# ผลของธัยรอยด์ฮอร์โมนต่อการป้องกันการหนาตัวของผนังหลอดเลือดในกระต่าย

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คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

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PROTECTIVE EFFECTS OF THYROID HORMONES ON

ATHEROSCLEROTIC FORMATION IN RABBITS

Miss Kingkarn Boonsuya

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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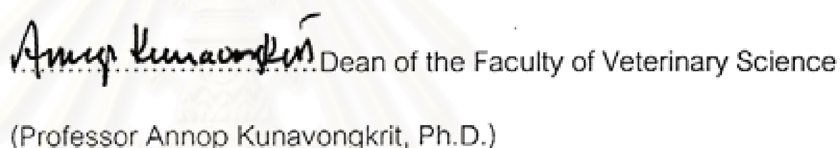

...............................Dean of the Faculty of Veterinary Science

(Professor Annop Kunavongkrit, Ph.D.)

THESIS COMMITTEE

...............................Chairman

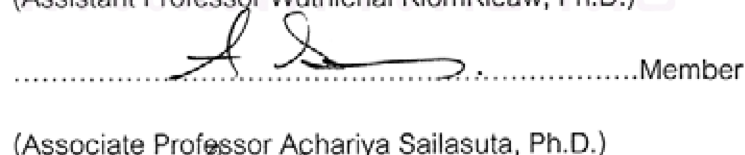
(Associate Professor Kris Angkanaporn, Ph.D.)

...............................Thesis Advisor

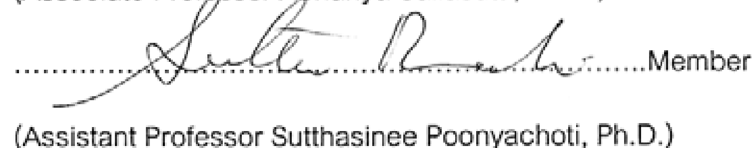
(Assistant Professor Suwanakiet Sawangkoon, Ph.D.)

...............................Thesis Co-advisor

(Assistant Professor Wuthichai KlomKleaw, Ph.D.)


...............................Member

(Associate Professor Achariya Sailasuta, Ph.D.)


...............................Member

(Assistant Professor Sutthasinee Poonyachoti, Ph.D.)

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จุฬาลงกรณ์มหาวิทยาลัย

กิ่งกานต์ บุญสุยา: ผลของธัยรอยด์ฮอร์โมนต่อการป้องกันการหนาตัวของผนังหลอดเลือดในกระต่าย (PROTECTIVE EFFECTS OF THYROID HORMONES ON ATHEROSCLEROTIC FORMATION IN RABBITS): อ.ที่ปรึกษา: ผศ.น.สพ.ดร.สุวรรณเกียรติ สว่างคุณ อ. ที่ปรึกษาร่วม: ผศ.น.สพ.ดร.วุฒิชัย กลมเกลียว, 96 หน้า, ISBN 974-14-2341-1

การวิจัยครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลของธัยรอยด์ฮอร์โมนต่อการป้องกันการหนาตัวของผนังหลอดเลือดในกระต่าย โดยใช้กระต่าย เพศผู้ น้ำหนักโดยเฉลี่ย 2.0-2.5 กิโลกรัม เป็นสัตว์ทดลอง แบ่งกลุ่มการทดลองออกเป็น 5 กลุ่มๆ ละ 5 ตัว กลุ่มที่ 1 เป็นกลุ่มควบคุมที่ได้รับอาหารพื้นฐาน กลุ่มที่ 2 เป็นกลุ่มที่ได้รับอาหารที่เหนี่ยวนำให้เกิดการหนาตัวของผนังหลอดเลือด (อาหารพื้นฐานที่ผสม 4% น้ำมันมะพร้าว และ 1% โคเลสเตอรอล) กลุ่มที่ 3 เป็นกลุ่มที่ได้รับอาหารที่เหนี่ยวนำให้เกิดการหนาตัวของผนังหลอดเลือดร่วมกับการให้ propylthiouracil ในขนาด 20 มิลลิกรัม/กิโลกรัม/วัน กลุ่มที่ 4 เป็นกลุ่มที่ได้รับอาหารที่เหนี่ยวนำให้เกิดการหนาตัวของผนังหลอดเลือด ร่วมกับการให้ propylthiouracil ในขนาด 20 มิลลิกรัม/กิโลกรัม/วัน และ L-thyroxine ($T_4$) ขนาด 50 ไมโครกรัม/น้ำหนักตัว/วัน กลุ่มที่ 5 เป็นกลุ่มที่ได้รับอาหารที่เหนี่ยวนำให้เกิดการหนาตัวของผนังหลอดเลือด ร่วมกับการให้ propylthiouracil ในขนาด 20 มิลลิกรัม/กิโลกรัม/วัน และ Triiodothyronine ($T_3$) ขนาด ไมโครกรัม/น้ำหนักตัว/วัน ซึ่งสัตว์ทุกตัวจะได้รับอาหารในปริมาณ 80 กรัม/น้ำหนักตัว/วัน ตั้งแต่เริ่มจนกระทั่งสิ้นสุดการทดลอง ทำการชั่งน้ำหนักและวัดอุณหภูมิทุกสัปดาห์, เก็บตัวอย่างเลือดเพื่อวัดค่า triglyceride, cholesterol, Total $T_3$, และTotal $T_4$ ก่อนและหลังการทดลอง เมื่อครบกำหนด 12 สัปดาห์ทำการวัดค่า malondialdehyde จากพลาสม่า และ Low density lipoprotein (LDL) และภายหลังจากการทำการุญฆาตทำการจัดเก็บตัวอย่างชิ้นเนื้อในส่วนของหลอดเลือด aortic arch, thoracic aorta และ abdominal aorta เพื่อศึกษาการเปลี่ยนแปลงของเนื้อเยื่อจากลักษณะทางกายวิภาคและจุลกายวิภาค โดยดูจากอัตราส่วนของพื้นที่ของรอยโรคที่เกิดจากการหนาตัวของผนังหลอด, ค่าเฉลี่ยของความหนาของหลอดเลือดส่วน intimal, และส่วนประกอบของเซลล์และเนื้อเยื่อในรอยโรค จากผลการทดลองพบว่า ค่าของ malondialdehyde ในพลาสม่าและLDL ของ กลุ่มที่ 4 มีค่าลดลงเมื่อเทียบกับกลุ่มที่ชักนำให้เกิดผนังหลอดเลือดหนาตัวในกลุ่มที่ 2 และ 3 นอกจากนี้ยังพบว่าอัตราส่วนของพื้นที่รอยโรคที่เกิดการหนาตัวของผนังหลอดเลือดของกลุ่มที่ 4 และ 5 มีค่าลดลงเมื่อเทียบกับกลุ่มที่ 2 และ 3 จากการศึกษานี้แสดงให้เห็นว่า ธัยรอยด์ฮอร์โมนสามารถป้องกันการหนาตัวของผนังหลอดเลือดในกระต่ายได้ ซึ่งจากผลการทดลองจะเป็นข้อมูลที่มีความสำคัญต่อการศึกษาเพื่อหาวิธีการป้องกันการเกิดการหนาตัวของผนังหลอดเลือดในอนาคตต่อไป

ภาควิชา.....................สรีรวิทยา........................ ลายมือชื่อนิสิต..........................................

สาขา..........................สรีรวิทยาการสัตว์........... ลายมือชื่ออาจารย์ที่ปรึกษา..................................

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จุฬาลงกรณ์มหาวิทยาลัย

## 4575552031: MAJOR ANIMAL PHYSIOLOGY

KEYWORD: ATHEROSCLEROSIS/THYROID HORMONE/OXIDATION/CHOLESTEROL

KINGKARN BOONSUYA: PROTECTIVE EFFECTS OF THYROID HORMONES ON ATHEROSCLEROTIC FORMATION IN RABBITS. THESIS ADVISOR: ASST. PROF. SUWANAKIET SAWANGKOON, Ph.D., THESIS CO-ADVISOR: ASST. PROF. WUTHICHAI KLOMKLEAW, Ph.D., 96 pp. ISBN 974-14-2341-1

The objective of this investigation was to study the protective effectives of thyroid hormones on atherosclerotic formation in rabbits. This experiment was performed in twenty-five male New Zealand White rabbits weighing between 2.0-2.5 kg. The animals were randomly divided into five groups, each group containing 5 animals. The rabbit model of atherosclerosis was induced with a period of twelve weeks. Group I, rabbits were fed with standard chow daily. Group II, rabbits were fed with lipid rich food (containing: standard chow, 4% coconut oil, 1% cholesterol). Group III, rabbits were fed with lipid rich food plus propylthiouracil (PTU) at the dose of 20 mg/kg body weight daily. Group IV, rabbits were fed with lipid rich food, 20 mg/kg of PTU, and 50 µg/kg of L- thyroxine ($T_4$) daily. Group V, rabbits were fed with lipid rich food, 20 mg/kg of PTU, and 0.6 µg/kg of Triiodothyronine ($T_3$) daily. Body weight and body temperature were measure weekly. Blood samples were collected for measurements of serum lipid profile, thyroid hormones and lipid oxidation. At the end of the experiment, animals were euthanized and vascular tissues were collected for measurement of histological analysis. Our results showed that animals in group IV had significant decreases in malondialdehydes in plasma and low density lipoprotein (LDL) contents, compared to animals in group II and III. Animals in group IV and V had lower ratio of areas of atherosclerotic lesion and compositions of atherosclerotic lesions, compared to animals in group II and III. In conclusion, the present study demonstrates that thyroxine and triiodothyronine have protective effect by inhibiting atherosclerotic formation in a rabbit model.

Department ..................Physiology........................ Student's signature..............................

Field of study ................Animal Physiology...............Advisor's signature..............................

Academic year...............2005............................... Co-advisor's signature..............................

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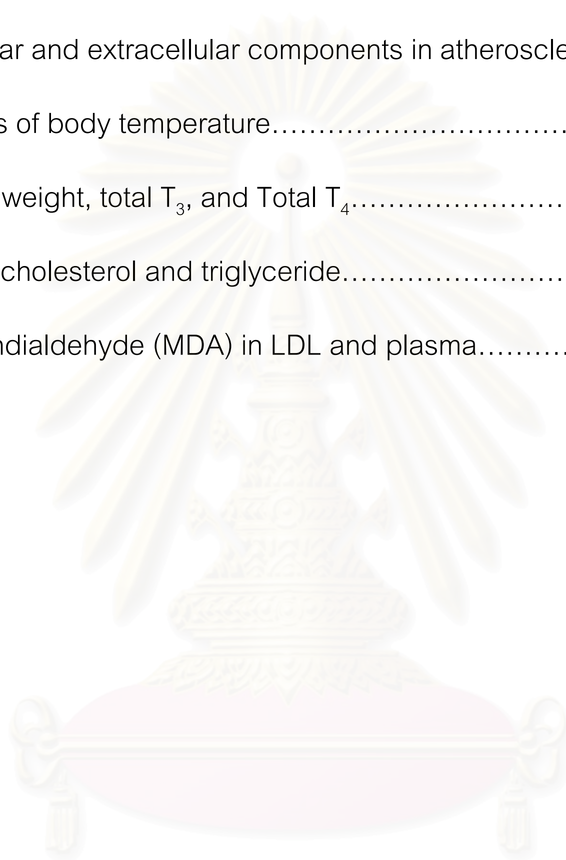

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# ABBREVIATION

ANOVA = Analysis of Variance

apo B = apolipoprotein B

BSA = Bovine serum albumin

CHD = coronary heart disease

g = gram

HDL = High density lipoprotein

hr = hour

KBr = potassium bromide

kg = kilogram

LDL = Low density lipoprotein

ml = milliliter

mm = millimeter

nm = nanometer

nmol = nanomole

PBS = phosphate buffer saline

PUFAs = polyunsaturated fatty acids

rpm = round per minute

SD = standard deviation

TBARS = thiobarbituric acid reactive substance

$T_3$ = Triiodothyronine

$T_4$ = 3,5,3′,5′-tetraiodo-L-thyronine , Thyroxine

μM = micromolar

μm = micrometer, micron

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# CHAPTER I

# INTRODUCTION

Cardiovascular diseases are still the leading causes of death to be chronic, and see in populations both developed countries and developing countries even in Thailand. In 1998, the Ministry of Public Health of Thailand has presented leading causes of death especially cardiovascular diseases about 70 per 100,000 of population (or 45,000 persons a year). The major problem may be due to feeding behavior in Thailand which becomes change to high lipid diets. Diets high in calories may lead to hypertriglyceridemia and hypercholesterolemia, and are considered a risk factor for the development of atherosclerosis. Atherosclerosis is the predominant cause of cardiovascular diseases—diseases of the heart and blood vessels, and it has pathologic lesion of the build-up of fatty deposits, called plaque, on the inside walls of arteries, which are blood vessels that carry oxygen and blood to organs, tissues, and every parts of the body. Arteriosclerotic arteries lose most of their distensibility, and because of the degenerative areas in their walls, arteriosclerotic arteries are easily ruptured. Where the plaques protrude into the flowing blood, the roughness of their surfaces causes blood clots to develop, resulting in thrombus or embolus formation, and blocking all blood flow in the artery suddenly (Guyton, 1996). Atherosclerosis may lead to clogged arteries in any part of the body. Where the coronary arteries are affected, angina (chest pain) or a heart attack may occur. Where the arteries on the leg are affected, legs pain may be experienced. Atherosclerosis of the arteries to the brain may also cause strokes. Atherosclerosis is

common in the United States. It often starts in childhood and the arteries become narrowed or clogged over many years (The National Women's Health Information Center; NWHIC, 2002).

The importance, risk factors of progressive atherosclerotic cardiovascular diseases such as heredity, male, sex, and age, cannot be changed. However, the risk factors including smoking, higher blood pressure, and high blood cholesterol levels can be alleviated (Porth, 1990). In a study, the relationship of atherosclerosis and thyroid hormone shows that hypothyroidism increases risks of progressive atherosclerotic cardiovascular diseases via increases in circulating levels of highly atherogenic low-density lipoprotein (LDL) cholesterol particles, induction of diastolic hypertension, alteration of coagulability, and direct effects on vascular smooth muscle (Cappola and Ladenson, 2003). Interestingly, in the study of relationship between LDL cholesterol and progressive atherosclerotic cardiovascular diseases has shown that elevated levels of total cholesterol, and LDL cholesterol particles is a major risk factor for atherosclerosis(Staub et al., 1992).

There is a study that evaluated the degree of oxidative stress in subjects with hypothyroidism or hyperthyroidism compared to when they returned to the euthyroid state. The total and LDL cholesterol levels were higher in hypothyroidism than in euthyroidism, and were lower in hyperthyroidism than in the euthyroid state (Sundaram et al., 1997). They conclude that LDL has more susceptibility to oxidation in both hypothyroid and hyperthyroid states. Thus, the enhanced LDL oxidation may play a role in the cardiac disease process in both hypothyroidism and hyperthyroidism. Additionally, hyperthyroidism reduces the LDL cholesterol

concentration without modifying high-density lipoprotein (HDL) or triglyceride concentrations. It is possible that effects of LDL oxidation may require a threshold level of LDL to induce atherosclerosis. It is also possible the hyperthyroidism may induce anti-atherosclerosis factors that require further investigation (Sundaram et al., 1997). In 1998, Diekman et al had also shown that hypothyroidism causes qualitative changes in circulating lipoproteins that increase their atherogenicity. This study has shown that LDL is more susceptible to oxidation in patients with hypothyroidism, which normalization after restoration of the euthyroid state. In this study, the effect of different levels of thyroid and metabolic activity on LDL oxidation was investigated. Both hypothyroidism and hyperthyroidism are presented with higher levels of LDL oxidation when compared with normolipidemic control subjects. In hyperthyroid patients, the increased lipid peroxidation was strictly related to free thyroxine levels, whereas in hypothyroidism it was strongly influenced by serum lipids (Costantini et al., 1998). A study on the effect of thyroxine on oxidation of LDL in hypothyroid patients has been shown an increased LDL levels in plasma. After replacement by 3,5,3′,5′-tetraiodo-L-thyronine ($T_4$), the LDL levels had returned to normal (O' Brien et al.,1997; Chomard et al.,1998; Diekman et al.,1998; Martinez Triguero et al., 1998). The relationship of $T_4$ and ascorbic acid on the protection of endothelial cells has been demonstrated. The study showed that $T_4$ was reduced oxidative modification of LDL. Additionally, a study in miniature pigs has yielded the same data (Hanna et al., 1993; Hanna et al., 1995; Eder and Stangl., 2000).

