocorticoid levels [13]) and in intracellular components (increased xanthine- [14] and NADPH-oxidase activity [15] and/or reduced superoxide dismutase activity [16]) have been indicated to promote cellular oxidative stress, but it is still uncertain which factor (or several of them) is responsible for enhanced cellular oxidative stress in hypertension.

The primary aim of the present study is to investigate what mechanism may be involved in the alteration of cellular redox state and programmed cell death in the SHR, intrinsic cellular properties or extracellular environment. Using peripheral blood lymphocytes from hyper- and normotensive animals, we examine the apoptotic response to serum withdrawal, a simple method for predisposing the cells to oxidative stress [17–19]. The effects of blood plasma and organ extract on cell survival and redox state are also examined, because they might contain some humoral factors modulating cell apoptosis and oxidative stress. We demonstrate that intrinsic factors seem to dominate the oxidative stress and consequent apoptosis in SHR lymphocytes.

Materials and methods

All protocols were approved by the Animal Subject Committee of the University of California at San Diego. Ten- to sixteen-week-old male SHR, normotensive Wistar–Kyoto (WKY) rats, and their progenitor Wistar strain (Charles River Breeding Laboratories, Wilmington, MA, USA) were given general anesthesia (Xylazine, 10 mg/kg body wt im; Nembutal, 10 mg/kg body wt im). After separation of peripheral blood mononuclear cells (PBMC) from arterial blood samples, we identified apoptotic and oxidized fractions by flow cytometry with lymphocyte gating. Temporal changes in cell apoptotic levels and oxidative stress status during serum-free incubation were examined. In addition, blood plasma and tissue homogenates from kidney and adrenal gland, two important organs in hypertension, were collected from the SHR and WKY rats, and their effects on apoptosis and cellular redox state were evaluated using lymphocytes from Wistar rats. All reagents and chemicals were purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA) unless stated otherwise.

Blood sample preparation

Under general anesthesia, catheters (PE-50; Clay-Adams, Parsippany, NJ, USA) were introduced into the femoral artery and vein, to measure systemic arterial pressure and euthanize the animals after blood sample collection. Fifteen minutes after the cannulation, systemic arterial pressure was digitally recorded over 15 min (MacLab System, ADInstruments Pty Ltd., Colorado Springs, CO, USA) and mean values were computed. After that, 8 ml of arterial blood was gently withdrawn into a heparinized syringe. The whole blood was overlaid on 1.076 and 1.086 g/ml Percoll layers and centrifuged at 800*g* for 30 min at 4°C. Blood plasma, the top layer of the density gradients, was frozen in liquid nitrogen and stored in a −80°C freezer until ready for use. After aspiration of the lymphocyte/monocyte fraction from the interface of two Percoll layers, contaminating red cells were removed with cold lysis buffer (1.5 mol/L NH_4Cl, 0.14 mol/L $NaHCO_3$, and 0.63 mmol/L $EDTA_2Na$; pH 7.3). The cells were then washed twice (4°C, 500*g*, 5 min) with culture medium (123 mmol NaCl, 4.3 mmol KCl, 8.1 mmol Na_2HPO_4, 1.5 mmol KH_2PO_4, 1.3 mmol $CaCl_2$, 1.0 mmol $MgCl_2 \cdot 6H_2O$, 0.81 mmol $MgSO_4 \cdot 7H_2O$, 5.6 mmol glucose, 10 mg phenol red, 500 U heparin, and 1.0 g bovine serum albumin, per liter). A portion of the cells (1×10^5) was separated out for immediate apoptosis analysis; the remaining cells were transferred into six-well plates (1×10^5 cells/well) and incubated for 4 h at 37°C in a 5% CO_2 atmosphere. Water-soluble antioxidants L-cysteine (1 or 10 mmol/L) and Tempol (4-hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl, 1 or 10 mmol/L) were applied into selected wells in order to examine the involvement of oxidative mechanisms in cell apoptosis. The culture medium for the cells from Wistar rats, which was exactly the same medium as applied for WKY and SHR cells, was supplemented with blood plasma (10 vol%) or tissue homogenates (5 vol%) obtained from the SHR and WKY rats. The total volume of cell suspension in individual wells was adjusted to 6 ml with the above-mentioned serum-free medium.

Preparation of tissue homogenates

After blood sample collection, we euthanized the animals by intravenous injection (Nembutal, 120 mg/kg) and surgically excised kidneys and adrenals. The organs were minced into small fragments on ice and transferred into 2-ml tubes with the same weight of phosphate-buffered saline. The tissue was homogenized and spun down (10,000*g*, 10 min, 4°C), and the supernatant was stored in a freezer (−80°C).

Flow-cytometric analysis

Using a flow cytometer equipped with a 488-nm argon ion laser (FACSCalibur; Becton–Dickinson, San Jose, CA, USA), cell apoptosis and oxidative stress status of freshly isolated or incubated PBMC were analyzed. The green (FL1), orange (FL2), and red fluorescence (FL3) was collected through a 530/30, 585/42, and 650 long-pass filter, respectively. The cells were resuspended (2×10^4 in 100 μl of labeling buffer; 10 mmol/L HEPES, 140 mmol/L NaCl, and 2.5 mmol/L $CaCl_2$, pH 7.4), and exposed to annexin V (Alexa Fluor 488 conjugated; Molecular Probes, Inc., Eugene, OR, USA; 2 μl) and YO-PRO-1 (10 μmol/L; Molecular Probes, Inc.; 2 μl), agents that detect phosphatidylserine externalization or activation of cation channels on apoptotic cell membrane, respectively [20–22]. Although the two indicators may identify both apoptosis and necrosis [21,23], in our systems the cells labeled with those