EFFECTS OF CURCUMIN ON DIABETES-INDUCED LEUKOCYTE-ENDOTHELIUM INTERACTION: ROLES OF NOX2 ENZYME AND THIOREDOXIN-INTERACTING PROTEIN

Miss Natchaya Wongeakin

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science, Program in Medical Science

Faculty of Medicine

Chulalongkorn University

Academic Year 2011

ผลของเคอร์คูมินต่อการเกาะติดของเม็ดเลือดขาวกับเอ็นโดทีเลียมในภาวะเบาหวาน: บทบาทของเอนไซม์นอกซ์ทูและไทโอรีดอกซิน-อินเตอร์แรคติงโปรตีน

นางสาวณัฏฐ์ชญา วงศ์เอกอินทร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์การแพทย์
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2554

Thesis Title	EFFECTS OF CURCUMIN ON DIABETES-INDUCED LEUKOCYTE-ENDOTHELIUM INTERACTION: ROLES OF NOX2 ENZYME AND THIOREDOXIN-INTERACTING PROTEIN
By	Miss Natchaya Wongeakin
Field of Study	Medical Science
Thesis Advisor	Associate Professor Suthiluk Patumraj, Ph.D.
Thesis Co-advisor	Associate Professor Parvapan Bhattarakosol, Ph.D.

Accepted by Faculty of Medicine, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

…….. Dean of Faculty of Medicine
(Associate Professor Sophon Napathorn, M.D.)

THESIS COMMITTEE

…….. Chairman
(Associate Professor Vilai Chentanez, M.D.)

……. Thesis Advisor
(Associate Professor Suthiluk Patumraj, Ph.D.)

…….. Thesis Co-advisor
(Associate Professor Parvapan Bhattarakosol, Ph.D.)

…….. Examiner
(Professor Narisa Futrakul, M.D., Ph.D.)

…….. External Examiner
(Assistant Professor Amporn Jariyapongskul, Ph.D.)

ณัฏฐ์ชญา วงศ์เอกอินทร์ : ผลของเคอร์คูมินต่อการเกาะติดของเม็ดเลือดขาวกับเอ็นโดทีเลียมในภาวะเบาหวาน: บทบาทของเอนไซม์นอกซ์ทูและไทโอรีดอกซิน-อินเตอร์แรคติงโปรตีน (EFFECTS OF CURCUMIN ON DIABETES-INDUCED LEUKOCYTE-ENDOTHELIUM INTERACTION: ROLES OF NOX2 ENZYME AND THIOREDOXIN-INTERACTING PROTEIN) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. สุทธิลักษณ์ ปทุมราช, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ดร. ภาวพันธ์, 79 หน้า.