Thyroid hormones and structural analogues have been reported to protect LDL from lipid peroxidation in vitro. Oziol et al (2003) examined the effects of thyroid compounds on macrophage induced LDL oxidation. They suggested that the

physiochemical antioxidant capacity of thyroid compounds was modulated by their action on the intracellular redox systems of macrophage. Recently, there was an evidence had showed that thyroid hormones might protect against macrophage-induced LDL oxidation (Oziol et al., 2003). Overall cellular effects of Triiodothyronine ($T_3$) lead to a reduction of its antioxidant capacity whereas those of $T_4$ increase it. Thus, $T_4$ might have antioxidant capacity for LDL against cellular oxidant in vivo more than $T_3$. Controversially, the discussion of the experiment that the macrophages was pre-incubated with the thyroid compounds and incubated with LDL showed that $T_3$ was reduced LDL oxidation more than $T_4$. Therefore, the effect of non-preincubation macrophages was directly opposite which assumed that the effect of different thyroid compounds on cellular oxidation status (Oziol et al., 2003).

The previous studies in vitro had been reported both $T_3$ and $T_4$ still had controversial on the protection of atherosclerosis. In the present study, we aimed to study the effect of $T_4$ and $T_3$ in vivo and presented the objectives of the study following:

1. To study the protective effects of thyroid hormones on atherosclerotic formation in the rabbit model.

2. To compare the protective effects between L-thyroxine ($T_4$) and Triiodothyronine ($T_3$) on atherosclerotic formation in the rabbit model.

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# CHAPTER II

# LITERATURE REVIEWS

## 2.1. ATHEROSCLEROSIS

Atherosclerosis is a common disease, and is the major cause of heart diseases and stroke which affects to arterial blood vessels or veins. The most attractive hypothesis has been proposed that atherosclerosis begins because the innermost layer of the artery, the endothelium, becomes damaged. The interactions between endothelial cells and oxidized low density lipoprotein (LDL), and between smooth muscle cells (SMCs) and oxidized LDL are important parts of atherosclerogenesis.

Atherosclerosis is a condition in which fatty materials are deposited along the walls of the arteries. These fatty materials thicken, harden and may eventually block the arteries. Atherosclerosis is just one of several types of “arterio-sclerosis”, which is characterized by thickening and hardening of artery walls, but both terms are often used to mean the same thing. Vascular lesions known as atheromas (or atheromata or atheromatous plaques) develop in the vessel wall, and may reduce or restrict blood flow in the lumen (stenosis) in late stages, as well as rupturing and leading to downstream tissue damage (Wang, 2001).

### 2.1.1. Anatomy of normal vessels

Understanding the pathogenesis of atherosclerosis first requires knowledge of the structure and biology of the normal artery. The basic constituents of the walls of blood vessels are cells, predominantly endothelial cells (ECs) and

smooth muscle cells (SMCs), and extracellular matrix including elastin collagen and glycosaminoglycans. The three concentric layers-intima, media, and adventitia-are most clearly defined in the larger vessels, particularly arteries. In normal arteries, the intima consists of a single layer of endothelial cells with minimal underlying subendothelial connective tissue. It is separated from the media by a dense elastic membrane called the internal elastic lamina. The smooth muscle cell layer of the media near the vessel lumen receives oxygen and nutrients by direct diffusion from the vessel lumen, facilitated by holes in the internal elastic membrane. The outer limit of the media of most arteries is a well – defined external elastic lamina. External to the media is the adventitia, consisting of connective tissue with nerve fibers and the vasa vasorum (see figure 2.1).

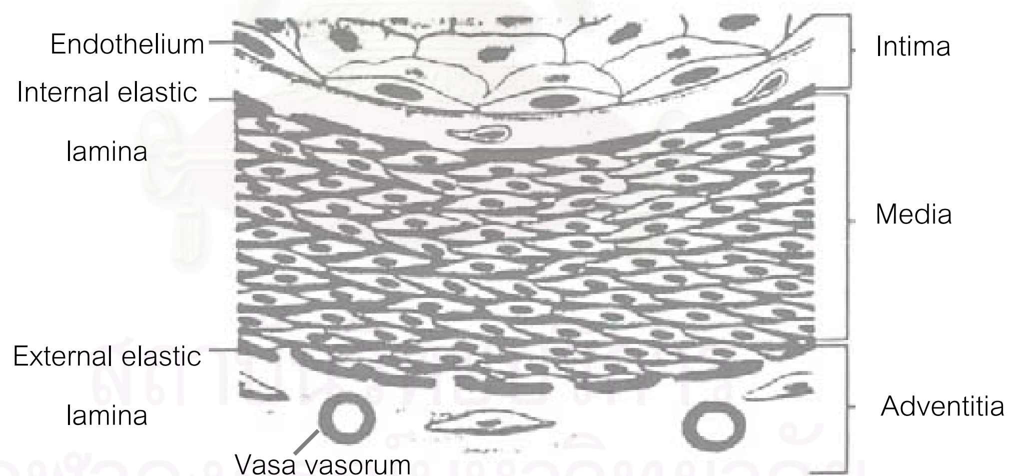


**Figure 2.1.** The diagram represents main components of vascular wall, seen in a muscular artery (Perkins, 2003).

### 2.1.2. Pathogenesis of atherosclerosis

Atherosclerosis literally means “hardening of the arteries”. It is a build up of cholesterol and other fat substances within the walls of the arteries. Atherosclerosis is a progressive disease and can develop in any arteries in the body. It is a common disorder of the arteries. Atherosclerosis is triggered by excess amounts of unstable particles known as oxygen free radicals, which bind to and alter other molecules, called oxidation. Atherogenesis is the developmental process of atheromatous plaques. It is characterized by a remodeling of arteries involving an accumulation of macrophage cells, forming atheroma or atheromatous plaques which contain an excess of fatty cellular membranes within the arterial wall. The plaques are usually located between the intima lining and muscular portion of the artery wall, typically without producing any narrowing, stenosis, of the arterial lumen.This concerns two major cell types of the arterial wall, endothelial cells and smooth muscle cells, as well as the extracellular matrix macromolecules that is synthesized. There are also other cell types that are involved in the process of atherosclerosis such as monocytes, macrophages and platelets, and other molecules such as chemotactic factors (Frohlich, 1989).

The first step of atherogenesis is the development of fatty streaks, small subendothelial deposits of lipid (figure 2.2). The exact cause for this process is unknown, and fatty streaks may appear and disappear. The initial damage to the blood vessel wall results in an inflammation response and monocytes enter the artery wall from the bloodstreams. The monocytes differentiate to be macrophages, which ingest oxidized cholesterol, slowly turning into large "foam cells" ,described from their

changed appearance resulting from numerous internal cytoplamic vesicles. Foam cells eventually die, and further propagate the inflammatory process (Mcphee, 1986).

Intracellular microcalcifications is formed within vascular smooth muscle cells of the surrounding muscular layer, specifically in the muscle cells adjacent to the atheromas. In time, as cells die, this leads to extracellular calcium deposits between the muscular wall and outer portion of the atheromatous plaques. Cholesterol is delivered into the vessel wall by cholesterol-containing low density lipoprotein (LDL) particles. To attract and stimulate macrophages, the cholesterol must be released from the LDL particles and oxidized, a key step in the ongoing inflammatory process. The process is worsened if there is insufficient high density lipoprotein (HDL), the lipoprotein particle that removes cholesterol from tissues and carries it back to the liver. A protective fibrous cap normally forms between the fatty deposits and the artery lining (the intima). These capped fatty deposits, called atheromas, produce enzymes which cause the artery to enlarge over time. As long as the artery enlarges sufficiently to compensate for the extra thickness of the atheroma, then no lumen narrowing or stenosis of the opening occurs. When disease is advanced, the artery becomes expanded and egg shaped, but still with a circular opening. If the enlargement is beyond proportion to the atheroma thickness, then an aneurysm is created (Porth, 1990).

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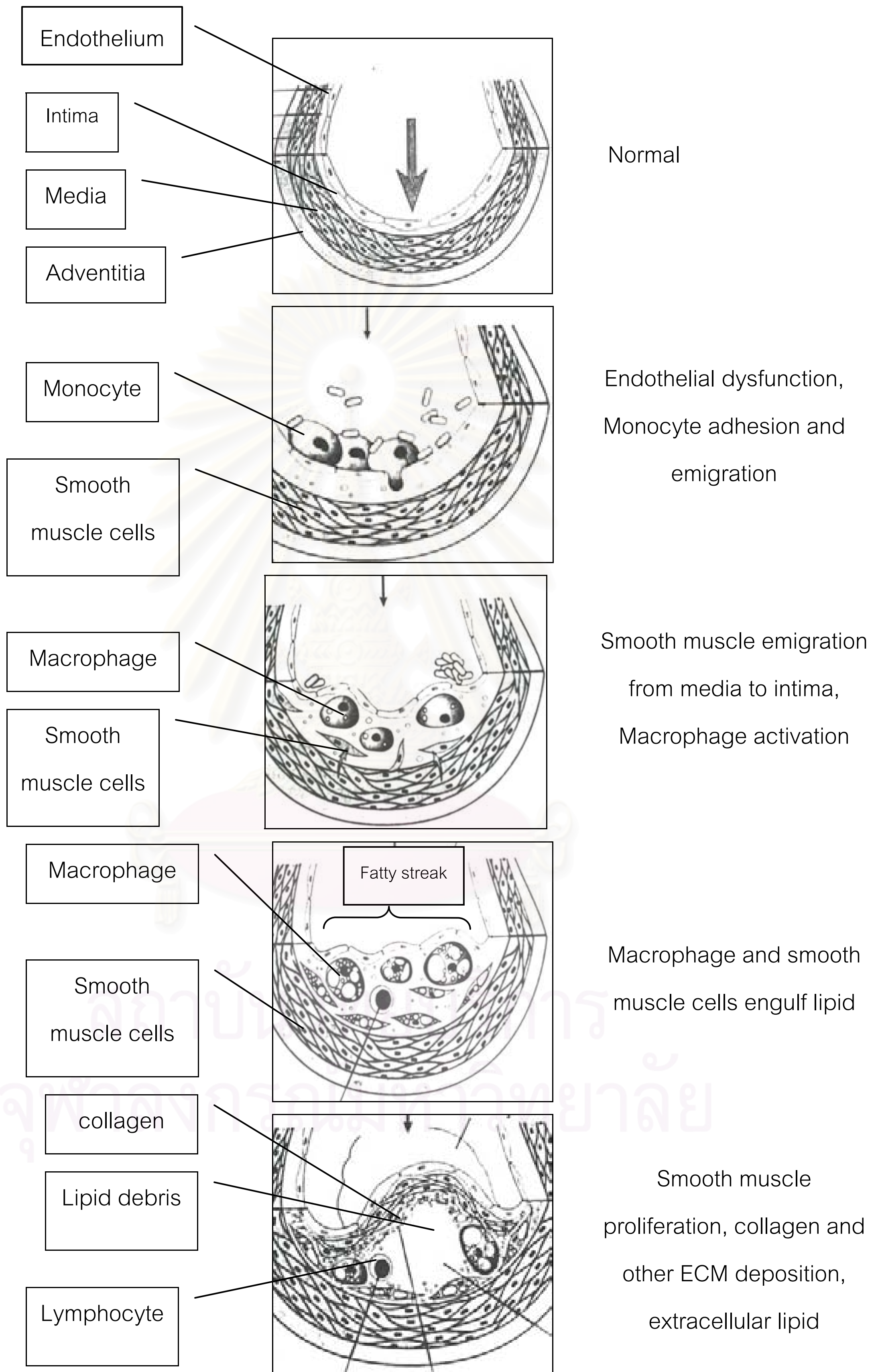


**Figure 2.2.** Processes in the response to injury hypothesis (Perkins, 2003).

### 2.1.3. Low density lipoprotein (LDL)

Low-density lipoprotein (LDL) is the major cholesterol-carrying particle in blood plasma. There is wide agreement that increased levels of LDL are causally related to atherosclerosis and the development of coronary heart disease (CHD). There is increasing evidence that LDL in its ‘native' state is not harmful; however, when it is altered by oxidation, it becomes a real threat within the arterial wall. The susceptibility of LDL to oxidation is determined by both internal (endogenous) and external (exogenous) factors. Half of the cholesterol in the blood is carried in LDL, which is a spherical fat-protein particle consisting of an outer monolayer containing the protein apolipoprotein B (apo B), which surrounds a core containing triglycerides and/or cholesterol esters (non-polar fats). One LDL particle contains about 3600 fatty acids, half of which are polyunsaturated fatty acids (PUFAs). LDL oxidation (or peroxidation) is a chain reaction initiated by free radicals, mainly reactive oxygen species. PUFAs are very susceptible to lipid peroxidation and breakdown to a variety of by-products which bond to LDL apo B. LDL can be oxidized in vitro by exposing them to smooth muscle and endothelial cells, macrophages, or metal ions (Copper or Iron). LDL oxidation processed in in vivo is poorly understood, and it may be reduced by the presence of anti-oxidants, e.g. ascorbic acid (vitamin C) in plasma (Buettner, 1993). Therefore, It is likely that LDL oxidation occurs in the artery wall rather than in the blood stream.

### 2.1.4. Lipid (LDL) oxidation and atherosclerosis

A high concentration of circulating LDLs is believed to be a major risk factor for atherosclerosis. The main physiological function of LDL is to deliver

cholesterol to peripheral cells. The essential step in the development of atherosclerosis begins as LDLs filter into the arterial wall and become entrapped in the intima, where they may undergo oxidative modification. Macrophages avidly take up this modified LDL, which contributes to their transformation to foam cells. The accumulation of foam cells in the intima results in the formation of fatty streaks. These may not produce significant obstruction of the artery, but they may be gradually converted into fibrous plaques by a mechanism similar to scar formation. These, in turn, are gradually transformed into atherosclerotic lesions which underlie most clinical events. In addition to lipid transport, LDL can effectively stimulate vascular smooth muscle cell (SMC) proliferation, a key event in the development of atherosclerosis (Ross and Glomset, 1973).

## 2.2. THYROID HORMONE

### 2.2.1. Anatomy

The thyroid gland is composed of two lobes, and is a shield-shaped structure located immediately below the larynx in the anterior middle portion of the neck. The thyroid gland consists of two types of cells, follicular and parafollicular (figure 2.3). Follicular cells are arranged spherically in a single layer with an apical end facing the center of the follicle and a basal end facing the interstitium. Follicular cells produce thyroid hormones, which are then stored in the central portion of the spherical follicles in a material called thyroid colloid. The interstitium contains the blood supply and parafollicular cells. Parafollicular cells secrete the hormone, calcitonin. For this reason they are called parafollicular C-cells or simply C-cells (Porth, 1990).

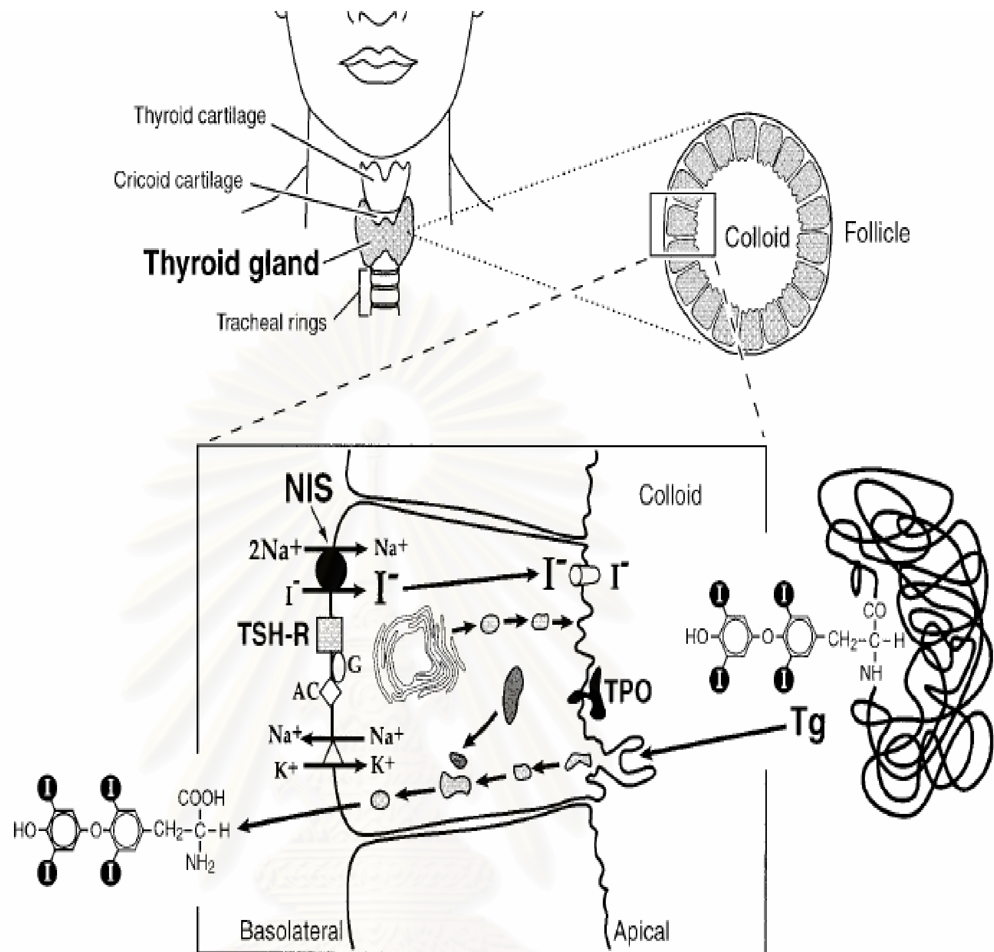


Figure 2.3. The thyroid gland and the follicular structure in humans (De La Vieja et al., 2000).

