Enhanced DNA fragmentation in the thymus of spontaneously hypertensive rats

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Suzuki, Hidekazu, Frank A. Delano, Neema Jamshidi, Dan Katz, Mikiji Mori, Kenjiro Kosaki, Roberta A. Gottlieb, Hiromasa Ishii, and Geert W. Schmid-Schönbein. Enhanced DNA fragmentation in the thymus of spontaneously hypertensive rats. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H2135–H2140, 1999.—The mechanisms contributing to organ injury in hypertension have been incompletely defined. The thymus gland of the spontaneously hypertensive rat (SHR) shows significant atrophy at the age of 15 wk compared with its normotensive control, the Wistar-Kyoto rat (WKY). The aim of the present study was to examine the thymus of SHR for evidence of DNA nicking as one of the mechanisms for thymic atrophy. SHR and WKY were subjected to adrenalectomy or sham surgery at 12 wk and studied at 15 wk. Adrenalectomy served to normalize the blood pressure in the SHR. DNA nicking was detected by in situ nick-end labeling (ISEL) of fixed tissue sections. Tissue sections were treated with proteolysis, and terminal deoxynucleobonucleotidyl transferase was used to incorporate biotinylated deoxynucleotides into DNA nick end in situ. DNA fragmentation was evaluated by measuring the level of released mono- and oligonucleosomes to the cytoplasm. A higher number of thymic ISEL-positive cells and a higher level of cytoplasmic mono- and oligonucleosomes were observed in SHR than in WKY. After adrenalectomy the enhanced level of ISEL and cytoplasmic mono- and oligonucleosomes in SHR was reduced to the level in WKY. Dexamethasone treatment (0.05 mg·kg⁻¹·day⁻¹) in WKY serves to decrease the thymus weight and significantly elevate the level of mono- and oligonucleosomes. Thus increased DNA fragmentation represents one of the mechanisms associated with thymic atrophy, a feature that reflects immune suppression in SHR.

in situ deoxyribonucleic acid nick-end labeling; adrenalectomy; glucocorticoid; apoptosis

A SUBSTANTIAL BODY of evidence has accumulated to suggest that the immune system may be compromised in many forms of hypertension. In the spontaneously hypertensive rat (SHR), several indexes of immune function are depressed (31). For example, in SHR the antibody response to sheep red blood cells is about one-tenth and the blastogenic responses of lymphocytes to phytohemagglutinin A and concanavalin A are less than one-fifth of those of normotensive Wistar-Kyoto rats (WKY). Furthermore, these mitogenic responses continue to be further reduced with age in the SHR. Delayed hypersensitivity, skin allograft rejection, and the ability of the T lymphocytes to cooperate with B lymphocytes in antibody production are also depressed (14, 31). A key feature in the SHR syndrome is the abnormal oversecretion of adrenal glucocorticoids (2, 11), hormones that are among the major determinants of apoptosis in several cell types, including thymocytes (21, 33).

Recently, Hamet et al. (10) reported a higher level of apoptosis in the heart of SHR than of WKY (10). The identification and quantification of apoptosis depend on the availability of suitable markers. Biochemical studies have suggested that the characteristic condensation of nuclear chromatin during apoptosis is the result of activation of endonucleases and formation of large and small chromatin fragments, sometimes of oligonucleosomal size (3, 33). Several groups have focused on the possibility of detecting DNA fragmentation in situ through end labeling of single- or double-strand DNA breaks with use of DNA polymerase or terminal deoxynucleobonucleotidyl transferase (TdT) (7, 8, 32). The latter appeared particularly promising, because double-strand breaks typically occur during apoptotic DNA fragmentation. As DNA ends are generated, TdT catalyzes template-independent addition of labeled deUTPs, which can be visualized using immunohistochemical stains. Residues of digoxigenin nucleotide are catalytically added to the DNA by TdT (19), an enzyme that catalyzes a template-independent addition of nucleotide triphosphate to the 3'-OH ends of double- or single-strand DNA. In addition, the photometric measurement of the levels of cytoplasmic mono- and oligonucleosomes (25) was also used in the present study to compare the level of DNA fragmentation biochemically in the thymus among groups. In the present study we have applied the in situ TdT-mediated dUTP-digoxigenin nick-end-labeling technique to the thymus tissue of SHR as well as WKY with or without adrenalectomy. Adrenalectomy serves to normalize blood pressure as well as oxygen radical formation in the SHR (26), suggesting that adrenal hormones may play a significant role in apoptosis.

