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Hypothyroidism and the Aorta. Evidence of Increased Oxidative DNA Damage to the Aorta of Hypothyroid Rats

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Abstract. *Background: Although it has been suggested that the hypometabolic state is associated with a decrease in oxidative stress, literature data are controversial, revealing an individuality of oxidant status in relation to tissue properties and responsiveness. Hypothyroidism has profound direct and indirect actions on the vascular system, inducing characteristic hemodynamic changes while the aorta represents an important determinant of vascular performance. This study aims to examine the oxidant status on the aorta in chronic experimental hypothyroidism. Materials and Methods: Chronic hypothyroidism was successfully induced in 20 male Wistar rats by administration of 0.05% 6-n-propyl 2-thiouracil in their drinking water for 8 weeks. Age-matched euthyroid rats were used as controls. Lipid peroxidation in the serum was determined by the end-product malondialdehyde (MDA). Oxidative damage to genomic DNA of aortic tissue and serum was investigated by measuring 8-oxo-dG, one of the base modifications produced in DNA by the reaction of reactive oxygen species. Serum lipids measurement was performed. Results: A hypothyroid state was confirmed by levels of serum thyroid hormones, lipidemic profile, clinical examination, pathological findings and cardiovascular hemodynamics parameters. Hypothyroidism was associated with a significant increase in lipid peroxidation. (MDA 1.44±0.93 vs. 0.64±0.53 nmol/ml, p<0.01). Levels of 8-oxo-dG on the aortic ring, expressing the oxidant damage on genomic DNA and in the serum, were observed to be significantly raised in the hypothyroid group compared to controls (8-oxodG_{serum} 29.22±17.78 vs. 17.56±4.44 ng/ml, p<0.01; 8-oxo-dG_{aorta} 11.58±2.70 vs. 4.09±1.27 ng/ml, p<0.001). A statistical correlation between measurements of 8-oxo-dG in the aorta and serum was found (correlation coefficient=0.36, p<0.05). A hyperlipidemic profile in hypothyroid animals was revealed. Conclusion: Vascular oxidative stress seems to play a pivotal role in the evolution of vascular pathology. Hypothyroidism was associated with increased DNA oxidative damage to the aorta. Hypercholesterolemia and an increase in mean arterial pressure associated with hypothyroidism may have a contributive role in the accumulation of damage in nuclear DNA of the vascular wall. 8-Oxo-dG is one of the mutagenic base modifications produced in DNA. Although clinical studies in other tissues have indicated a direct correlation between in vivo 8-oxo-dG formation and pathological processes, its role on the vascular wall needs further investigation.*

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It is well recognized from studies of patients with hypothyroidism that thyroid hormone has profound direct and indirect actions on the heart and the cardiovascular system. The basis for these effects is the ability of the thyroid hormone to exert a cellular action on cardiac myocytes, vascular smooth muscle cells and endothelium (1-3). The hemodynamic changes characteristic of hypothyroidism include a decrease in cardiac contractility, cardiac output, heart rate and left ventricular compliance, as well as an increase in total peripheral vascular resistance (1, 2). The bradycardia, reduced contractility and reduced ventricular filling due to diastolic dysfunction cause a low cardiac output, but as overall oxygen demand is reduced due to a decrease in cellular metabolism, this does not usually result in inadequate oxygen delivery to the peripheral tissues.

Accumulating evidence has suggested that the hypometabolic state induced by hypothyroidism is associated with a decrease in free radical production and in lipid peroxidation products (4, 5). However, it is not clear whether the induced decrease in oxidative stress in hypothyroidism is confined to some tissues. The aorta has a fundamental mechanical-hemodynamic role and represents an important determinant of blood regulation and cardiovascular performance (1, 6, 7). Hypothyroidism is associated with

augmentation of central aortic pressure and central arterial stiffness leading to an increased cardiovascular risk (8).

Oxidative stress plays a major role in several aspects of vascular biology, while current studies have suggested a potential implication in signaling mechanisms leading to vascular pathologies (9, 10). The objective of this study was to examine the oxidant status in the aorta of a rat model with chronic hypothyroidism. To the best of our knowledge, there is no reported study that has examined the oxidant status on the aorta in experimental hypothyroidism.