2.2.2. Thyroid physiology

The thyroid gland has its main function to produce and secrete metabolically active hormones that are essential for the regulation of various metabolic processes. Thyroid hormones are produced within the cells of the follicles from the amino acid, tyrosine, and the halogen element, iodine. The two most important thyroid hormones are thyroxine ($T_4$), which contains four iodine atoms, and triiodothyronine ($T_3$), which contains four iodine atoms.

The thyroid gland produces and secretes iodothyronines. About 90% of the output is 3, 5, 3’, 5’-tetraiodothyronine (thyroxine or $T_4$), while 3, 5, 3'-triiodothyronine ($T_3$) accounts for the remaining 10%. $T_4$ is considered to have little or no intrinsic bioactivity. $T_3$ is the most important bioactive thyroid hormone and virtually all the biological effects of $T_4$ elicited in vivo can be ascribed to its conversion into $T_3$. The total $T_4$ concentration in human serum is 75-135 nM, while total T3 is present at a much lower concentration of 1.35–2.60 nM. Finally, about 1% of the output of the thyroid gland is the inactive iodothyronine 3, 3’, 5’-triiodothyronine (r $T_3$). Molecular structures of the iodothyronines are shown in figure 2.4(Yen, 2001).

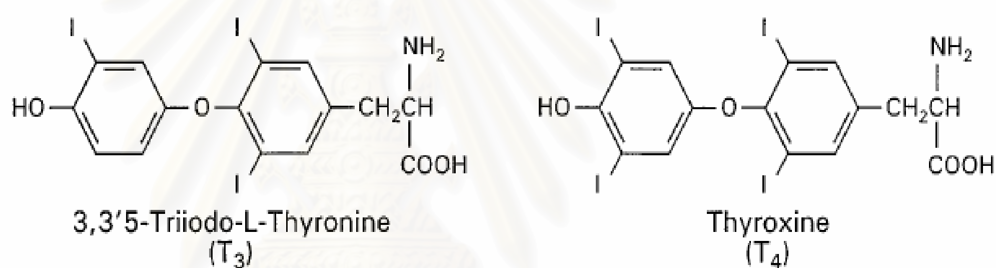


**Figure 2.4.** Structures of triiodothyronine ($T_3$) and L-thyroxine ($T_4$)

Thyroid hormones, $T_4$ and a more potent $T_3$, are synthesized in the thyroid gland (figure 2.3). Iodide is actively transported and concentrated into the thyroid by NIS. The trapped iodide is oxidized by TPO in the presence of hydrogen peroxide and incorporated into the tyrosine residues of a 660-kDa glycoprotein, Tg. This iodination of specific tyrosine located on Tg yields monoiodinated and diiodinated residues (MIT, monoiodo-tyrosine; DIT, diiodo- tyrosine) are enzymatically coupled to form $T_4$ and $T_3$. The iodinated Tg containing MIT, DIT, $T_4$, and $T_3$, then is stored as an extracellular storage polypeptide in the colloid within the lumen of thyroid follicular cells. Genetic defects along the synthetic pathway of thyroid hormones have been

described in humans and are major causes of congenital hypothyroidism in iodine-replete environments. The secretion of thyroid hormones requires endocytosis of the stored iodinated Tg from the apical surface of the thyroid follicular cell. The internalized Tg is incorporated in phagolysosomes and undergoes proteolytic digestion, recapture of MIT and DIT, and release of $T_4$ and $T_3$ into the circulation via the basal surface. Only 0.03% of the total serum $T_4$ is unbounded by proteins, but the remainder is bounded to carrier proteins such as thyroxine binding globulin (TBG), albumin, and thyroid binding prealbumin. Approximately 0.3% of the total serum $T_3$ is in the free form, and the remainder bounds to TBG and albumin. Only the free form enters target cells and generates a biological response (Yen, 2001).

**2.2.3. Mechanisms of thyroid Hormones**

The synthesis and secretion are exquisitely regulated by a negative-feedback system that involves the hypothalamus, pituitary, and thyroid glands [Hypothalamic/pituitary/ thyroid (HPT) axis] (figure 2.5).

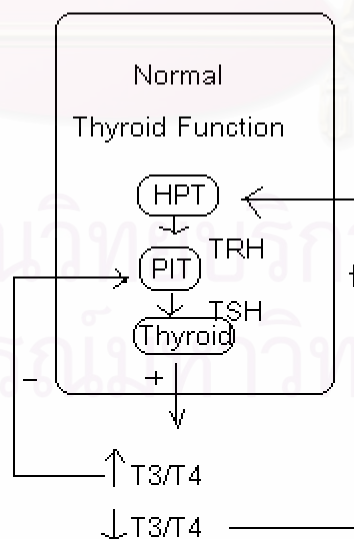


**Figure 2.5.** Hypothalamic/pituitary/ thyroid (HPT) axis

The action of $T_3$ on metabolism includes an increase in the basal rate of oxygen consumption and heat production that is generated via stimulation of ATP-requiring cellular processes such as the activity of enzymes and ion transport systems as well as the synthesis of DNA, RNA and other cellular components (Clausen et al., 1991; Guyton and Hall, 2000; Yen, 2001). Thyrotropin releasing hormone (TRH) is a tripeptide (PyroGlu-His-Pro), synthesized in the paraventricular nuclei of the hypothalamus. It is transported via axons to the median eminence and then to the anterior pituitary gland via the portal capillary plexus. TRH binds to TRH receptors in pituitary thyrotropes, a subpopulation of pituitary cells that secrete thyroid stimulating hormone (TSH). TRH receptors are members of the seven-transmembrane spanning receptor family and are coupled to Gq11. TRH stimulation leads to synthesis and release of TSH in thyrotropes. TSH is the primary regulator of thyroid hormone release and secretion. It also has a critical role in thyroid growth and development. TSH is a 28-kDa glycoprotein composed of $\alpha$- and $\beta$-subunits designated as glycoprotein hormone (Yen, 2001). Both TRH and TSH secretion are negatively regulated by thyroid hormones. An important mechanism for the negative regulation of TSH may be the intrapituitary conversion of circulating $T_4$ to $T_3$ by deiodinase type II (Maeda et al., 2003; Yen, 2001). The major production pathway for $T_3$ is via 5´-deiodination of the outer ring of $T_4$ by deiodinase enzymes, and accounts for the majority of the circulating $T_3$. Deioidinase type I is found in peripheral tissues such as liver and kidneys, and is responsible for the conversion of the majority of $T_4$ to $T_3$ in circulation. Deioidinase type I activity is known to decrease in the hypothyroid state and plays a critical role in maintaining circulating $T_3$ level. Deioidinase type II activity, in contrast, increases in the hypothyroid state and plays a critical role in

providing local intracellular $T_3$ (St. Germain and Galton, 1997; Koehrle, 1999; Bianco et al., 2002).

In this study, the deiodinase Type II is found in brain, pituitary, and brown adipose tissue that primarily convert $T_4$ to $T_3$ for intracellular use. These deiodinases have recently been cloned and demonstrated to be selenoproteins. 5´-Deiodination by deiodinase type I and deioidinase type III, which are found primarily in placenta, brain, and skin, lead to the generation of r $T_3$, an inactive form. r $T_3$ and $T_3$ can be further deiodinated in the liver, and are conjugated before excretion in the bile (Bianco et al., 2002; Yen, 2001). There is an enterohepatic circulation of thyroid hormones where intestinal flora deconjugate some of these compounds, and promote the reuptake of thyroid hormones. Although thyroid hormones may exert their effects on a number of intracellular loci, their primary effects are on the transcriptional regulation of target genes (Yen, 2001).

Early studies showed that the effects of thyroid hormones at the genomic level are mediated by nuclear TRs, which are associated with chromatin and bind to thyroid hormones with high affinity and specificity (Brent, 1994; Yen, 2001). Thyroid-stimulating hormone (TSH; thyrotropin) and TSH receptor (TSHR) are key proteins in the control of thyroid function. TSH synthesis in the anterior pituitary is stimulated by thyrotropin-releasing hormone (TRH) and inhibited by thyroid hormone in a classical endocrine negative-feedback loop. TSH controls thyroid function upon its interaction with the G protein-coupled TSHR (Szkudlinski et al., 2002). Then, somatostatin and dopamine from the hypothalamus can inhibit TSH secretion. TSH binds to the TSH receptor (TSHr), which also is a seven-transmembrane spanning receptor coupled to Gs (Parmentier et al., 1989; Yen, 2001). The activation of TSHr by TSH or

autoantibodies (in Graves' disease) leads to an increase in intracellular cAMP and stimulation of protein kinase A mediated pathways. A number of thyroid genes, including Na1/I2 symporter (NIS), thyroglobulin (Tg), and thyroid peroxidase (TPO), are stimulated by TSH and promote the synthesis of Thyroid hormones. Moreover, activating mutations in TSHr and Gs have been described in autonomously functioning thyroid nodules and familial congenital hyperthyroidism (Yen, 2001).

At first, Thyroid hormones action at the cellular level, and their metabolites are transport across the cell membrane by direct diffusion. In the cell, $T_3$ associates with cytosolic binding proteins, mitochondrial binding proteins and a specific nuclear receptor (Fig. 2.6) (Ichikawa & Hashizume, 1995; Yen., 2001). Association with cytosolic proteins is regarded as an intracellular reservoir and also allows transfer of hormones in the cytosol.

Interestingly, $T_3$ is also essential for normal growth and development. Most actions of $T_3$ are mediated via the nuclear receptor (TR). TR is a member of a large superfamily that includes receptors for steroids, retinoic acid, prostaglandins and fatty acids. In vitro, $T_3$ receptors bind to DNA as homodimers or as heterodimers with other nuclear protein that in most cases TR acts as a heterodimer with the retinoic acid receptor (RXR) (Brent, 1994; Yen, 2001). Furthermore, it is a ligand-modulated transcription factor capable to activate or repress gene expression (Muñoz & Bernal, 1997; Wu & Koenig, 2000; Lazar., 1993). Similar to steroid hormones that bind to nuclear receptors, thyroid hormones enters the cell and proceeds to the nucleus (figure 2.6).

The actions of thyroid hormone are primary the result of the interaction of $T_3$ with nuclear receptors for $T_3$ that bind to regulatory regions of genes (thyroid

hormone-response elements) and modify their expression (Glass et al.,1990; Brent et al., 1991a, 1994b).The formation of ligand-bound TR complexes that are also bound to TREs is the critical first step in the positive or negative regulation of target genes and the subsequent regulation of protein synthesis. Given their abilities to bind both ligand and DNA as well as their ability to regulate transcription, TRs can be regarded as ligand-regulatable transcription factors (Pantos et al., 2004).

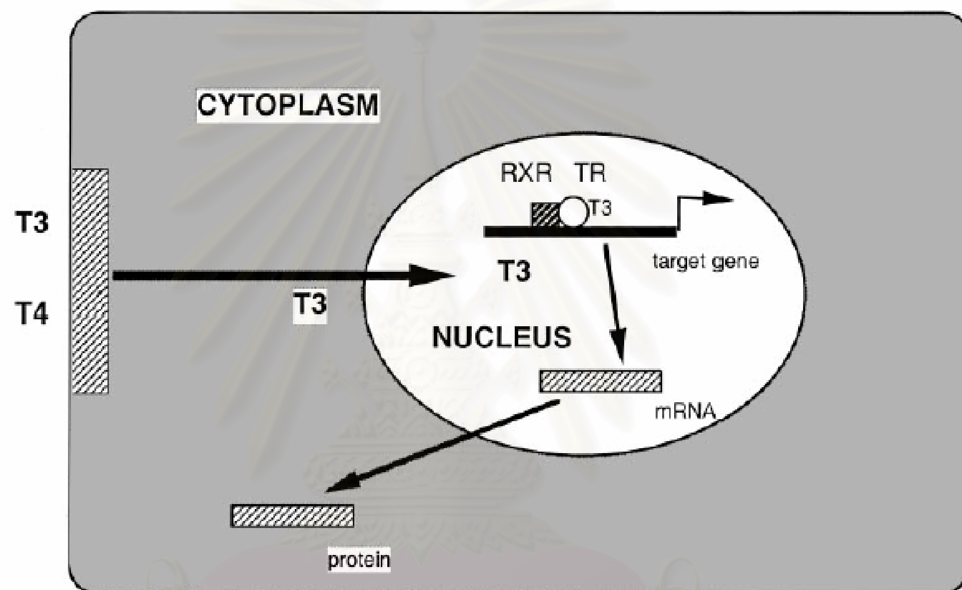


**Figure 2.6.** General model for thyroid hormone action in the nucleus. TR, thyroid hormonereceptor; RXR, retinoid X receptors(Yen, 2001).

Thyroid hormone receptors are two $T_3$-receptor genes, α-receptor and ß-receptor, located on chromosomes 17 and 3, respectively. Several receptor subtypes have been described of which the α-receptor and ß1-receptor are expressed in the heart (Lazar, 1993; Muñoz & Bernal, 1997; Brent, 1994). Transcription is modulated by binding of the unligand TR to specific DNA acceptor sites known as $T_3$ response elements (TRE), resulting in a repression of gene transcription. Association of $T_3$ with TR leads to a conformational change, thereby relieving the repression, resulting in

induction of gene transcription. In addition to the action of thyroid hormones mediated through nuclear receptors, direct and non-genomic effects have been described. Segal et al. (1989a; 1989b) showed that $T_3$ increases cellular amino acid and sugar uptake in presence of a protein-synthesis inhibitor. These non-genomic actions occur primarily at the plasma membrane (Segal, 1990; Huang et al., 1999) and the mitochondria (Wrutniak-Cabello et al., 2001). In a study of Goglia et al. (1999), high affinity binding sites for $T_3$ have been identified in the mitochondria and it was shown that $T_3$ has a direct effect on mitochondrial ATP production (see figure 2.7).