MATERIALS AND METHODS

Animal preparation. All animal procedures were previously reviewed and approved by the University of California, San Diego, Animal Subject Committee. SHR and WKY (n = 41 each; Charles River Breeding Laboratories, Wilmington,
MA) were used at 13–14 wk of age. A cohort of SHR and WKY (n = 16 each) was subjected to bilateral adrenalectomy at 12 wk (27). The cohort of bilaterally adrenalectomized SHR and WKY was fed standard rat chow and water ad libitum (1, 26, 27, 29). The animals appeared alert and in good health. All experiments were carried out at the same time of the day (11:00 AM–2:00 PM). Completeness of the adrenalectomy was verified by postmortem examination of the suprarenal region. To explore the relationship of adrenal cortical secretions to the observed vascular sequelae, we resorted to adrenalectomy in SHR with and without replacement therapy.

Blood pressure measurement. The rats were maintained under general pentobarbital sodium anesthesia (40 mg/kg im) as part of the routine for in vivo microscopy, arranged on a heating pad, and covered with a blanket at 37°C. A catheter (PE-50 tubing, Clay Adams, Parsippany, NJ) was inserted into the femoral artery, and mean arterial blood pressures were measured for 30 min (35). The group average values of the mean arterial blood pressures in each animal are presented in Table 1.

Glucocorticoid treatment. A group of 18 WKY were used to examine the effect of exogenous glucocorticoid on thymic apoptosis. Six WKY received dexamethasone (Sigma Chemical, St. Louis, MO; 0.05 mg/kg body wt−1·day−1 in 0.1% ethanol sc) for 3 days, and four WKY were treated for 5 days. The remaining eight WKY received 0.1% ethanol alone for 3 days as controls. The thymus glands were excised and weighed. Thereafter, the thymus tissues were treated with lysis buffer for the photometric determination of cytoplasmic mono- and oligonucleosomes as an index of DNA fragmentation.

In situ nick-end labeling. Thymus glands were fixed with 1% glutaraldehyde and then embedded in an Araldite resin (Polysciences, Washington, PA). Glass knives were prepared on a knife maker (LKB Broma 2, 2178), and 1-µm sections were cut on a microtome (LKB Broma Ultratome, NOVA). In situ DNA nick-end labeling was utilized with some modification, as described previously (7, 8, 32). Briefly, tissue sections were transferred to PBS (pH 7.4) for 10 min and digested for 15 min with 20 µg/ml proteinase K (Sigma Chemical) in PBS. Endogenous peroxidase was inactivated by covering the sections with 2% H2O2 for 5 min at room temperature. The sections were transferred to PBS (pH 7.4) for 10 min and digested for 15 min with 20 µg/ml proteinase K (Sigma Chemical) in PBS. Endogenous peroxidase was inactivated by covering the sections with 2% H2O2 for 5 min at room temperature. The sections were transferred to PBS (pH 7.4) for 10 min and digested for 15 min with 20 µg/ml proteinase K (Sigma Chemical) in PBS. Endogenous peroxidase was inactivated by covering the sections with 2% H2O2 for 5 min at room temperature.

Assay for cytoplasmic mono- and oligonucleosomes. DNA fragmentation was also evaluated by photometric enzyme immunoassay of cytoplasmic mono- and oligonucleosome (histone-associated DNA fragments) determination (Boehringer Mannheim) (25). Briefly, thymus tissues were treated with lysis buffer for 120 min and centrifuged at 9,000 g for 10 min. The supernatant of the cell lysate was transferred to a streptavidin-precoated 96-well microplate. Biotinylated anti-histone antibody, which binds to histone H1, H2A, H2B, H3, or H4, and peroxidase-labeled anti-DNA antibody were then added. Because mitochondrial DNA is not covered by histones (17), the present value of cytoplasmic mono- and oligonucleosomes addresses specific damage of nuclear DNA that is covered by histones. Two hours later, the supernatant was discarded and then incubated with substrate solutions. The absorbance (405 nm) was determined by a microplate reader (Bio-Rad Laboratories, Hercules, CA). The light absorbance levels were normalized to the thymus weight, and the index of cytoplasmic mono- and oligonucleosomes was expressed as optical density units per gram of tissue.

Statistical analysis. Statistical comparison among groups was determined by one-way ANOVA and Fisher’s multiple-comparison test. Values are means ± SE. P < 0.05 was considered to be statistically significant.