ภาวะเบาหวานทำให้มีการแสดงออกของไทโอรีดอกซินอินเตอแรคติงโปรตีน (Txnip) มากขึ้น ส่งผลให้ภาวะสมดุลของเซลล์เสียไป และการเพิ่มขึ้นของไทโอรีดอกซินอินเตอแรคติงโปรตีน ยังไปกระตุ้นให้มีการแสดงออกของแอดฮีชั่นโมเลกุล เช่น ไอแคมวัน (ICAM-1) เพิ่มขึ้นด้วย ส่งผลให้มีการเกาะติดกันของเม็ดเลือดขาวและเอ็นโดทีเลียมมากขึ้นทำให้เกิดการอักเสบของหลอดเลือด และการที่มีเซลล์เม็ดเลือดขาวมาชุมนุมกันในบริเวณหลอดเลือดมาก ยังส่งผลไปกระตุ้นปฏิกิริยานอกซ์ทูเอนไซม์ (NOX2 enzyme) ของเซลล์เม็ดเลือดขาว ทำให้มีการสร้างซุปเปอร์รอกไซด์เพิ่มขึ้นส่งผลให้หลอดเลือดถูกทำลายมากขึ้น มีรายงานมากมายพบว่าเคอร์คูมินมีฤทธิ์ต่อการต้านสารอนุมูลอิสระอีกทั้งยังมีฤทธิ์ต้านการอักเสบ การทดลองนี้จึงสนใจศึกษากลไกการออกฤทธิ์ของเคอร์คูมินในการป้องกันการเกาะติดของเม็ดเลือดขาวกับเอ็นโดทีเลียม โดยศึกษาผ่านกลไกนอกซ์ทูเอนไซม์และการแสดงออกของไทโอรีดอกซินอินเตอแรคติงโปรตีน การทดลองนี้ใช้หนูวิสต้า (Wistar rat) เพศผู้ โดยแบ่งออกเป็น 4 กลุ่ม 1.กลุ่มควบคุม (CON) 2.กลุ่มควบคุมที่ได้รับเคอร์คูมิน (CONCUR) 3.กลุ่มเบาหวาน (DM) และ 4.กลุ่มเบาหวานที่ได้รับเคอร์คูมิน (DMCUR) หลังจากเหนี่ยวนำให้เป็นเบาหวานเป็นเวลา 12 สัปดาห์ นำหนูมาทำการวัดอัตราการไหลเวียนของหลอดเลือดที่บริเวณม่านตาด้วยเครื่องเลเซอร์ดอปเปลอร์และการทำการศึกษาการเกาะติดของเม็ดเลือดขาวกับเอ็นโดทีเลียมผ่านภายใต้กล้องฟลูออเรสเซนต์ เมื่อเสร็จการทดลองได้ทำการเก็บเลือดมาวัดระดับน้ำตาลและฮีโมโกลบินเอวันซี (HbA1c) และทำการเก็บลูกตามาศึกษาการแสดงออกของไทโอรีดอกซินอินเตอแรคติงโปรตีนและพีฟอร์ตี้เซเว่นฟอกซ์ (p47phox, มาร์กเกอร์ของนอกซ์ทูเอนไซม์) ด้วยวิธีเวสเทิร์นบลอท (Western blot) และวัดระดับมาลอนไดแอลดีไฮน์ (MDA, มาร์กเกอร์ของไลปิดเพอรอกซิเดชั่น) ด้วยวิธีทีบาร์แอสเส (TBAR assay) ผลการทดลองพบว่าน้ำหนักและอัตราการไหลเวียนของหลอดเลือดที่บริเวณม่านตาในกลุ่มเบาหวานและกลุ่มเบาหวานที่ได้รับเคอร์คูมินลดลงอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มควบคุม (P<0.001)แต่ระดับน้ำตาลในเลือดและฮีโมโกลบินเอวันซีในกลุ่มเบาหวานและกลุ่มเบาหวานที่ได้รับเคอร์คูมินสูงขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มควบคุม (P<0.001) และที่สำคัญพบว่าการเกาะติดของเม็ดเลือดขาวกับเอ็นโดทีเลียม, ระดับมาลอนไดแอลดีไฮน์ และการแสดงออกของพีฟอร์ตี้เซเว่นฟอกซ์ในกลุ่มเบาหวานที่ได้รับเคอร์คูมินลดลงอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มเบาหวาน (P<0.05) สรุปการทดลองครั้งนี้ได้ว่า เคอร์คูมินความเข้มข้น 300 มก./น้ำหนักตัวกก.สามารถลดการเกิดกระบวนอักเสบของหลอดเลือดในภาวะเบาหวาน โดยลดการสร้างสารอนุมูลอิสระ ยับยั้งการเกาะติดของเม็ดเลือดขาวกับเอ็นโดทีเลียม และยังลดการกระตุ้นนอกซ์ทูเอนไซม์ภายในเซลล์เม็ดเลือดขาว แต่อย่างไรก็ตามเคอร์คูมินไม่สามารถที่จะลดระดับน้ำตาลและยับยั้งการแสดงออกของไทโอรีดอกซินอินเตอแรคติงโปรตีน รวมถึงยังไม่สามารถป้องกันการลดลงของการไหลเวียนเลือดในม่านตาได้

สาขา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต.....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก..... .....

5174772530 : MEDICAL SCIENCE

KEYWORDS : DIABETES / CURCUMIN / NOX2 / TXNIP / ENDOTHELIAL DYSFUNCTION / LEUKOCYTE ADHESION

NATCHAYA WONGEAKIN: EFFECTS OF CURCUMIN ON DIABETES-INDUCED LEUKOCYTE-ENDOTHELIUM INTERACTION: ROLES OF NOX2 ENZYME AND THIOREDOXIN-INTERACTING PROTEIN. ADVISOR: ASSOC.PROF. SUTHILUK PATUMRAJ, Ph.D., CO-ADVISOR: ASSOC.PROF. PARVAPAN BHATTARAKOSOL, Ph.D., 79 pp.