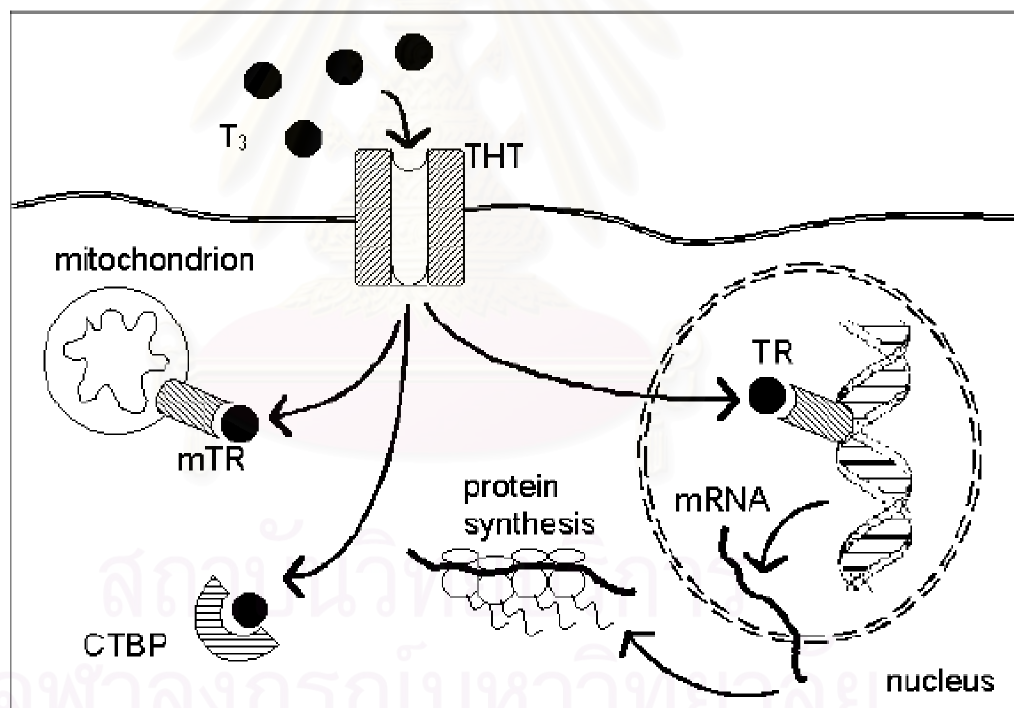


**Figure 2.7. $T_3$ action in the cell.** $T_3$ enters the cell via a carrier-mediated mechanism located in the plasma membrane, and binds to nuclear $T_3$ receptors to modulate transcription of genes. It can also associate with cytosolic binding proteins or with sites at the mitochondria. THT, plasma membrane thyroid hormone transporter;

CTBP, cytosolic binding protein; TR, nuclear thyroid hormone receptor; mTR, mitochondrial thyroid hormone receptor (Ichiwaka et al., 1995).

2.2.4. Transport of thyroid hormones

2.2.4.1. In blood

The action of thyroid hormones reach their sites by binding to serum proteins. The three major binding proteins in serum are thyroxine-binding globulin (TBG), transthyretin (TTR) and albumin (Bartalena, 1990; Schussler, 2000). Human TBG circulates at a concentration of only 0.4 – 0.8 μM. Nevertheless, because of its high affinity, TBG carries most of the serum $T_4$ (75% in humans) and $T_3$ (38-80% in humans). TTR, a binding protein of much more ancient lineage than TBG, is present in fish and higher vertebrates (Bartalena, 1990). TTR is synthesized in the liver and the concentration of TTR in plasma is about 5 μM (Visser & de Jong, 1998). In addition, choroid epithelial cells produce and secrete TTR, and it is suggested that this is an important source for brain $T_4$ (Bartalena, 1990; Schussler, 2000). Both TBG and TTR are considered to function as an extrathyroidal storage and as a buffer to protect cells from excessive hormone entry. The third binding protein for thyroid hormones and a variety of other compounds e.g. fatty acids in serum is albumin, it circulates at a concentration of 640 μM and binds $T_4$ with a 10- fold higher affinity than $T_3$. With the lowest affinity for thyroid hormones and the fastest thyroid hormone release compared to TBG and TTR, albumin may promote quick exchange of thyroid hormones with tissue sites (Curry et al., 1998; Krenning et al., 1979; Pardridge, 1987).

2.2.4.2. In tissues

Forty years ago, it was postulated that thyroid hormones

would pass the lipid bilayer of the cell membrane by passive diffusion (Lein & Dowben, 1961; Pardridge & Mietus, 1980). This was based on the fact that thyroid hormones are lipophilic and, as the plasma membrane is constituted of a lipid bilayer, there seemed no need for any other mechanism of translocation than that of diffusion. Today, it is clear that this assumption is not correct and that cells exhibit carrier-mediated mechanisms for the entry of thyroid hormones.

The criteria for the existence of these mechanisms are included of:

1) The mechanisms are specific; only structurally related compounds are transported or compete with the mechanism.

2) It is saturable and has limited capacity and no significant diffusion.

3) It is subject to regulation. The number of transporters, energy status and possibly the Na+ gradient may be important factors in regulation.

4) Finally, thyroid hormone uptake is rate limiting for subsequent metabolism (Yen, 2001; De La Vieja et al., 2000).

In patients with non-thyroidal illness (NTI) or during starvation, plasma $T_3$ production, converted from $T_4$, in the liver is decreased (Docter & Krenning., 1990), due to reduced cellular uptake and not because of defective 5'-deiodination of $T_4$ (Hennemann et al., 1993).Thus, transmembrane transport of thyroid hormones is important in the overall body thyroid hormone bioactivity.

2.2.5. Effect of thyroid hormone on the heart and vessels

The relationship between the cardiovascular system and thyroid hormones had shown that the heart is very sensitive to the action of thyroid hormones. Cardiovascular manifestations are a frequent finding in hyperthyroidism and hypothyroidism. Measurable changes in cardiac performance are detected with

small variations in thyroid hormone serum concentrations (Polikar et al.,1993; Rijnberk, 1996; Fadel et al., 2000; Klein & Ojamaa, 2001). Alterations in cardiac performance include an increase in resting heart rate (a chronotropic effect), contractility (an inotropic effect), left ventricular muscle mass, and predisposition to atrial arrhythmias. These effects are mediated by thyroid hormone regulation of transcription of cardiac-specific genes (Polikar et al., 1993; Klein & Ojamaa, 2001). The study showed that $T_3$ administration in animals enhances myocardial contractility by stimulating the synthesis of the fast $\alpha$ myosin heavy chain and inhibiting the expression of the slow $\beta$ isoform (Zähringer & Klaubert, 1982; Korecky et al., 1987; Williams & Ianuzzo, 1988; Dillmann, 1990). It also causes an increase in sarcoplasmic reticulum (SR) Ca2+ adenosine triphosphatase (ATPase) and a decrease in the expression of the Ca2+ ATPase regulatory protein phospholamban (Zähringer & Klaubert, 1982; Simonides et al., 1996; Muller et al., 1997). Additionally, the cardiac changes typical of hypothyroidism are opposite to those of hyperthyroidism, but they are accompanied by fewer signs and symptoms (Rijnberk, 1996; Klein & Ojamaa, 2001). Finally, thyroid hormone induced cardiac hypertrophy can also result from changes in the blood pressure and flow rate (Simpson et al., 1982; Klein et al., 1992). Thyroid hormone decreases blood pressure and flow rate by dilating the resistant arterioles of the peripheral circulation via effects on the vascular smooth muscle cells and by increasing blood volume (Klein & Ojamaa, 2001).

Thyroid hormones (THs) exert effects on cardiac function that result from direct effects of the hormones on the cardiac myocyte as well as effects on the peripheral vasculature (Klein & Ojamaa, 2001). The latter effect is best demonstrated by the characteristically high systemic vascular resistance (SVR) observed in

patients and experimental animals with hypothyroidism, which are rapidly reversed with TH supplement. Hyperthyroidism produces a marked decrease in SVR, which in turn facilitates an increase in cardiac output and augments peripheral blood flow characterized of this disease state.

### 2.2.6. Pharmacological and clinical usage of thyroid hormones

#### 2.2.6.1. Hypothyroidism

The clinical state of hypothyroidism develops whenever insufficient amounts of thyroid hormone are available to tissues. Hypothyroidism can result from various conditions. It often follows surgical thyroidectomy or therapy with radioiodine for treatment of Graves' disease. Developmental abnormalities and tumors and other infiltrative disorders that displace and destroy thyroid tissue can occasionally cause hypothyroidism. Congenital defects in hormonogenesis and the effect of drugs can also result in hypothyroidism. Pituitary and hypothalamic disease are rare conditions that occasionally lead to hypothyroidism caused by inadequate TRH or TSH secretion. Thyroid hormone is an important modulator of intermediary metabolism, as exemplified by the hypercholesterolemia of hypothyroidism. Hypothyroidism is a common clinical condition with increasing prevalence with advancing age and has been suggested to be a risk factor for atherosclerosis and cardiovascular diseases. Hypothyroidism is contributed to a proatherogenic lipid profile with greater effects at higher thyroid stimulating hormone levels (Hak et al., 2000; Ineck et al., 2003). Hypothyroidism is related with altered lipoprotein metabolism, inducing an increase in serum concentrations of intermediate-density lipoprotein (IDL) and low density lipoprotein (LDL) cholesterol. The altitude of

elevation in the serum cholesterol concentration is correlated with the degree of hypothyroidism (Packard et al., 1993; Wiseman et al., 1993).

#### 2.2.6.2. Hyperthyroidism

Hyperthyroidism is explained due to excessive delivery of thyroid hormones to the peripheral tissue (Porth, 1990). The reported overall prevalence of subclinical hyperthyroidism in humans ranges between 0.5 and 6.3%, and the prevalence being higher in patients over 65 years (Biondi, 2004). Hyperthyroidism is accompanied by multiple metabolic abnormalities, with increased energy expenditure and excessive mobilization and utilization of metabolic substrates. However, a study in rats which was experimentally induced to be hyperthyroid has shown that the hypermetabolic state leads to increases in productions of oxygen free radicals and lipid peroxidation (Fernandez et al., 1985). Additionally, the enhanced LDL oxidation is found in both the hypothyroid and hyperthyroid states (Sundaram et al., 1997).

### 2.2.7. Thyroid hormones and Atherosclerosis

Thyroid hormones have various effects on lipid metabolism that potentially could be the cause of atherosclerosis and ischemic heart disease. In fact, low density lipoprotein (LDL) receptor expression can be decreased after thyroxine treatment. (Ness et al., 1990), and thyroid hormones and their derivatives may have LDL-antioxidant properties. Their importance may be related to their 49-hydroxy diphenyl ether structure and depending upon the nature and the position of substituents in this structure (Chomard et al., 1998). Thyroid analogue with specific action on this receptor has been recently developed as an effective cholesterol lowering agent (Trost et al., 2000). Interestingly, the reported evidence shows that

thyroid hormone might protect against macrophage- induced LDL oxidation (Oziol et al., 2003).

2.2.7.1. Effects of L-thyroxine ($T_4$) on atherosclerotic formation

The data shown that elevated the serum LDL cholesterol concentration in hypothyroid patients and the response to thyroxine treatment are strongly correlated with the genotype of the LDL – receptor gene.(Wiseman et al., 1993) This presented that the increased LDL susceptibility to oxidation in hypothyroidism is a risk factor for atherosclerosis. Increases both the lipid [total fatty acids (FA)] and apolipoprotein (apoB-100) contents of the low density lipoprotein (LDL) are in accordance with overt hypothyroidism. Moreover, an increase in LDL oxidation is secondary to less $T_4$ available for binding to LDL, since $T_4$ functions as an anti-oxidant of LDL in vitro. This data concluded that subclinical hypothyroidism deserves substitutive $T_4$ therapy (Dieckman et al., 1997; Sundaram et al., 1997; Hanna et al., 1993; Hanna et al., 1995). The recent evidence has been shown that increased $T_4$ concentrations in pigs fed with oxidized oil were associated with reduce plasma cholesterol concentrations (Eder and Stangl., 2000).

2.2.7.2. Effects of triiodothyronine ($T_3$) on atherosclerotic formation

According to data in human fibroblasts verified that the $T_3$-induced increase in LDL degradation was mediated through an increase in LDL receptor number, without any change in the affinity of LDL for its receptor. A specific effect of thyroid hormone on the LDL receptor was suggested by a lack of $T_3$ effect on LDL concentration in cultured cells without LDL receptors (Chait et al., 1979).

# CHAPTER III

# MATERIALS AND METHODS

## Experimental Animals

This experiment was performed on twenty-five male New Zealand White rabbits purchased form the Faculty of Veterinary Science, Chulalongkorn University weighing between 2.0-2.5 kg. They were individually caged in the Laboratory Animal Unit at the Department of Physiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. The environmental were conditioned in room temperature (27± 2 °C), a relative humidity of approximately 70%, under a 12 hr light/ 12 hr dark cycle. Water was allowed ad libitum, and 80 g/ kg body weight of food was provided.

## Experimental Protocol

The experimental animals were randomly divided into five groups, each group contained 5 animals. The animals were given specific food as described below for twelve weeks.

Group I: rabbits were fed with standard chow diet.

Group II: rabbits were fed with lipid-rich food (containing: standard chow, 4% coconut oil, 1% cholesterol in egg yolk).

Group III: rabbits were fed with lipid-rich food (containing: standard chow, 4% coconut oil, 1% cholesterol in egg yolk), and were given oral propylthiouracil (PTU) 20 mg/kg body weight daily.

Group VI: rabbits were fed with lipid-rich food (containing: standard chow, 4% coconut oil, 1% cholesterol in egg yolk), and were given oral PTU 20 mg/kg body weight daily plus oral L-thyroxine ($T_4$) 50 μg/kg body weight daily.

Group V: rabbits were fed with lipid-rich food (containing: standard chow, 4% coconut oil, 1% cholesterol in egg yolk), and were given oral PTU 20 mg/kg body weight daily plus oral triiodothyronine ($T_3$) 2 μg/kg body weight daily (Van Der Wal., 1998).

Body weight and rectal temperature were measure weekly. Blood samples of the animals were drawn before start the experiment and at the twelve week for measurements of thyroid hormones, triglyceride, and cholesterol. At the end of the experiment, the animals were anesthesized with sodium pentobarbital at a dose of 25 mg/kg IV, and the heart and great vessels were rapidly excised. The vessel tissues were divided for measurement of LDL oxidation and histological analysis.

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**Determination of triglyceride, total cholesterol, $T_3$ and $T_4$**

Triglycerides and total cholesterol were determined by enzymatic assays. Briefly, blood samples were collected from marginal ear vein, and serum samples were prepared by centrifugation at 1500 g for 15 minutes at 4 °C. Serum sample each 100 μl were taken to determine the triglycerides and total cholesterol concentrations by using commercially available kits with enzymatic assays kit

(Meguro et al., 2003; Mitani et al., 2003). Serum sample each 100 µl were taken to determine total $T_3$ and total $T_4$ by chemiluminescence method. Measurement of serum thyroxine and triiodothyronine concentration are generally regarded as an important in vitro diagnostic test for assessing thyroid function. The principle of the test was competitive enzyme immunoassay. (Immulite, DPC Co, Ltd.)

**Determination of LDL Oxidation**

1. **Plasma LDL oxidation**

Blood samples were collected from marginal ear vein, and placed in EDTA – coated tubes. Plasma samples were separated by centrifugation at 1750 g for 10 minutes. All samples were stored at -80 °C until being analyzed (Quiles et al., 2002). For each sample, 2 ml of frozen plasma was rapidly thawed and used for isolation of LDL. The preparation of low density lipoprotein (LDL; density (d) =1.019 -1.063 g/ml) was isolated by sequential ultracentrifugation at 4 °C(Van de Vijver et al., 1998).The plasma density was adjusted to d =1.019 g/ml by adding solid KBr. The plasma were transferred to Beckman Optiseal tubes (Beckman instruments) for the Beckman optima XL70 near-vertical rotor. The tubes were capped and centrifuged at 65,000 rpm for 4 hrs at 4 °C (Morena et al., 2000). After centrifugation, the upper layer of supernatant was removed. The infranate was adjusted to d =1.063 g/ml by adding solid KBr and transferred to a new tube and were capped. Recentrifugation was performed at 65,000 rpm for 4 hrs at 4 °C. After that, the LDL fraction, which floats at a relative density of 1.063 g/ml, was collected from the top layer. The degree of LDL oxidation was determined by using the thiobarbituric acid reactive substances assay (Kaplan et al., 2001).

2. TBARS determination

Lipid peroxidation of LDL was assessed by thiobarbituric acid-reactive substances (TBARS) formation. Briefly, samples were incubated with 0.5 ml of 20% acetic acid, pH3.5, and 0.5 ml of 0.78% aqueous solution of thiobarbituric acid. After heating at 95 ºC for 45 minutes, the samples were centrifuged at 4,000 rpm for 5 minutes. The supernatant fraction was estimated by spectrophotometric absorbance at 532 nm. The TBARS concentration in the samples was measured against the malondialdehyde (MDA) standard curve. The protein content of the LDL was measured by the Lowry's method, using bovine serum albumin as a standard protein. Results were expressed as nmole of MDA /mg LDL protein (Scoccia et al., 2001).

Histological analysis

1. Preparation of histological sections

After thoracotomy, tissue samples were collected from the aortic arch, the thoracic aorta, the abdominal aorta, and the proximal portion of the left coronary artery. They were separated into two parts, the first part was dissected about 5 mm long for analyzing of atherosclerotic lesions and the second part was dissected about 3 mm long for analyzing of intimal thickening and compositions of the lesions.

The first parts of the aortic arch, the thoracic aorta, and the abdominal aorta, were dissected and fixed with 4% formaldehyde for a day before studying atherosclerotic lesions.