**RESULTS**

Mean arterial blood pressure levels were significantly higher in SHR than in WKY (Table 1). Adrenalectomy served to significantly lower the blood pressure in SHR but not in WKY (Table 1), in line with our previous observations (26, 27, 29).

The average weight of thymus glands was significantly decreased in SHR compared with WKY (Table 2). This characteristic reduction of thymus weight in SHR was no longer present after adrenalectomy (Table 2).

Figure 1a (WKY thymus) and Fig. 1c (SHR thymus) show the sections stained with toluidine blue, in which all nuclei in the fields are visible.
labeling of each serial section was enhanced in the thymus gland of SHR (Fig. 1d) compared with WKY (Fig. 1b). The high-power view serves to demonstrate nick-end-labeled nuclei in individual cells of the SHR thymus (Fig. 1d).

Semiquantitative analyses showed that the number of in situ nick-end-label (ISEL)-positive cells in the thymus was higher in SHR than in WKY (Fig. 2). Adrenalectomy led to a decrease in the number of ISEL-positive cells in the thymus of SHR to levels comparable to those of WKY (Fig. 2). In WKY, adrenalectomy had no significant effect on the number of ISEL-positive cells in the thymus (Fig. 2).

Figure 3 depicts the level of release of mono- and oligonucleosomes to cytoplasm of thymus tissues. The cytoplasmic mono- and oligonucleosomes increased significantly in the thymus of SHR compared with WKY. The elevated value in SHR was significantly lowered after adrenalectomy (Fig. 3). In WKY, adrenalectomy had no significant effect on the formation of cytoplasmic mono- and oligonucleosomes in the thymus (Fig. 3).

The thymus weight in WKY significantly decreased after 3 and 5 days of dexamethasone treatment (Fig. 4A). Treatment for 5 days, compared with only 3 days, further reduced the thymus weight in WKY. The weight loss was accompanied by a significantly increased level of cytoplasmic mono- and oligonucleosomes in the thymus of dexamethasone-treated WKY (Fig. 4B). Treatment for 5 days further increased the cytoplasmic mono- and oligonucleosomes.

### Table 2. Thymus weight normalized with respect to body weight

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<tr>
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<th>WKY</th>
<th>SHR</th>
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<tr>
<td></td>
<td>Sham (n = 19)</td>
<td>Adrenalectomy (n = 16)</td>
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<td>Thymus wt, mg/100 g body wt</td>
<td>115.0 ± 33.6</td>
<td>123.6 ± 30.9</td>
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Values are means ± SD; n, number of animals. *P < 0.05 compared with sham-operated WKY; †P < 0.05 compared with sham-operated SHR.

![Fig. 1. Representative micrographs of in situ nick-end labeling (ISEL) in thymus recorded by ×100 objective lens. a and c: bright-field image of toluidine blue staining. b and d: ISEL. Thymus sections were from sham-operated Wistar-Kyoto rat (WKY) with toluidine blue staining (a), sham-operated WKY with ISEL (b), sham-operated spontaneously hypertensive rat (SHR) with toluidine blue staining, and sham-operated SHR with ISEL (d). Arrows indicate nick-end-labeled nuclei.](http://ajpheart.physiology.org/)

![Fig. 2. ISEL-positive cells. Values are means ± SE for 6 animals in each group. *P < 0.05 compared with sham-operated WKY; †P < 0.05 compared with sham-operated SHR.](http://ajpheart.physiology.org/)

![Fig. 3. Thymus cytoplasmic mono- and oligonucleosome levels expressed in optical density (OD) units per gram of tissue. Values are means ± SE. *P < 0.05 compared with sham-operated WKY; †P < 0.05 compared with sham-operated SHR.](http://ajpheart.physiology.org/)
DISCUSSION

There are several indicators that SHR have an increased adrenal glucocorticoid secretion (2, 11), mainly corticosterone, which may play an important role in the pathogenesis of the hypertensive syndrome. We previously reported that the leukocyte adhesion to microvascular endothelial cells induced by histamine or H$_2$O$_2$ stimulation was attenuated in SHR because of an impairment of the selectin-mediated adhesion pathway (20, 24) and that the impaired adhesion response was restored by adrenalectomy (27). Modification of the glucocorticoid levels in SHR by adrenalectomy served to normalize the elevated levels of arteriolar tone (28, 29), a major determinant of peripheral resistance. Although in WKY the average blood pressure after adrenalectomy tends to decrease, it was not statistically significant, possibly because of the biologic variability in these strains (15). These results are in line with our previous data, which showed mean arterial blood pressure under general anesthesia in WKY with (83.2 ± 10.5 mmHg) or without (102.0 ± 11.1 mmHg) adrenalectomy (29).