Materials and Methods

Animal preparation and induction of hypothyroidism. Forty male Wistar rats weighing 250-300 g were maintained in the Laboratory for Experimental Surgery and Surgical Research, Medical School of Athens University, in European standard cages, in a controlled environment at 20±2˚C, 55% relative humidity, with central ventilation (15 air changes/h) and an artificial 12-h light:dark cycle. All experiments were performed in accordance with local and national guidelines covering animal experimentation. Twenty rats (Group B) were rendered hypothyroid by the administration of 0.05% 6-*n*-propyl-2-thiouracil (PTU; Sigma Chemicals, St Louis, MO, USA) in their drinking water for 8 weeks. Twenty rats (Group A) acted as age-matched controls, housed under the same conditions to those of the hypothyroid group, without receiving any drugs.

6-*n*-Propyl 2-thiouracil is a thiolated uracil derivative, known as an antihyperthyroid agent, which inhibits the deidonation of thyroxine to triiodothyronine. The concentration of the administrated solution with PTU was determined in a pilot study in our Laboratory along with related data from the literature for induction of experimental hypothyroidism (11, 12). Treatment with the above mentioned concentration results in a long-term moderate hypothyroidism (13). The drinking solution for the animals was prepared daily. The daily consumption of water was measured for each animal during the entire period of the experiment. During the study, all rats were on the same diet. Clinical examination of the animals of both groups during the entire experimental period included weekly measurement of body weight (Navigator Balance, Ohaus Corporation, USA), and rectal temperature. Assessment of pressure and cardiac rhythm were performed with the Coda 6 Non Invasive Blood Pressure Acquisition System (Kent Scientific Corporation, Torrington, CT). Necropsy findings included measurement of the animal's body and heart weight.

Surgery and tissue preparation. All animals of both groups were sacrificed 8 weeks after the initiation of the study. Following sedation of the animal with a combination of an intramuscular injection of ketamine (90 mg/kg) and xylazine (5 mg/kg), the abdominal wall was entered through a midline incision, the inferior vena cava was dissected, and blood was withdrawn with a heparinized syringe. Blood samples were centrifuged at 3000×g for 15 min and then serum aliquots were stored frozen at –80˚C until analyzed. Following the cessation of respiration and heart function, the thoracic cavity was opened through a median sternotomy. The heart was excised, washed from blood and weighed. The descending aorta from the left subclavian artery to the diaphragm, with the surrounding loose periaortic tissue, was excised with extreme care to avoid damage to the aortic wall. A complete aortic ring of 2 to 3 mm length, at the level of the second intercostal artery, was carefully excised. For genomic DNA isolation and determinations, the aortic ring was snap-frozen in liquid nitrogen and kept at –80˚C until analysed. Animals in the control group were treated by the same procedures described above.

Measurement of thyroid hormones. Serum total triiodothyronine (tT3), free triiodothyronine (fT3) and total thyroxine (tT4) were measured every 14 days during 8 weeks of treatment through to the day of sacrifice. Serum tT3 and fT3 concentrations were measured by the Microparticle Enzyme Immunoassay (Abbott autoanalyzer IMX System; Abbott Diagnostics Division, Abbott Laboratories, Abbott Park, IL 60064, USA) and serum tT4 concentrations were determined by a Fluorescence Polarization Immunoassay (Abbott autoanalyzer IMX System).

Serum lipids measurement. Blood samples for determination of lipids levels were taken after an overnight fast, 2 and 8 weeks after initiation of the experiment. Plasma was separated by low-speed centrifugation (3500×g for 15 min). Serum cholesterol and high-density lipoprotein (HDL)–cholesterol were determined enzymatically by the CHOD-PAP method using a commercially available kit (Biosis, Hellas). Serum triglycerides were measured by the enzymatic GPO-PAP method using a commercially available kit (Biosis). LDL–Cholesterol was estimated by the Friedewald formula. HDL was isolated by precipitating chylomicrons, very low density lipoproteins (VLDL) and low density lipoproteins (LDL) by adding phosphotungstic acid and magnesium ions to the samples.