The second parts of the aortic arch, the thoracic aorta, the abdominal aorta, and proximal portion of left coronary artery were dissected and fixed with 4%

formaldehyde for four days. After that, the tissues were trimmed and embedded in paraffin from which further 5 μm cross-sections.

**2. Quantitative analysis of atherosclerotic lesions**

The aortic arch was dissected and removed into segments. The thoracic aorta was dissected and removed from proximal portion of thoracic aorta. The abdominal aorta was dissected and removed from proximal portion of abdominal aorta. Each vessel was dissected and removed the adventitia and perivascular fat as much as possible to prevent errors resulting from Sudan IV staining of vessels. The vessel was opened longitudinally, pinned flat on styrofoam surface. After overnight fixation in 10% formalin and rinsed in 70% ethanol for 10 minutes, the tissue was stained with 0.5% Sudan IV in 35% ethanol and 50% acetone for 20 minutes. Destaining was carried out for 20 minutes in 80% ethanol (Staprans et al., 1998). After staining with Sudan IV, atherosclerotic lesions were photographed. The extent of sudanophilia (red color), described as the atherosclerotic lesion on the aortic surface, was determined as the ratio of the lesion surface area to the total intimal surface area of the vessel by planimetry with computer-assisted morphometry system (Kwon et al., 2003; Meguro et al., 2003; Mitani et al., 2003). (as shown in figure 3.1) The details were used to calculate the intimal thickening as the ratio between the intima and the intima plus media areas (Jacobsson et al., 2004).

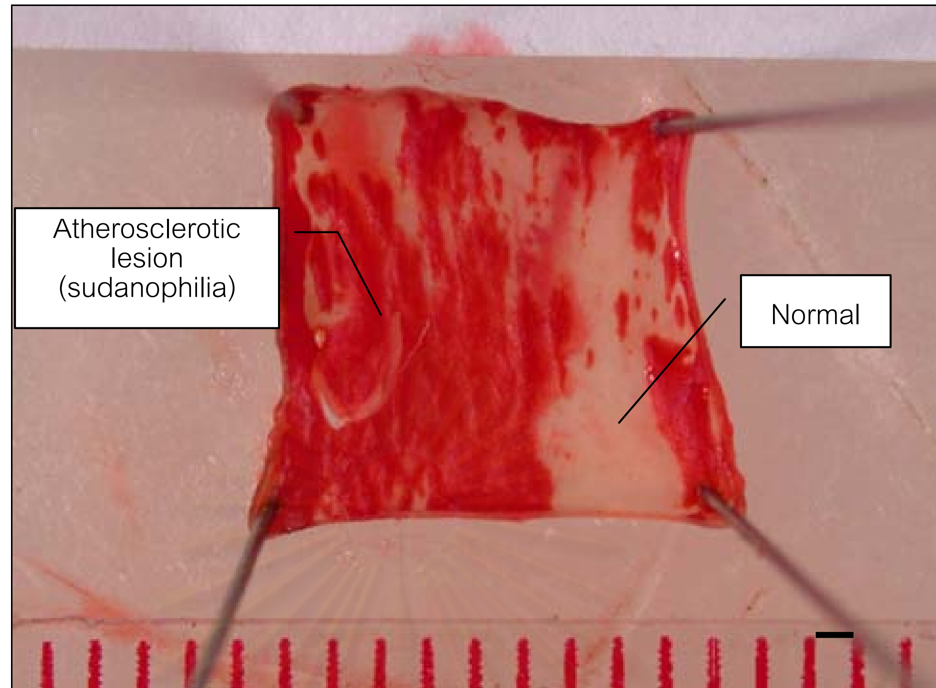


Figure 3.1. A photograph of the pinned-out thoracic segment of the aorta, stained with Sudan IV (10X) from New Zealand white rabbit (Bar =1000 μm).

3. Quantitative analysis of intimal thickening

The intimal thickening of vessel was determined from the areas of tunica intima and tunica media of the cross-sections, stained with hematoxylin and eosin. After lesions were photographed, pictures of vascular lesions were analyzed using a color image analysis system from a light microscope and computer-assisted morphometry system. The mean thickeness of the intima (area of the intimal lesion / length of media) were measured by an image analysis software (Image Tool), as shown in figure 3.2 (Meguro et al., 2003; Mitani et al., 2003).

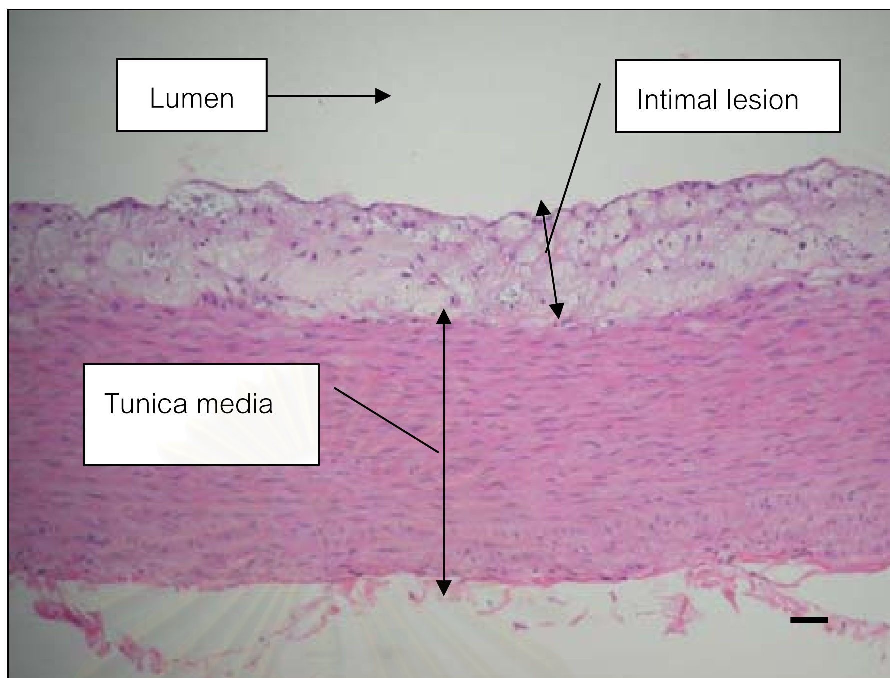


**Figure 3.2.** Microscopic analysis of the intimal thickness lesion of the thoracic aorta. The representative photograph was stained with hematoxylin and eosin (Bar = 50 μm).

**4. Quantitative analysis of lesional composition**

To examine the cellular composition of atherosclerotic lesion, tissues were stained immunohistochemically, and Masson's trichrome. The serial cross-sections of aortic arch, thoracic, and abdominal aorta were stained immunohistochemically with monoclonal antibodies against rabbit macrophage (RAM 11, DAKO CO., CA, USA) and human smooth muscle cell actin (HHF35, DAKO). After that, target tissues were visualized with Vectastain Elite ABC Mouse IgG kit (Vector, CA, USA) and 3, 3' – diaminobenzidine (DAKO) as described below.

At frist, the serial cross-sections were deparafinized with xylene for 3 times-10 minutes each, xylene plus absolute alcohol for 2 minutes, absolute alcohol for 2 times-2minutes each, 95% alcohol for 2 minutes, running water for 5 minutes, distilled water for 5 minutes and PBS for 5 minutes. For the antigen

retrieval method, the tissue section was performed with the microwave retrieval technique by heating with microwave for 5 minutes in Tris EDTA pH 9, and allowed to cool for 30 minutes. After that, the tissue section was washed with PBS for 5 minutes, 3 times. For blocking endogenous peroxidase, the tissue section were incubated with 0.3% $H_2O_2$ for 30 minutes at room temperature, then washed in distilled water for 5 minutes, followed by PBS for 5 minutes, 3 times. For blocking serum, the tissue section was incubated for 30 minutes at 37 °C with diluted normal blocking serum which was prepared from the species in which the secondary antibody was made, then washed in PBS for 5 minutes, 3 times. Next step, the tissue section was incubated overnight at 4 °C with primary monoclonal antibodies diluted in sterilized PBS. After that, the section was allowed to warm for 15 minutes at room temperature, and washed in PBS for 5 minutes, 3 times. The section was then incubated with diluted biotinylated secondary antibody solution, followed by PBS for 5 minutes, 3 times. After that, the section is incubated with Vectastain Elite ABC reagent(Vector, CA, USA) for 30 minutes at 37 °C, washed in PBS for 5 minutes, 3 times, and then was incubated in 3, 3' – diaminobenzidine (DAB peroxidase substrate solution) (DAKO) for 30 seconds before the section was rinsed with distilled water. The section was counter-stained with Mayer's hematoxylin solution. Finally, the section is underwent the processed of dehydration, clearing, and mounting (figures 3.3 and 3.5).

The Masson's trichrome staining was performed to determine collagen stain blue element and extracellular lipid deposits were stained slightly blue or light (figure 3.4). The stained areas of each cellular and extracellular components (macrophage, smooth muscle cell, collagen, and extracellular lipid deposit) were determined by an

image analysis software (Image Tool); then, expressed as a percent of each staining area in the cross-sectional lesion area of the artery examined.

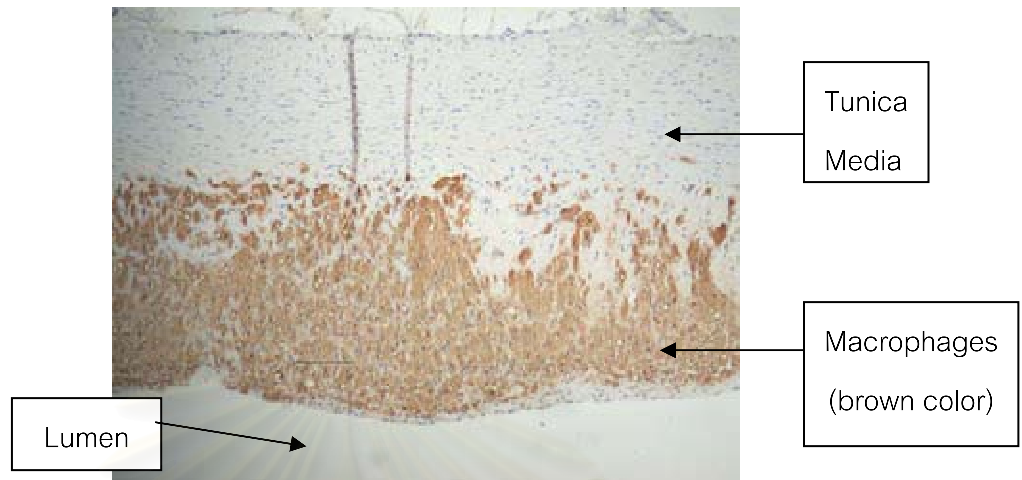


**Figure 3.3.** Immunohistochemical analysis of macrophages in the atherosclerotic lesion of the aorta. The representative micrographs stained with RAM11 monoclonal antibody (DAB) (Bar = 200 μm).

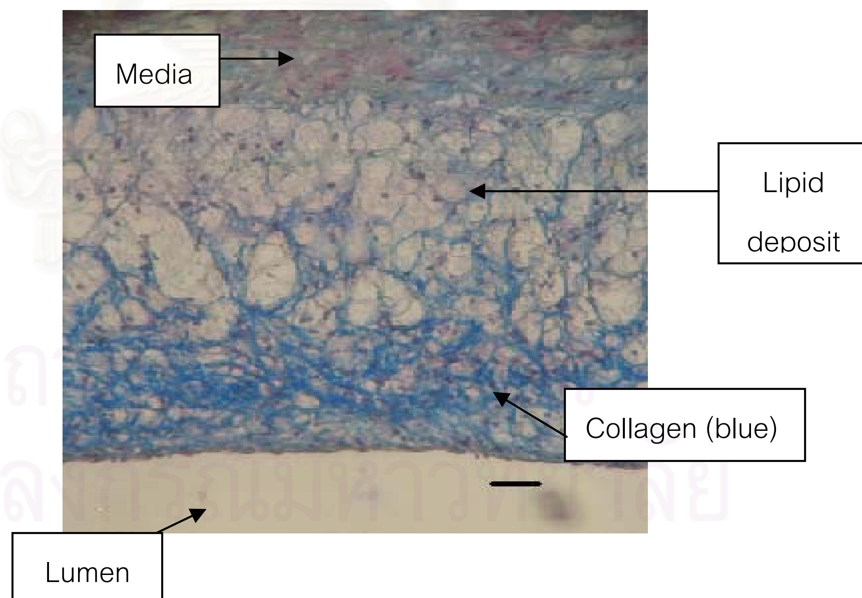


**Figure 3.4.** Microscopic analysis of collagen and lipid of the intimal lesion of the aorta. The representative micrographs stained with Masson’s trichrome (Bar = 40 μm).

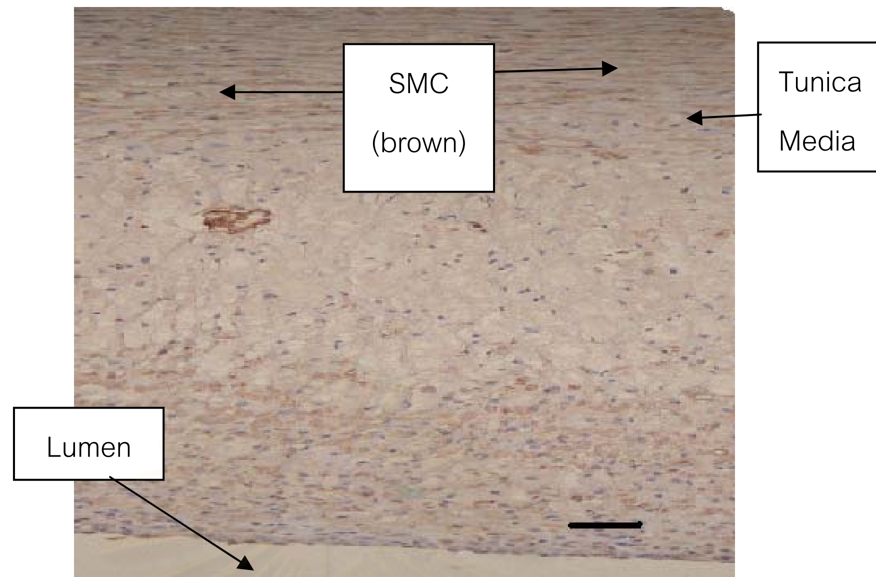


Figure 3.5. Immunohistochemical analysis of smooth muscle cells (SMC) in the atherosclerotic lesion of the aorta. The representative micrographs stained with HHF35 monoclonal antibody (DAB) (Bar = 40 μm).

## Statistical Analysis

All data are presented as means ± SD. The effects of treatments were analyzed using one-way Analysis of Variance (ANOVA) followed by the Post hoc, Student-Newman Keuls method. Differences between means were considered significantly at $P<0.05$.

# CHAPTER IV

# RESULTS

## Body weight and body temperature.

The body temperatures are shown in figure 4.1. Body temperatures were significantly declined in group 2 and 3 compared to group 1 (the control). The significant increases in body temperature were also observed in groups 4 and 5 compared to other groups at the twelve week.

Body weights of the animals are shown in figure 4.2 as % change of values at the end of experiment compared to starting the experiment. There were no significant differences in body weight changes among groups excepted in group 4 (lipid-rich food plus PTU and T4) and group 5 (lipid-rich food plus PTU and T3) which were significantly decreased, compared to group III (lipid-rich food plus PTU).