The present study demonstrates enhanced levels of DNA fragmentation in the thymus glands of SHR and its normalization after adrenalectomy (Figs. 2 and 3). Such a change in the levels of DNA damage was well correlated with the shift in thymus wet weight (Table 2).

The morphological and biochemical characteristics of thymocyte apoptosis have been primarily established using cultured immature thymocytes treated in vitro with various glucocorticoids (4, 16, 33). Glucocorticoids are also believed to induce thymic atrophy directly or indirectly in vivo through enhancement of apoptosis (5, 34). According to Sun et al. (21), evidence of thymocyte apoptosis was first seen 2–4 h after dexamethasone administration and reached a maximum at ~8 h. In the present study, dexamethasone treatment induced a significant reduction of thymus weight (thymic atrophy) as well as an increase in cytoplasmic mono- and oligonucleosomes (DNA fragmentation).

According to the report by Keyton et al. (13), basal plasma corticosterone concentrations are about twice as high in young SHR as in WKY. We previously measured the level of plasma corticosterone concentration in animals surgically treated according to the same regimens used in the present study (23). According to these measurements, the level of plasma corticosterone was significantly higher in sham-operated SHR than in sham-operated WKY: 423 ± 34 and 357 ± 12 ng/ml, respectively (P < 0.05). After adrenalectomy the plasma corticosterone level was significantly lowered: 8.6 ± 4.9 and 17.3 ± 14.6 ng/ml in SHR and WKY, respectively. These results indicate an enhanced level of plasma glucocorticoid in SHR and a normalization of these values after adrenalectomy.

To evaluate apoptosis in the target organs of hypertensive animals, Hamet and colleagues (9, 10) developed an assay for in situ TdT labeling of tissue slices in which the density of labeling corresponds to the amplification of oligonucleosomal DNA fragments. They determined the amount of DNA fragments in whole tissue sections by densitometry (6) and applied these techniques especially to the heart of SHR. According to their observations, an increase in apoptosis in SHR is levels of mono- and oligonucleosomes compared with 3 days of treatment.
observed in the ventricular portion of the heart compared with age-matched normotensive WKY controls. To confirm the observations obtained by the in situ labeling technique, they also demonstrated oligonucleosomal laddering in DNA extracted from the heart of 8 wk-old SHR but not in age-matched normotensive WKY controls (10). They reported enhanced levels of apoptosis in the heart and kidney of the hypertensive mouse by performing in situ DNA end labeling in tissue sections with TdT and digoxigenin nucleotide (10), similar to the present study in the rat thymus.

Exogenous H₂O₂ evokes apoptotic cell death in a dose-dependent manner (12). Transfected cells with Bcl-2, which is localized at intracellular sites of oxygen radical generation and acts as an antioxidant, were protected from the lethal effects of H₂O₂ (12). Our previous studies show that the average level of oxygen radicals in circulating leukocytes (18) as well as in the microvascular endothelium (22, 26) of SHR was significantly elevated compared with that in WKY and that this enhanced level of oxygen radicals in SHR could be normalized by adrenalectomy (26), suggesting a parallel shift in oxygen radical formation and the level of thymocyte apoptosis.

Takechi et al. (31) reported a suppression of T lymphocyte function and detected the existence of a natural thymocytotoxic autoantibody, which in the SHR occurs as early as 4 wk of age. The thymocytotoxic autoantibody is cytotoxic for thymocytes but is weakly cytotoxic or reactive on spleen cells, bone marrow cells, and lymph node cells. According to their report, the weight of the thymus in SHR showed a slight but insignificant reduction until 16 wk of age and a significant reduction at 24 wk compared with WKY (30). These values were expressed by the actual weight of the thymus, without normalization with respect to body weight (Table 2). The reduction of thymus weight and the enhancement of DNA nicking suggest that the presence of apoptosis in the thymus gland serves as a significant mechanism that prevents full development of this organ as well as lymphocyte maturation. Exogenous glucocorticoid, dexamethasone, dose dependently induced apoptosis of thymus cells, suggesting a possible adrenal signaling pathway to induce apoptosis in the thymus.

In conclusion, the levels of thymic cellular apoptosis were significantly enhanced in SHR together with an enhanced corticosterone secretion and oxygen radical formation in this hypertensive strain.

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REFERENCES