Malondialdehyde (MDA) and 7, 8-dihydro-8-oxo-2'-deoxyguanosine, (8-oxo-dG) measurement. Lipid peroxidation was determined by assessment of end product malondialdehyde (MDA), as evaluated by reaction with thiobarbituric acid (14). The samples were preserved in tubes with anticoagulant and antioxidative agent (BHT 0.2% in methanol), to measure 2-thiobarbituric acid reactive substances (TBARS). Plasma was separated by low-speed centrifugation (3500×g/min for 15 min). A 0.5 ml aliquot of each sample was added to a tube containing 3 ml of 0.05 N HCl and mixed. In each tube, 1 ml of 46 mmol/l l2-thiobarbituric acid (TBA) was added. The tubes were boiled for 30 min and allowed to cool. These mixtures were then added to tubes containing 4 ml methanol/butanol 3:17 and mixed. After centrifugation at 2500×g/min for 20 min, 1.5 ml of each supernatant was taken and the absorbance at 535 nm was measured. The TBARS were calculated as (MDA) equivalents, using freshly diluted MDA *bis* (1,1,3,3-tetraethoxypropane) as the standard. Malondialdehyde was prepared by hydrolysis of 1,1,3,3-tetraethoxypropane with concentrated H_2SO_4.

Genomic DNA was extracted from aortic rings using Genomic DNA Purification Kit from tissue (Macherey-Nagel, Germany). DNA was digested with nuclease P1, phosphodiesterase I and alkaline phosphatase to yield free deoxynucleosides.

The amount of 8-oxo-dG was measured by using competitive ELISA (Highly Sensitive 8-OhdG ELISA Kit, Japan Institute for the Control of Aging, Fukuroi, Japan).

Serum 8-oxo-dG levels were measured using a competitive enzyme-linked immunosorbent assay (ELISA) (New 8-OhdG ELISA Kit; Japan Institute for the Control of Aging).

Statistical analysis. Results are expressed as mean±standard deviation or standard error of the mean. SPSS 12 (SPSS Inc. Chicago, Illinois, USA) was used for analyses, including linear and

Table I. *Values of thyroid hormones in both groups 2 and 8 weeks after initiation of the experiment respectively. Clinical and necropsy findings. Values are expressed as mean±standard error of the mean.*

2nd Week

	Hypothyroid (n=20)	Control (n=20)	*P-Value*
Total T4 (ng/ml)	19.94±2.58	39.31±6.3	<0.05
Total T3 (ng/ml)	<0.05	0.34±0.02	<0.05
Free T3 (pg/ml)	0.27±0.02	0.57±0.07	<0.05
Temperature (range ºC)	35.8-36.7	36.9-37.4	-
Body weight (g)	280±24	330±18	<0.05

8th Week

	Hypothyroid (n=19)	Control (n=19)	*P-Value*
Total T4 (ng/ml)	18.86±2.44	41.1±6.9	<0.05
Total T3 (ng/ml)	<0.05	0.38±0.03	<0.05
Free T3 (pg/ml)	0.29±0.04	0.63±0.06	<0.05
Temperature (range ºC)	35.7-36.6	37.0-37.5	-
Body weight (g)	365±34	448±23	<0.05
HR (beats/min)	310±10	360±8	<0.05
Mean Pressure (mmHg)	84±6	74±6	<0.05

LVW/BW: Left ventricular weight/body weight ratio; n.s.=non significant; HR: heart rate.

power regressions. The results of the hypothyroid group were compared to those of control group using one way ANOVA (applying robust tests of equality of means: Welch and Brown – Forsythe) and the non-parametric Mann–Whitney test. Correlations between quantitative normally distributive parameters were assessed with Pearson's two-way test or Spearman's rho otherwise. A *p*-value less than 0.05 was considered statistically significant.