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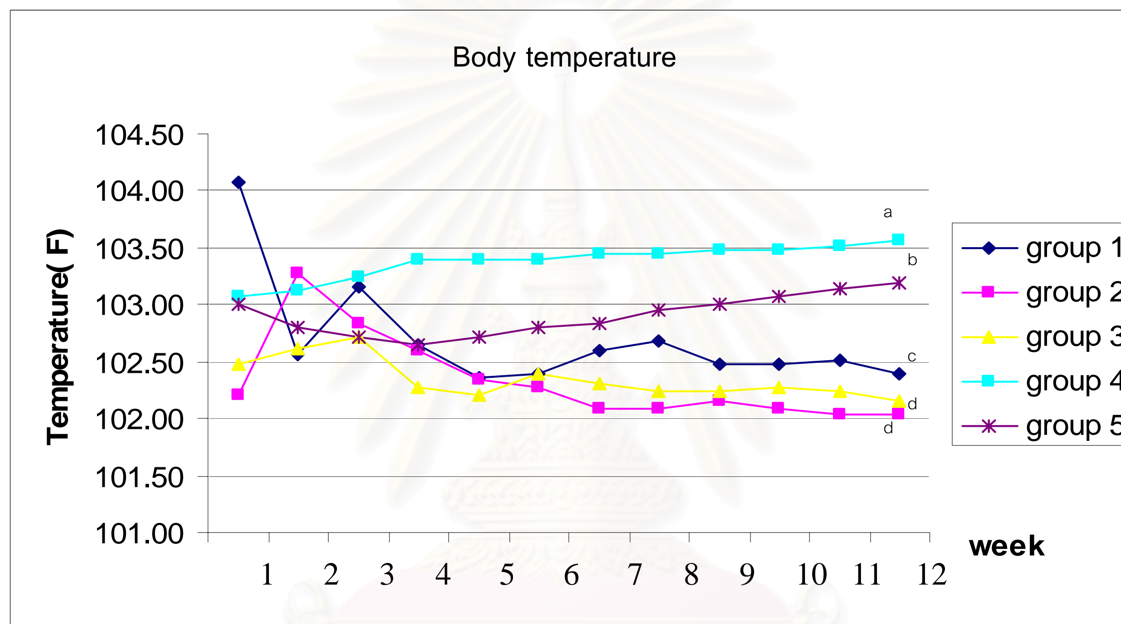


Figure 4.1. Weekly mean values for body temperature in five groups of rabbits throughout the experimental period.

Group 1; control, Group 2; lipid-rich food, Group 3; lipid-rich food +PTU, Group 4; lipid-rich food +PTU+T4, Group 5; lipid-rich food +PTU+T3.Different superscripts represent statistical significant differences at the twelve week ($P < 0.05$)

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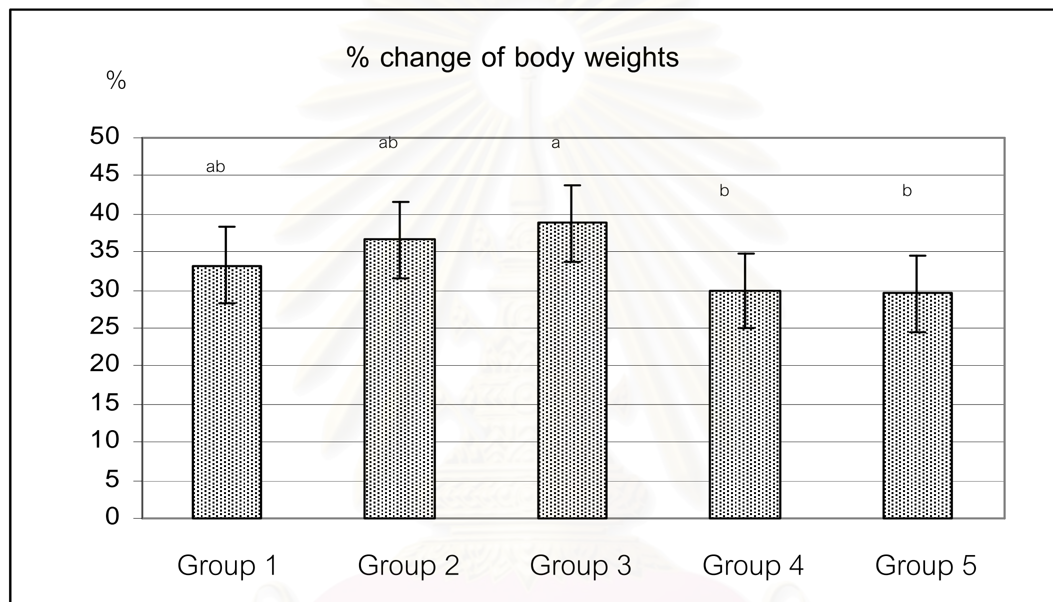


Figure 4.2. Effect of Thyroid hormone on percent changes of body weights in five groups. Group 1; control, Group 2; lipid-rich food, Group 3; lipid-rich food +PTU, Group 4; lipid-rich food +PTU+T4, Group 5; lipid-rich food +PTU+T3. Different superscripts represent statistical significant differences ($P < 0.05$)

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**Concentrations of triglyceride and total cholesterol.**

Serum triglyceride and total cholesterol in each group are presented in figure 4.3. After 12 weeks of treatments, there was a significant decrease in percent changes of triglycerides of animals in group 3 compare to group 1. Total cholesterols were also significantly increased in groups 2, 3 and 4 compared to group 1. Additionally, total cholesterol was also significantly decreased in group 5 compared to group 2.

**Concentrations of triiodothyronine (total T3) and thyroxine (total T4).**

Percent changes of triiodothyronine (total T3) and thyroxine (total T4) in serum of each group are presented in figure 4.4. There were no significant differences of triiodothyronine (total T3) among groups except group 5 which were significantly increased. On the other hand, thyroxine (total T4) was also significantly decreased in group 5 compared to the others. Although, thyroxine levels in group 4 were not significant difference, but they tended to have the highest values compared to all groups.

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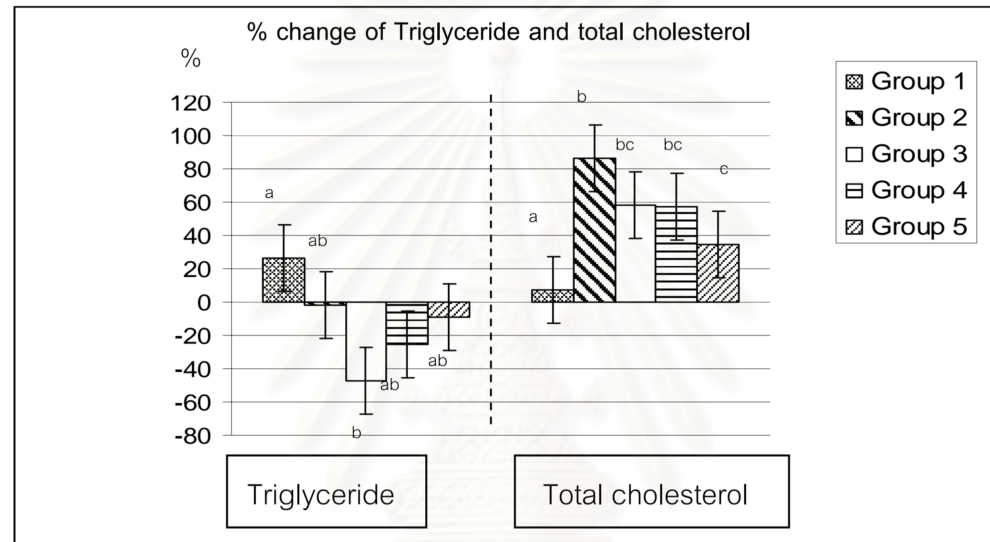


Figure 4.3. Percent change of mean values triglyceride and percent change of mean values total cholesterol in experimental groups. Group 1; control, Group 2; lipid-rich food, Group 3; lipid-rich food +PTU, Group 4; lipid-rich food +PTU+T4, Group 5; lipid-rich food +PTU+T3. Different superscripts represent statistical significant differences ($P < 0.05$)

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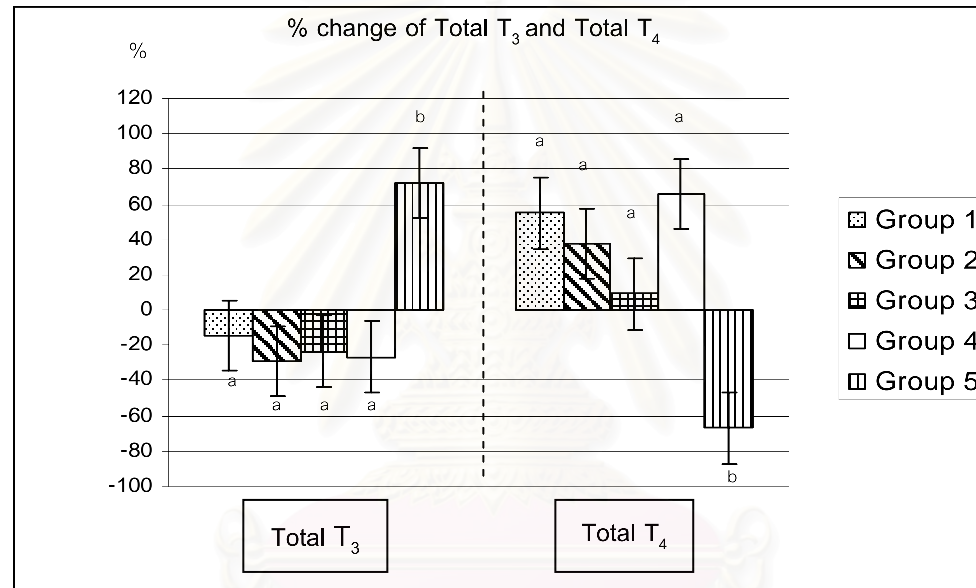


**Figure 4.4.** Percent change of mean values total $T_3$ and total $T_4$ in experimental groups. Group 1; control, Group 2; lipid-rich food, Group 3; lipid-rich food +PTU, Group 4; lipid-rich food +PTU+T4, Group 5; lipid-rich food +PTU+T3. Different superscripts represent statistical significant differences ($P < 0.05$)

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**Effect of Thyroxine and Triiodothyronine on malondialdehyde (MDA) in low density lipoprotein (LDL) content and plasma.**

The plasma MDA of Group 2 (lipid-rich food), was significantly increased, compared to group 1(control), group 3 (lipid-rich food plus PTU), and group 4 (lipid-rich food plus PTU and T4). Moreover, plasma MDA of group 5 (lipid-rich food plus PTU and T3) was significantly increased compared to group1 (control) and group 3 (lipid-rich food plus PTU). Additionally, LDL- MDA of group 4 (lipid-rich food plus PTU and T4) was significantly decreased, compared to group 1 (control), group 2(lipid-rich food), group 3 (lipid-rich food plus PTU) and group 5 (lipid-rich food plus PTU and T3) (figure 4.5).

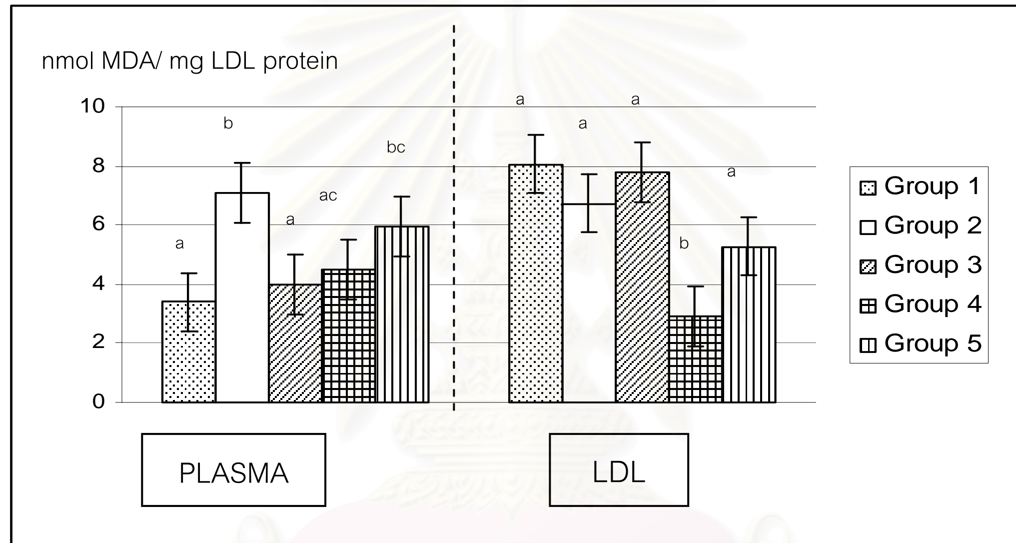


Figure 4.5. Effect of Thyroxine and Triiodothyronine on malondialdehyde (MDA) in LDL and MDA in plasma.The data were show as mean ± SD; n =5 (LDL; nmol MDA/ mg LDL protein, Plasma; nmol MDA/ml) Group 1; control, Group 2; lipid-rich food, Group 3; lipid-rich food +PTU, Group 4; lipid-rich food +PTU+T4, Group 5; lipid-rich food +PTU+T3. Different superscripts represent statistical significant differences ($P<0.05$)

Atherosclerotic lesion area

Figure 4.6 shows photographs of Sudan IV-stained aortic arch, thoracic aorta and, abdominal aorta, and table 4.1 shows the ratio of atherosclerotic lesion area in the aortic arch, the thoracic aorta, and the abdominal aorta from each group.

The ratio of aortic atherosclerotic lesions in all areas of groups 2, 3, 4, and 5 were significantly higher compared to group 1, but degrees of atherosclerotic increments of animals in groups 2 and 3 were significantly higher compared to groups 4 and 5 especially the aortic arch and the thoracic aorta.

Intimal thickening

The intimal thickening of the aortic arch, the thoracic aorta, and the abdominal aorta of groups 2, 3, 4, and 5 were significantly increased, compared to group 1. There was no significant difference among groups 2, 3, 4, and 5 as shown in table 4.2 and figure 4.7.

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Table 4.1. Atherosclerosis lesion in the experimental groups.

| Experimental group | Ratio of atherosclerotic lesion (mean ± SD) | | |
|---|---|---|---|
| | Aortic arch | Thoracic aorta | Abdominal aorta |
| Group 1 | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ |
| Group 2 | 0.82±0.19$^{b}$ | 0.70±0.09$^{b}$ | 0.62±0.38$^{b}$ |
| Group 3 | 0.83±0.18$^{b}$ | 0.63±0.12$^{bc}$ | 0.44±0.34$^{b}$ |
| Group 4 | 0.40±0.22$^{c}$ | 0.54±0.07$^{c}$ | 0.41±0.19$^{b}$ |
| Group 5 | 0.53±0.09$^{c}$ | 0.55±0.05$^{c}$ | 0.51±0.06$^{b}$ |

Different superscripts in the same column represent statistical significant differences ($P<0.05$)

The surface area of atheromatous lesions were measured by Image Tool analyzer. Group 1; control, Group 2; lipid-rich food, Group 3; lipid-rich food +PTU, Group 4; lipid-rich food +PTU+T4, Group 5; lipid-rich food +PTU+T3.

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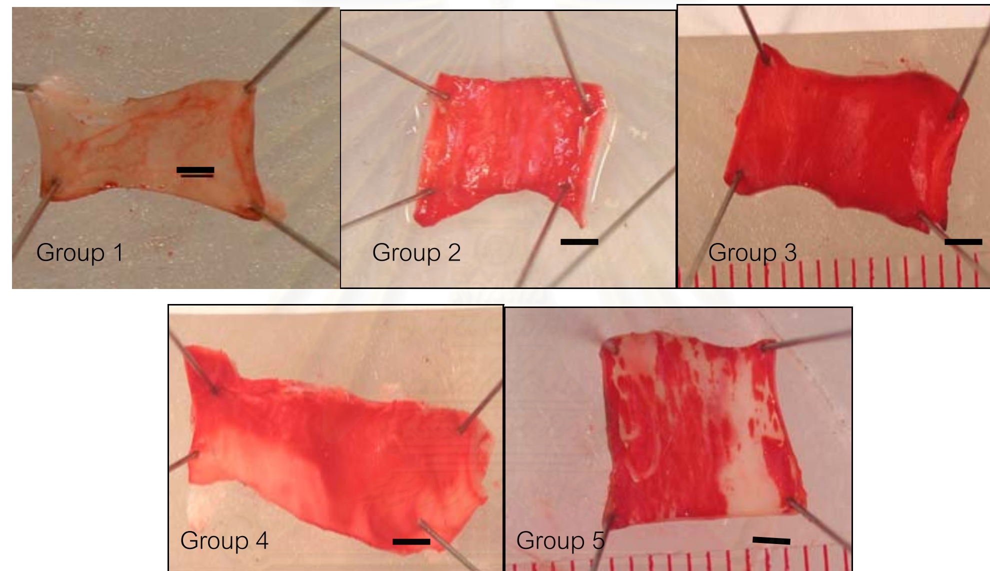


**Figure 4.6.** Photographs of the pinned-out subintimal surface of thoracic aorta from new zealand white rabbit stained with Sudan IV. The surface area of atheromatous lesions were measured by Image Tool analyzer (UTHSCSA; version 3.0) (Bar =0.2 mm).

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Table 4.2. Intimal thickening in the experimental groups.

| Experimental group | Intimal thickening (mean ± SD) | | |
|---|---|---|---|
| | Aortic arch | Thoracic aorta | Abdominal aorta |
| Group 1 | $0.00 \pm 0.00^{a}$ | $0.00 \pm 0.00^{a}$ | $0.00 \pm 0.00^{a}$ |
| Group 2 | $1.66 \pm 0.45^{b}$ | $1.20 \pm 0.85^{b}$ | $0.60 \pm 0.48^{b}$ |
| Group 3 | $1.53 \pm 1.55^{b}$ | $0.86 \pm 0.27^{b}$ | $0.61 \pm 0.24^{b}$ |
| Group 4 | $1.63 \pm 0.97^{b}$ | $0.94 \pm 0.59^{b}$ | $0.88 \pm 0.57^{b}$ |
| Group 5 | $1.08 \pm 0.35^{b}$ | $0.96 \pm 0.38^{b}$ | $0.83 \pm 0.26^{b}$ |

Different superscripts in the same column represent statistical significant differences ($P < 0.05$)

Intimal thickening were measured by Image Tool analyzer. Group 1; control, Group 2; lipid-rich food, Group 3; lipid-rich food +PTU, Group 4; lipid-rich food +PTU+T4, Group 5; lipid-rich food +PTU+T3.

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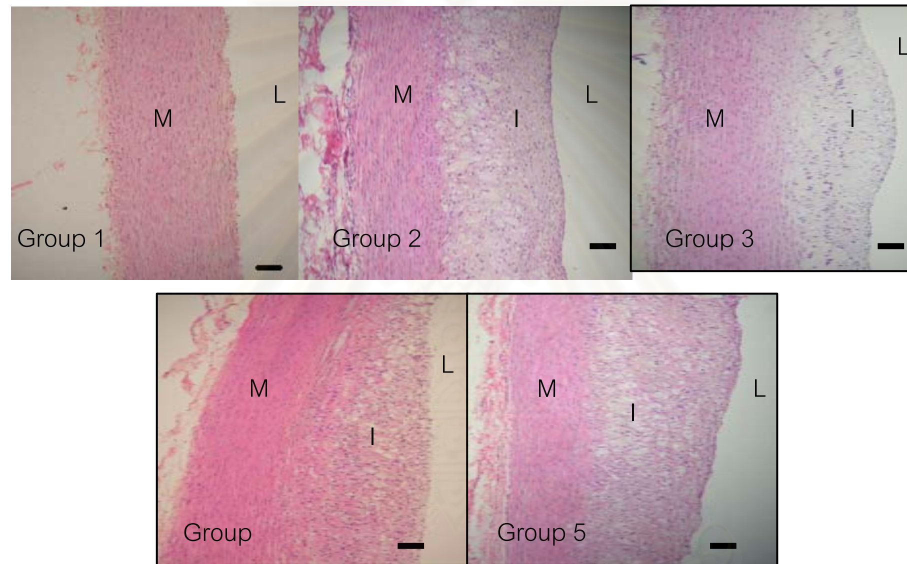


Figure 4.7. Microscopic demonstration of the intimal thickness lesion of the thoracic aorta. The micrographs were stained with hematoxylin and eosin (Bar = 10 μm). L; lumen, M; tunica media, I; intimal lesion.Group 1; control, Group 2; lipid-rich food, Group 3; lipid-rich food +PTU, Group 4; lipid-rich food +PTU+T4, Group 5; lipid-rich food +PTU+T3.

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Lesional composition

The cellular compositions of atherosclerotic lesions of each group are shown in table 4.3 and figures. 4.8, 4.9, and 4.10. The areas of macrophage, SMC, collagen and lipid were significantly increased in groups 2, 3, 4, and 5 compared to group 1. The areas of macrophage in groups 4 and 5 were also significantly lower than the areas of macrophage in groups 2 and 3. The areas of SMC, collagen, and lipid in groups 2, 3, 4, and 5 were significantly increased, compared to group 1. The areas of collagen and lipid were also highest in group 3 compared to the others.

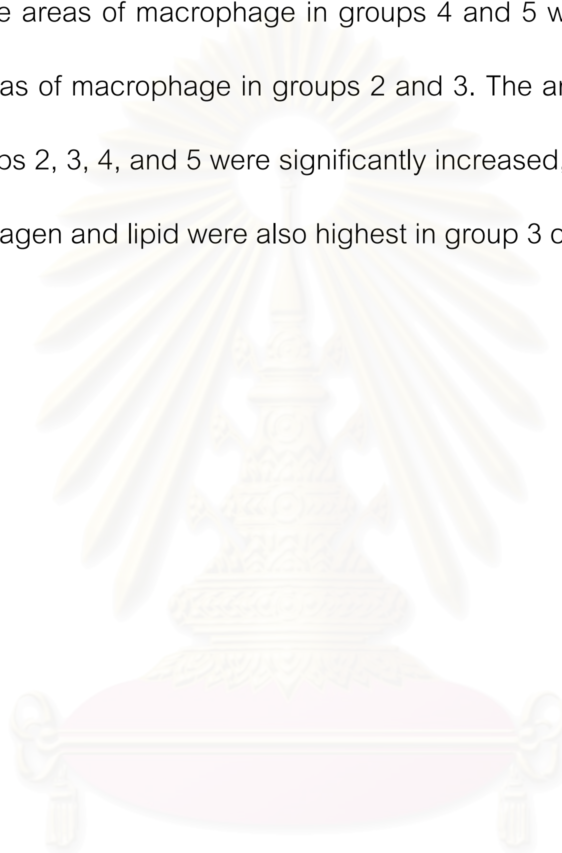

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Table 4.3. Cellular and extracellular components in atherosclerotic lesions.

| Experimental group | Lesion Components (%) (mean ± SD) | | | |
|---|---|---|---|---|
| | Macrophage | SMC | collagen | lipid |
| Group 1 | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 4.776 ±1.01$^{a}$ |
| Group 2 | 32.72±1.79$^{b}$ | 11.41±0.86$^{b}$ | 21.44± 0.94$^{b}$ | 43.60 ±1.14$^{b}$ |
| Group 3 | 33.43±1.43$^{b}$ | 11.95±0.63$^{b}$ | 22.85± 0.49$^{c}$ | 46.00 ± 0.79$^{c}$ |
| Group 4 | 26.06±0.93$^{c}$ | 11.99±0.54$^{b}$ | 20.42± 0.47$^{d}$ | 43.19 ± 0.87$^{b}$ |
| Group 5 | 27.47±2.28$^{c}$ | 12.13±0.61$^{b}$ | 21.15±0.58$^{b,d}$ | 44.62 ± 0.97$^{b}$ |

Different superscripts in the same column represent statistical significant differences ($P < 0.05$)

Group 1; control, Group 2; lipid-rich food, Group 3; lipid-rich food +PTU, Group 4; lipid-rich food +PTU+T4, Group 5; lipid-rich food +PTU+T3.

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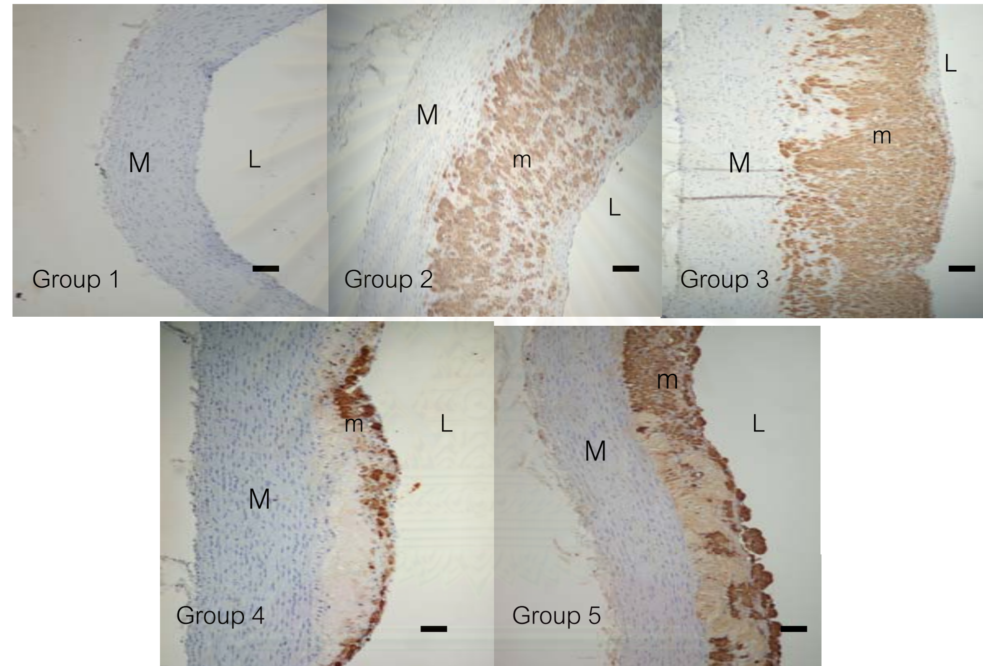


Figure 4.8. Immunohistochemical staining of macrophages infiltration in the atherosclerotic lesion in the tunica intima of the thoracic aorta. Representative micrographs were stained with RAM11 monoclonal antibody (DAB) (Bar = 10 μm). L; lumen, M; tunica media, m; macrophage. Group 1; control, Group 2; lipid-rich food, Group 3; lipid-rich food +PTU, Group 4; lipid-rich food +PTU+T4, Group 5; lipid-rich food +PTU+T3.

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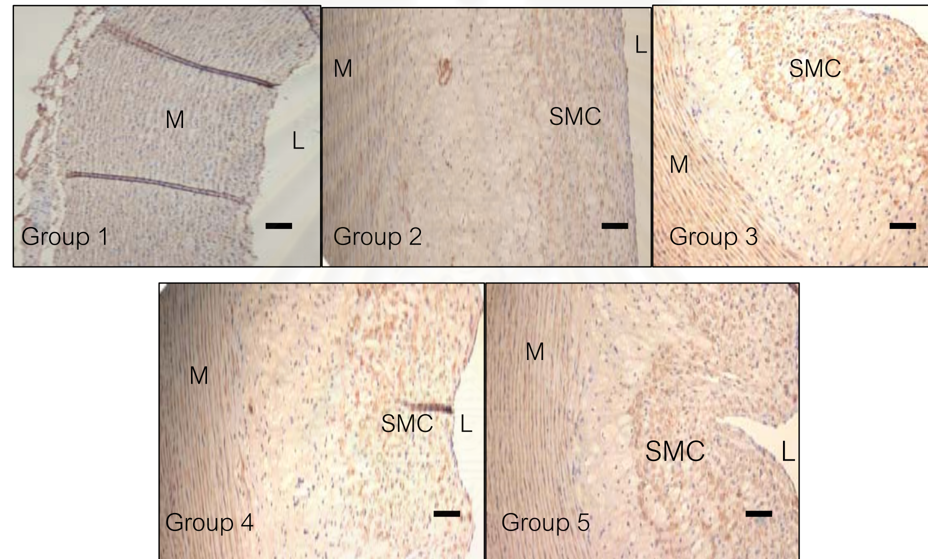


Figure 4.9. Immunohistochemical staining of smooth muscle cells (SMC) in the atherosclerotic lesion of the thoracic aorta. The micrographs were stained with HHF35 monoclonal antibody (DAB) (Bar = 10 μm). L; lumen, M; tunica media.Group 1; control, Group 2; lipid-rich food, Group 3; lipid-rich food +PTU, Group 4; lipid-rich food +PTU+T4, Group 5; lipid-rich food +PTU+T3.



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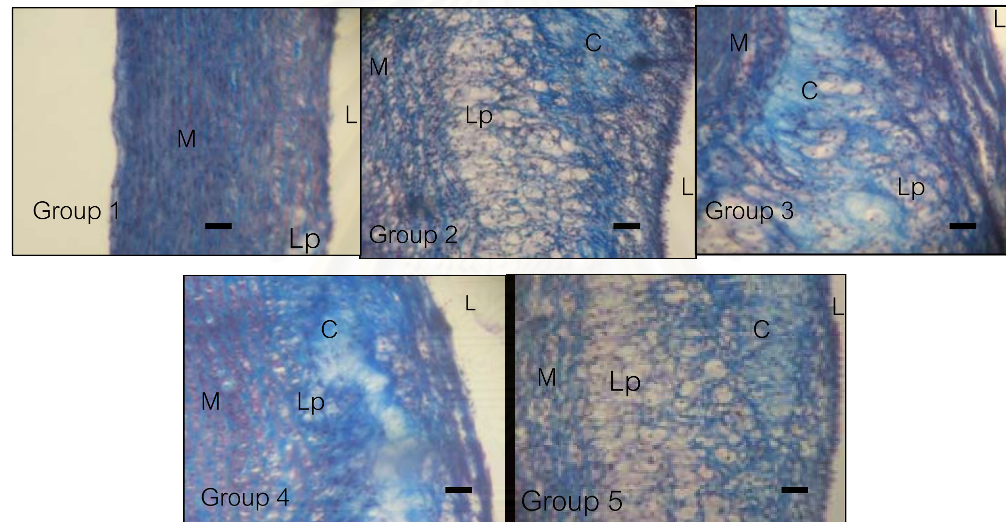


Figure 4.10. Microscopic demonstration of collagen and lipid of the atherosclerotic lesion of the thoracic aorta. The micrographs were stained with Masson's trichrome (Bar = 10 μm). L; lumen, M; tunica media, C; collagen (blue), Lp; lipid (white). Group 1; control, Group 2; lipid-rich food, Group 3; lipid-rich food +PTU, Group 4; lipid-rich food +PTU+T4, Group 5; lipid-rich food +PTU+T3

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CHAPTER V

# DISCUSSION

As we know, thyroid hormones have multiple effects on the cardiovascular system (Klein and Ojamaa., 2001) especially atherosclerosis. Atherosclerosis involves a complex interaction between the cells of the arterial wall and various blood components, such as lipoproteins, platelets, and monocyte-derived macrophages. The pathogenesis of atherosclerosis is generally accepted as being a reaction to injury. In response to vascular injury, such as in the condition of hypercholesterolemia, vascular smooth muscle cells (VSMCs) can migrate and proliferate to the intimal layer. These proliferating VSMCs deposit the extracellular matrix and accumulate lipid in the intimal layer. A high-fat diet has been reported to induce hypercholesterolemia, thus promoting atherogenesis in both humans and experimental animals( Chan et al., 2004).The alteration of thyroid hormone showed that hypothyroidism may result in accelerated risk for atherosclerotic vascular disease, presumably because of the associated hypercholesterolemia (Diekman et al., 1998; Klein and Ojamaa., 2001), which is also supported by our data that animals received atherogenic diet for 12 weeks develop a high degree of atherosclerotic lesions. Additionally, Hypothyroidism is frequently associated with an elevation of serum cholesterol, generally reflecting an increase in low-density lipoprotein (LDL) cholesterol, which can be normalized after substitution therapy with thyroid hormones.

In the present study, animals in group 4 (lipid-rich food plus PTU and T4 group) and group 5 (lipid-rich food plus PTU and T3) had less body weight gain as compared to the other groups. These may be due to effects of thyroid hormones on facilitation of energy utilization and catabolic process (Guyton, 2000). The increase in body weight of animals in group 3 (lipid-rich food plus PTU) as compared to group 2 (lipid-rich food) may be due to decreases in thyroid hormone action which turn out to be less energy utilization and decrease metabolic rate. However, these effects may not always occur because thyroid hormones also increase the appetite, and this may counterbalance the change in the metabolic rate (Guyton, 2000).

The results of mean values for body temperature showed that animals in group 4 (lipid-rich food plus PTU and T4) and group 5 (lipid-rich food plus PTU and T3) had higher body temperatures compared to other groups. These may be due to the effect of thyroid hormones on basal metabolic rate which may promote body heat production. Once receiving thyroid supplement for long periods of time may have effects on thermoregulation, and is possible to increase the body temperature (Saliva, 2003). In addition, the relationship of thermogenesis to food intake and lipogenesis suggests that the biological role of thyroid hormones on thermogenesis in temperature homeostasis, not in dumping calories to protect the body from obesity. On the other hand, the depression of thyroid function associated with the so-called euthyroid sick syndrome indicates that thermogenesis is sacrificed when food availability is reduced or when the preservation of body mass is needed in catabolic states(Klein and Ojamaa., 2001; Saliva, 2003).

Animals in group 2 (lipid-rich food) had significant increases in total cholesterol compared to other groups, and tended to be declined of cholesterol levels in groups 4 and 5. This study presented the risk of high fat diet which is

frequently associated with an elevation of serum cholesterol, generally reflecting increased low-density lipoprotein (LDL) cholesterol, which may be normalized after substitution therapy with thyroid hormone. These may be due to the decreased plasma cholesterol level despite an enhanced synthetic rate and is thus related to increased catabolism of cholesterol by the excess thyroid hormones (Cachefo et al., 2001; O'Brien et al., 1993).