Results

Biological variables. The thyroid state of different groups of animals was characterized by the data reported in Table I. One rat from the hypothyroid group was excluded from the study due to infection of the respiratory system, while one rat from the control group died during blood sampling. The values indicate the effectiveness of 6-*n*-propyl 2-thiouracil administration. The effectiveness of the experimental treatment was ascertained by the measurement of serum thyroid hormones. There was no difference in thyroid hormone blood levels between the two groups at the beginning of the study. The animals that received 6-*n* propyl 2-thiouracil became hypothyroid by day 15 of treatment, as manifested by a decrease in serum thyroid hormone levels (Table I), which remained diminished for the duration of the treatment. These values were significantly lower ($p<0.05$) than those of the control group. In order to ascertain the effectiveness of the experimental treatment, rectal temperature was measured during the 8 weeks of treatment

Table II. *Oxidative stress parameter values and plasma lipid levels in hypothyroid and control animals 8 weeks after initiation of the experiment. Values are expressed as mean±standard deviation.*

Oxidative stress parameters	Hypothyroid (n=19)	Control (n=19)	*P-value*
8-oxo-dG aortic ring (ng/ml)	11.58±2.7	4.09±1.27	<0.001
8-oxo-dG serum (ng/ml)	29.22±17.78	17.56±4.44	<0.01
Malondialdehyde (nmol/mL)	1.44±0.93	0.64±053	<0.01
C-Reactive protein	28.32±4.72	23.37±3.15	<0.001
Lipidemic profile values	**Hypothyroid (n=19)**	**Control (n=19)**	***P-value***
Total cholesterol (mg/dL)	117.74±12.17	97.32±18.36	=0.001
Triglycerides (mg/dL)	66.42±17.69	67.32±14.61	NS
LDL (mg/dL)	50.3±6.78	41.06±14.16	<0.05
HDL (mg/dL)	54.16±11.8	42.79±9.84	<0.01

LDL, Low-density lipoprotein; HDL, high-density lipoprotein.

and showed an increase in Group B, while it did not show any changes in control animals. Rectal temperature decreased in Group B, while no changes were noticed in controls animals. At the end of the second week, rectal temperature was significantly lower in Group B (range 35.8-36.7˚C) than in the control (range 36.9-37.4˚C) group. Hypothyroid animals exhibited a lower heart rate (310±10 *vs*. 360±8 beats/min, $p<0.05$), and a lower final body weight (2nd week 290±34 *vs*. 330±18 g, $p<0.05$, 8th week 375±44 *vs*. 448±23 g, $p<0.05$).

Oxidative DNA damage. The two groups differed significantly in the 8-oxo-dG measurements (Table II). Specifically, 8-oxo-dG serum levels (Group A: 17.56±4.44 *vs*. B: 29.22±17.78 ng/ml, $p<0.01$) and 8-oxo-dG levels measured in the aortic ring (A: 4.09±1.27 *vs*. B: 11.58±2.7 ng/ml, $p<0.001$) were observed to be raised in the hypothyroid group and the differences were found to be statistically significant (Figure 1). 8-Oxo-dG measurements in the aortic ring and in the serum were positively correlated in the hypothyroid animals (correlation coefficient(c.c.)=0.36, $p<0.05$) (Figure 1). Multivariate analysis revealed a significant correlation between levels of T3, T4 and 8-oxodG in the aortic ring (T3: c.c.=0.73, $p<0.001$; T4: c.c.=0.4, $p<0.05$) and between levels of T3 and 8-oxodG in the serum (T3: c.c.=0.4, $p<0.05$).

Lipid peroxidation. The two groups differed significantly in all the parameters concerning lipid peroxidation and their lipidemic profile (Table II). Plasma MDA (nmol/ml) levels were significantly augmented in the hypothyroid group compared with levels of control rats. (Group A: 0.64 ±0.53 *vs*. B: 1.44±0.93 nmol/ml, $p<0.01$). Total serum cholesterol (Group A: 97.32±18.36 *vs*. Group B: 117.74±12.17 mg/dl