In the present study, animals in group 5 (lipid-rich food plus PTU and T3) and group 4 (lipid-rich food plus PTU and T4) have the same effect on total cholesterol levels. This cannot be explained by the cholesterol lowering effects of T4, and this have prompted a number of attempts, so far unsuccessful, to develop an analogue that selectively decreases serum cholesterol; the differential affinity of T3-receptor isoforms for T3 anlogues suggests that this may be possible (Schueler et al., 1990).

The hypothesis that oxidative stress plays an important role in the pathogenesis of atherosclerosis has gained considerable support. Although there are many determinants in the development of atherosclerotic lesions, a substantial in vitro evidence links LDL oxidation to potentially atherogenic process at the molecular and cellular level (Diaz et al., 1997). The oxidized LDLs produced in the arterial intima are intensively taken by scavenger receptors on the macrophages, which directly contributes to foam cell formation. These cells, loaded with cholesteryl esters, are characteristic of the precocious lesions of the atherosclerotic plaques. Moreover, oxidized LDL may affect many other aspects of arterial wall metabolism and thus contribute to the atherogenic process (Parthasarathy et al., 1992). However, the mechanism of the initiation and progression of LDL oxidation is presently unclear in vivo and needs much research to be proven. In the present

study, animals receiving triiodothyronine and thyroxine have lower LDL oxidation compared to hypothyroid and atherosclerotic animals. It is possible that pathophysiological implication of a decrease in lipid peroxidation of $T_4$ replacement may involve the entry of $T_4$ in to cells. Moreover, LDL also contains three binding sites for $T_4$ localized on apolipoprotein B-100 (Benvenga et al 1990). These may support that thyroid hormones can bind to LDL and exert their antioxidant effects as demonstrated in vitro (Hanna et al, 1993, Hanna et al, 1995), and less availability of oxidized LDL to be taken up by macrophages (Steinberg, 1997). Because of LDL oxidation are taken up by macrophages at an enhanced rate via scavenger receptors, leading to the formation of lipid-laden foam cells and accelerated atherosclerosis (Aviram, 1996; Steinberg, 1997). According to macrophage function, cholesterol accumulation leading to foam cell formation is the mark of early atherosclerosis (Kaplan et al., 2001). Therefore, thyroid hormone supplement may reduce the risk of atherosclerotic development in vivo as also demonstrated in our study.

In the present study, animals receiving atherogenic diet were associated with an increase in lipid peroxidation as demonstrated by increased plasma MDA, but animals receiving T3 and T4 had lower plasma MDA. These may be due to antioxidant effects of thyroid hormones themselves, and decreases in lipid peroxidation were also reported previously by some investigations (Hanna et al., 1995; Sundaram et al., 1997; Diekman et al., 1998; Benvenga, 1998). Moreover, our results also show that animals receiving T4 have much lower LDL-MDA than animals receiving T3. These may be due to the higher concentration of T4 exerts more antioxidant effects, and may be due to T4 possesses the differences of LDL affinity or antioxidant activity, compared to T3. However, some experiments have to be

done before these ideas are to be proved. Because of oxidative stress is believed to play an important role in early atherogenesis, efforts should be made to reduce oxidative stress (Aviram and Fuhrman., 1998; Maxwell and Lip., 1997).

As we expected, high fat, high cholesterol feeding raised plasma cholesterol levels, which led to the development of well-defined lesions. The lesion depth varies widely around the arterial wall, as has been noted in many studies (Minqin et al., 2003; Mitani et al., 2003; Meguro et al; 2003). In the present study, the ratio of the atherosclerotic lesion area in aortic arch and thoracic aorta of animals in group 4 (lipid-rich food plus PTU and T4) and group 5 (lipid-rich food plus PTU and T3) were lower than those of group 3 (lipid-rich food plus PTU) and group 2 (lipid-rich food). These results may be due to thyroid hormones inhibit atherosclerotic formation by inhibition of LDL oxidation and macrophage lipid peroxidation (Oziol et al., 2003). Interestingly, the presented available LDL uses the macrophage as a scavenge molecule that binds onto and internalizes the LDL. After that, internalised LDL may be oxidized in macrophages before turning macrophages to foam cells (Cabbold et al., 2002).

In addition, lesional compositions in the aortic arch, thoracic aorta of group 5 (lipid-rich food plus PTU and T3) and group 4 (lipid-rich food plus PTU and T4) had significantly decreases in macrophage content and extracellular lipid deposition in the cross-sectional area of the vessels as compared to group 3 (lipid-rich food plus PTU) and group 2 (lipid-rich food).The other reported oxidized LDL can attract monocytes that adhere to the intravascular endothelium and migrate to the subendothelial space where they differentiate into macrophages. Thus, cholesterol-loaded macrophages change into foam cells and invade the arterial wall inducing the fatty streak, an early step in atheromatous plaque formation (Cabbold et al.,

2002). The relatively substantial reduction of lipid peroxides content in macrophages may indicate that both thyroid hormones may have protective effects on the atherosclerotic formation.

Moreover, rabbits in group 3 (lipid-rich food plus PTU), had no significant difference in vascular smooth muscle cell content compared to group 2(lipid-rich food). Normally, PTU exerts its hypothyroid effect by inhibiting iodide oxidation, monoiodotyrosine iodination, and coupling steps in thyroxine production, as well as the peripheral conversion of thyroxine (T4) to triiodothyronine (T3). However, changes cannot be induced between group 2 and 3 that PTU cannot enhance the risk of lipid-rich food on atherosclerotic formation. This may be partially due to the protective effect of propylthiouracil independent of its hypothyroid effect on atherogenesis in rabbits model. A study has shown that PTU may have an inhibitory effect on VSMC proliferation and migration (Chen et al., 2004). The pathogenesis of atherosclerosis is generally accepted as being a reaction to injury. In response to vascular injury, such as in the condition of hypercholesterolemia, vascular smooth muscle cells (VSMCs) can migrate and proliferate into the intimal layer. Migration of VSMCs from the media to the intima, and their subsequent proliferation, is an important mechanism in atherogenesis. These proliferating VSMCs deposit the extracellular matrix and accumulate lipid in the intimal layer. If PTU can inhibited VMSCs proliferation and migration; therefore, PTU may not enhance the atherosclerotic formation as also shown in our study.

The mean thickness of the tunica intima in the aortic arch of animals in group 4 (lipid-rich food plus PTU and T4) and group 5 (lipid-rich food plus PTU and T3) were lower than those of animals in group 2 (lipid-rich food), The mean thickness of the tunica intima in the aortic arch of animals in group 5 (lipid-rich food plus PTU

and T3) tended to be lower than animals in group 4 (lipid-rich food plus PTU and T4) and group 3 (lipid-rich food plus PTU). These results may suggests that thyroid hormone may inhibit and prevent the macrophage accumulation in atherosclerotic lesion at sub-intimal layer, which is one of the key processes in the development of atherosclerosis representing the accumulation of cholesterol by macrophages in subendothelial space of the vessel wall (Kaplan et al., 2001; Staub et al., 1992; Diekman et al., 1997; Witzum., 1994).

Finally, the most relevant information concerning this mechanism has come from histological analysis and biochemical analysis of atherosclerosis lesion in aortic arch, thoracic aorta and, abdominal aorta alterations indicated a protective effect of thyroid hormones on atherosclerotic formation that to be considered by atherosclerotic lesion, intimal thickening, and lesional composition.

In conclusion, the present study demonstrates that thyroxine and triiodothyronine replacement therapy can inhibit atherosclerotic formation in a rabbit-model, and protective effects of thyroid hormones are also supported by:

1. Lipid peroxidation are decreased especially in animals receiving thyroxine by detectable lower MDA levels in plasma and LDL content, compared to atherogenic and atherogenic plus propylthiouracil-receiving animals.
2. The areas of atherosclerotic lesion especially aortic arch are decreased in thyroid hormone receiving animals, compared to atherogenic and atherogenic plus propylthiouracil-receiving animals.
3. The lesional components e.g. macrophage, collagen, and lipid are decreased in thyroid hormone receiving animals, compared to atherogenic and atherogenic plus propylthiouracil-receiving animals.

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# APPENDIX

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# APPENDIX


Table A-1. Means of Body Temperature.

Table A-2. Body weight, Total T3, and Total T4.

Table A-3. Total Cholesterol and Triglyceride.

Table A-4. Malondialdehyde (MDA) in LDL and plasma.

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**Table A-2.** Body weight, Total T3, and Total T4

| Rabbit No. | Body weight(g) | | Total T3 | | Total T4 | |
|---|---|---|---|---|---|---|
| | wk 0 | wk 12 | wk 0 | wk 12 | wk 0 | wk 12 |
| Group 1.1 | 1,680 | 2,500 | 59.5 | 42 | 0.1 | 0.8 |
| Group 1.2 | 1,760 | 2,720 | 24.6 | 30.9 | 0.6 | 3.3 |
| Group 1.3 | 1,800 | 2,640 | 32.8 | 56.6 | 0.7 | 4.8 |
| Group 1.4 | 1,840 | 2,650 | 67.4 | 35.9 | 3 | 4 |
| Group 1.5 | 2,000 | 2,600 | 85.6 | 35.9 | 2.1 | 2 |
| Group 2.1 | 1,900 | 2,800 | 55.5 | 42.7 | 0.9 | 3.7 |
| Group 2.2 | 1,900 | 2,900 | 70.6 | 55.5 | 1.8 | 1.8 |
| Group 2.3 | 1,750 | 2,850 | 86.1 | 54.6 | 1.5 | 5.1 |
| Group 2.4 | 1,700 | 2,900 | 55.7 | 58.5 | 1.5 | 2.6 |
| Group 2.5 | 1,900 | 3,000 | 78.9 | 25.3 | 1.4 | 1.4 |
| Group 3.1 | 1,680 | 3,000 | 59.3 | 91.4 | 2.2 | 5.8 |
| Group 3.2 | 1,900 | 3,120 | 51 | 44.7 | 3.6 | 3.9 |
| Group 3.3 | 2,100 | 3,160 | 82.2 | 52 | 3.5 | 3.8 |
| Group 3.4 | 1,880 | 3,180 | 74 | 31 | 2.6 | 0.9 |
| Group 3.5 | 2,100 | 3,300 | 54.7 | 29.8 | 4.2 | 6.3 |
| Group 4.1 | 2,000 | 3,000 | 28.9 | 34.6 | 0.9 | 9.8 |
| Group 4.2 | 2,200 | 3,000 | 80.3 | 54.4 | 4.7 | 6.8 |
| Group 4.3 | 2,100 | 2,800 | 52.9 | 51.5 | 0.5 | 5.6 |
| Group 4.4 | 2,200 | 3,350 | 83 | 36 | 1.7 | 3.8 |
| Group 4.5 | 2,100 | 3,000 | 60.5 | 25.4 | 3.6 | 8.9 |
| Group 5.1 | 2,100 | 2,900 | 61 | 152 | 1.5 | 0.5 |
| Group 5.2 | 2,200 | 3,100 | 57 | 282 | 1.3 | 0.5 |
| Group 5.3 | 2,200 | 2,800 | 61 | 165 | 2.5 | 0.5 |
| Group 5.4 | 2,200 | 3,050 | 42 | 297 | 1.1 | 0.5 |
| Group 5.5 | 1,800 | 2,800 | 68 | 245 | 1.8 | 0.5 |

Group 1; control, Group 2; lipid-rich food, Group 3; lipid-rich food +PTU, Group 4; lipid-rich food +PTU+T4, Group 5; lipid-rich food +PTU+T3.

Table A-3.Total Cholesterol and Triglyceride

| Rabbit No. | Triglyceride | | Cholesterol | |
|---|---|---|---|---|
| | wk 0 | wk 12 | wk 0 | wk 12 |
| Group 1.1 | 34.1 | 37.8 | 37 | 40.1 |
| Group 1.2 | 21.3 | 97.6 | 33.8 | 65.8 |
| Group 1.3 | 14.1 | 23 | 28.6 | 46.7 |
| Group 1.4 | 54.6 | 24.3 | 49.8 | 24.6 |
| Group 1.5 | 52.2 | 24.9 | 56.6 | 53 |
| Group 2.1 | 34.1 | 63.2 | 38.9 | 279.6 |
| Group 2.2 | 85.6 | 599.7 | 48.5 | 970.5 |
| Group 2.3 | 96.8 | 54 | 77.1 | 562.4 |
| Group 2.4 | 59.9 | 45.9 | 53.1 | 413.3 |
| Group 2.5 | 191.3 | 49.9 | 73.8 | 330.5 |
| Group 3.1 | 57 | 25.7 | 61.7 | 216 |
| Group 3.2 | 92.2 | 44.7 | 145.2 | 264.4 |
| Group 3.3 | 92.7 | 21.3 | 72.7 | 209.3 |
| Group 3.4 | 77.4 | 29.4 | 101 | 43.7 |
| Group 3.5 | 32.7 | 35.4 | 75.5 | 411.6 |
| Group 4.1 | 184.4 | 36.6 | 156.3 | 175.9 |
| Group 4.2 | 131.2 | 16 | 101.5 | 233.8 |
| Group 4.3 | 75.3 | 76.7 | 117.2 | 755.8 |
| Group 4.4 | 42.3 | 61.5 | 73.6 | 592.8 |
| Group 4.5 | 40.7 | 45 | 83.5 | 204.1 |
| Group 5.1 | 241 | 109 | 132 | 218 |
| Group 5.2 | 120 | 117 | 140 | 288 |
| Group 5.3 | 137 | 127 | 167 | 264 |
| Group 5.4 | 178 | 142 | 182 | 217 |
| Group 5.5 | 94 | 151 | 192 | 274 |

Group 1; control, Group 2; lipid-rich food, Group 3; lipid-rich food +PTU, Group 4; lipid-rich food +PTU+T4, Group 5; lipid-rich food +PTU+T3.

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Table A-4. Malondialdehyde (MDA) in LDL and plasma.

| Group | LDL MDA (nmol/mg LDL protein) | Plasma MDA (nmol/ml) |
|---|---|---|
| Group 1 | 8.06±0.78 [a] | 3.39±0.31 [a] |
| Group 2 | 6.73±0.66 [a] | 7.07±1.39 [c] |
| Group 3 | 7.78±0.82 [a] | 3.99±0.51 [a] |
| Group 4 | 2.89±0.33 [b] | 4.51±0.54 [ab] |
| Group 5 | 5.27±0.85 [a] | 5.95±1.87 [bc] |

Different superscripts in the same column mean significantly different ($P < 0.05$)

Group 1; control, Group 2; lipid-rich food, Group 3; lipid-rich food +PTU, Group 4; lipid-rich food +PTU+T4, Group 5; lipid-rich food +PTU+T3.

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Table A-1. Means of Body Temperature.

| Group | Week1 | Week2 | Week3 | Week4 | Week5 | Week6 | Week7 | Week8 | Week9 | Week10 | Week11 | Week12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 104.08 | 102.56 | 103.16 | 102.64 | 102.36 | 102.40 | 102.60 | 102.68 | 102.48 | 102.48 | 102.52 | 102.40 |
| 2 | 102.20 | 103.28 | 102.84 | 102.60 | 102.35 | 102.28 | 102.08 | 102.08 | 102.16 | 102.08 | 102.04 | 102.04 |
| 3 | 102.48 | 102.62 | 102.72 | 102.28 | 102.20 | 102.40 | 102.30 | 102.24 | 102.24 | 102.28 | 102.24 | 102.16 |
| 4 | 103.08 | 103.12 | 103.24 | 103.40 | 103.40 | 103.40 | 103.44 | 103.44 | 103.48 | 103.48 | 103.52 | 103.56 |
| 5 | 103.00 | 102.80 | 102.72 | 102.64 | 102.72 | 102.80 | 102.84 | 102.96 | 103.00 | 103.08 | 103.14 | 103.20 |

Group 1; control, Group 2; lipid-rich food, Group 3; lipid-rich food +PTU, Group 4; lipid-rich food +PTU+T4, Group 5; lipid-rich food +PTU+T3.

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# BIOGRAPHY

Miss Kingkarn Boonsuya was born on February 14, 1976 in Surin, Thailand. She graduated from the Faculty of Veterinary Medicine, Khon Kaen University, Thailand in 2001. She admitted with the degree of Master of Science, Department of Physiology, Faculty of Veterinary Science, Chulalongkorn University in 2002.

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