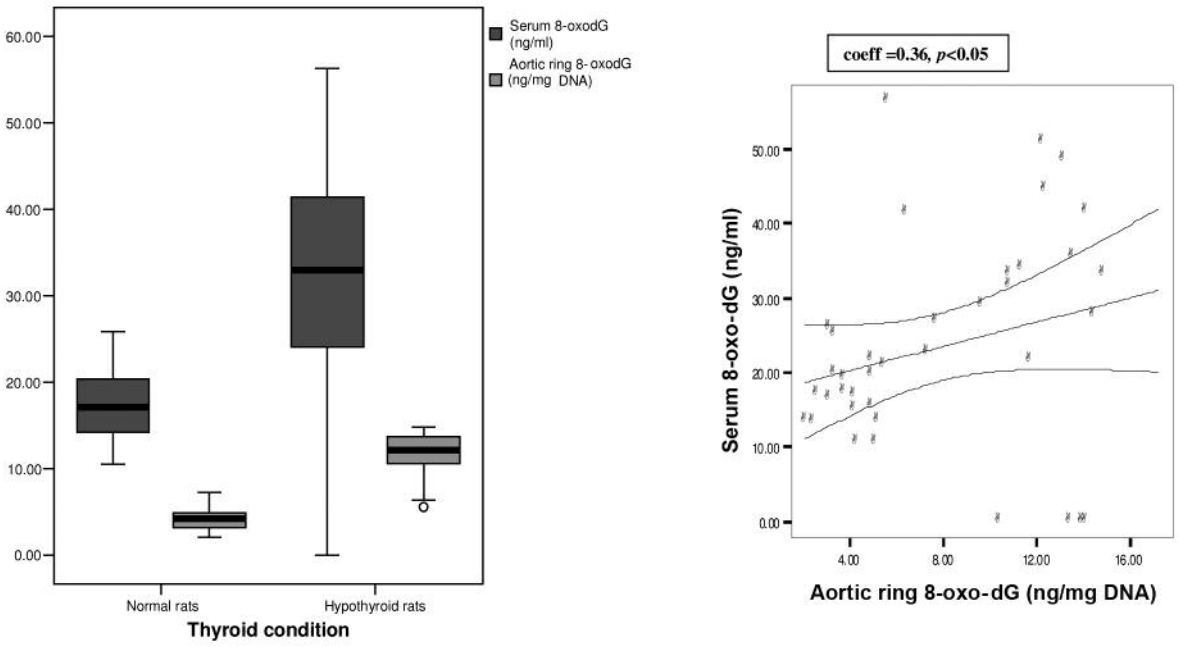

Figure 1. *The two groups differed significantly in the 8-oxo-dG serum and aortic ring measurements. 8-Oxo-dG measurements in the aortic ring and in the serum were positive correlated in the hypothyroid rats.*

p=0.001), LDL (Group A: 41.06±14.16 *vs*. Group B: 50.3±6.78, p<0.05) and HDL levels (Group A: 42.79±9.84 *vs*. Group B: 54.16±11.8 mg/dl, p<0.01) were found to be significantly lower in Group B compared to the control group. No significant differences were found in triglyceride levels.

Discussion

In our study, a hypothyroid state was confirmed by levels of serum thyroid hormones, lipidemic profile, clinical examination and pathological findings. Hypothyroidism was associated with a significant increase in lipid peroxidation. Levels of 8-oxo-dG on the aortic ring, expressing the oxidant damage to genomic DNA, and in the serum, were observed to be significantly raised in the hyporthyroid group. An augmented oxidant status was also revealed by statistical correlation analysis, showing a positive correlation between measurements of 8-oxo-dG in the aorta and serum (c.c.=0.361, p<0.05) and between levels of total triiodothyronine and 8-oxo-dG in the aorta ring and serum (c.c.=0.73, p<0.001 and c.c.=0.40, p<0.05 respectively).

Thyroid hormones are recognized to have a significant regulatory role in macromolecular catabolism by increasing the levels of many enzymes involved in metabolic reactions as well as mitochondrial enzymes involved in oxidative phosphorylation (15). Controversy exists in literature data concerning the influence of hypothyroidism in oxidative damage. Accumulating evidence has suggested that the hypometabolic state induced by hypothyroidism is associated with a decrease in free radical production and in lipid peroxidation products (4, 5). However, it is not clear if thyroid hormone-induced increase in lipid peroxidation is confined to some specific tissues. In fact, several organs are unresponsive to the effects of thyroid hormones, reflecting a different distribution pattern of thyroid hormone receptors, or autoregulation defensive mechanisms and different response to oxidative stress. Moreover, the response of the antioxidant systems to hypothyroidism has been fully investigated. Venditti *et al*. reported no modification of lipid peroxidation in liver, heart and skeletal muscle of hypothyroid rats and a significantly raised glutathione peroxidase activity in the heart and skeletal muscle (16). Similar studies have shown no effect of hypothyroid status on lipid composition or peroxidation in the mouse liver, while a significant increase in concentration of MDA has been found in eyes of hypothyroid rats (17, 18).

During recent years it has become clear that the nucleotide pool of cells is an important target for oxidative stress, as the free nucleotide forms are prone to oxidation. 8-Oxo-dG is one of the mutagenic base modifications produced in DNA by the reaction of reactive oxygen species. Its increased excretion has been equated to the presence of high levels of oxidative stress, since it is considered a sensitive biomarker of elevated DNA damage both *in vivo* and *in vitro* (19-22). 8-Oxo-dG is a mutagenic source, since it promotes the incorporation of dATP residues during replication and leads

to transversion mutation (GC→TA) (22, 23). It has been suggested that the chromatin structure of DNA in the nucleus with the association of nuclear proteins reduces its accessibility to free radicals and offers additional protection. Based on this observation, it has been proposed that DNA is better protected *in vivo* from oxidative stress than other macromolecules, incorporating and explaining its vital function as genetic material (24, 25). However, in our study, an increased level of lipid peroxidation and genomic DNA damage was found.

The biological significance of 8-oxo-dG is shown by the existence of repair pathways that are able to recognize and remove this lesion from both DNA and the nucleotide pool. Although observations in clinical studies of other tissues have indicated a direct correlation between *in vivo* 8-oxo-dG formation and pathological processes including inflammatory diseases and cancer (26-28), the clinical significance of an accumulation of damage in nuclear DNA of the vascular wall remains undetermined. Oxidative stress plays a major role in several aspects of vascular biology. Several enzymes expressed in the vascular tissue can contribute to the balance between antioxidants and the reactive oxygen species generating system. This observation has been strengthened by clinical and experimental studies documenting that oxidant products are involved in signalling mechanisms leading to vascular pathologies (9, 10, 29-31). An increase in oxidative stress has also been found to accompany progressive atherosclerosis and a number of cardiovascular risk factors including hypercholesterolemia and hypertension (11, 32, 33). Hypothyroidism is associated with hypercholesterolemia and an increase in total peripheral vascular resistance, a fact which was also confirmed in our study.

Previous reported study by the same scientific team, demonstrated increased oxidative damage to genomic DNA in aortic wall of hyperthyroid rats (22). The interpretation of this finding was attributed to the induced hyperdynamic cardiovascular state observed in hyperthyroidism. In the present study, an augmented oxidative stress on the aorta of hypothyroid animals was revealed. The understanding and analysis of the clinical significance of this finding is challenging and needs further investigation. The hypercholesterolemia and the increase in mean arterial pressure associated with hypothyroidism could justify in part and explain an increase in oxidative status. Thyroid hormones have pleiotropic effects on the vascular system, including a direct action in vascular smooth muscle tone and proliferation, in qualitative and quantitative modifications of plasma lipoproteins and an effect on plasma homocysteine concentration (34-37). The hemodynamic changes characteristic of hypothyroidism include a diastolic dysfunction causing a decrease in cardiac output and heart rate, as well as an increase in total peripheral vascular resistance (1, 2). Clinical studies based on chronic hypothyroidism oxidative stress effects on vascular wall are required. A critical approach to correlate the induced changes in oxidative stress with clinical parameters should be based on the molecular and hemodynamic consequences of the lack of thyroid hormone actions. In clinical practice, a possible association between hypothyroidism and aortic pathologies, such as aortic dissection and aortic aneurysm formation, has been suggested, based on the evidence of altered mucopolysaccharide turnover, disturbance of the mechanical properties and progression of atherosclerosis in hypothyroid individuals (38-40).

In conclusion, this study provides the first investigation of DNA oxidative stress on the descending aorta in chronic hypothyroidism. Hyporthyroidism was associated with increased DNA oxidative damage to the aorta. Vascular oxidative stress seems to play a pivotal role in the evolution of vascular pathology. Clinical studies and further investigation based on chronic hypothyroidism oxidative stress effects on vascular tissue are needed to determine the clinical significance of DNA oxidative damage to the aortic wall.

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