Hyperglycemia increases thioredoxin-interacting protein (Txnip) expression that causes cellular redox imbalance. In addition, the up-regulation of Txnip can enhance vascular cell adhesion molecules expression such as intercellular adhesion molecule-1 (ICAM-1) which leads to increase leukocyte-endothelium interaction to promote vascular inflammation. Moreover, the leukocyte accumulation in microcirculation causes endothelial damage by NADPH oxidase enzyme (NOX2). Curcumin is a polyphenolic compound which exhibits antioxidant, and anti-inflammatory properties. The present study aimed to study the mechanisms of curcumin on preventing diabetes-induced leukocyte-endothelium interaction in association with its actions on NOX2 enzyme and Txnip expressions. Male Wistar rats were divided into four groups: control-treated with corn oil group (CON), control-treated with curcumin group (CONCUR, 300mg/kgBW), diabetic-treated with corn oil group (DM; streptozotocin (STZ), i.v. 55 mg/kg BW), and diabetes-treated with curcumin (DMCUR). The supplementation of curcumin was started at 10 days after STZ-injection. Then on the 12th week after STZ injection, iris blood perfusion and leukocyte adhesion at iris of each rat was measured by using laser Doppler and intravital fluorescent microscopy, respectively. Plasma glucose and HbA1c were also determined by using enzymatic and turbidimetric immunoinhibition methods, respectively. P47phox expression (marker of NOX2 enzyme activation), Txnip expression, and malondialdehyde (MDA) level at eye fundus were determined by Western Blot analysis and TBAR assay, respectively. The results showed that body weight and iris blood perfusion of DM and DMCUR were decreased significantly as compared to CON ($P < 0.001$), but plasma glucose and HbA1c of DM and DMCUR were increased significantly as compared to CON ($P < 0.001$). Interestingly, the leukocyte adhesion, p47phox expression, and MDA level in DM were increased significantly as compared to CON ($P < 0.05$), but decreased significantly when compared to DMCUR ($P < 0.05$). It is concluded that 300 mg/kgBW curcumin could ameliorate diabetic vascular inflammation by decreasing ROS overproduction, reducing leukocyte-endothelium interaction, and also inhibiting NOX2 activation. However, it could not significantly reduce blood glucose, Txnip expression, and increase iris blood perfusion.

Field of Study: : Medical Science

Academic Year: 2011

Student's Signature……

Advisor's Signature……

Co-advisor's Signature……

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and deep appreciation to Associate Professor Suthiluk Patumraj, Ph.D., my advisor, for her valuable advice, helpful guidance, suggestion, encouragement, and kindness throughout this study. All of this has enabled me to carry out my study successfully.

I am greatly indebted to Associate Professor Parvapan Bhattarakosol, Ph.D. my co-advisors, for her valuable suggestions, helpful criticism, kindness and important support throughout this work.

I am very grateful to the members of the thesis committee for their magnificent comments and correction of this thesis.

I also wish to express my special thanks to Assistant Professor Supang Maneesri le Grand, Mr. Dusit Promrug, Miss Yadarat Suwanmanee, Miss Sheepsumon Viboolvorakul, and all my colleges and my friends, whose name cannot be fully listed for their experimental assistance, kind support, and cheerfulness throughout the study.

I would like to thank the Department of Physiology and the Department of Microbiology, Faculty of Medicine, Chulalongkorn University for their constant cooperation and support.

Finally, I would like to express my profound gratitude and appreciation to my parents and my sister for their encouragement, understanding, and loving support.

This study was supported by the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund).

CONTENTS

PAGE

LIST OF TABLES

LIST OF FIGURES

FIGURE PAGE

FIGURE PAGE

LIST OF ABBREVIATIONS

BCA	=	Bicinchoninic acid
ChREBP	=	Carbohydrate responsive element binding protein
DM	=	Diabetes mellitus
EC	=	Endothelial cell
FOXO1	=	Forkhead box protein O1
ICAM-1	=	Intercellular Adhesion Molecule-1
MDA	=	Malondialdehyde
NADPH oxidase	=	Nicotinamide adenine dinucleotide phosphate-oxidase
NF-kB	=	Nuclear factor κ B
NO	=	Nitric oxide
NRF2	=	Hormetic nuclear factor (erythroid-derived 2)-like 2
PVDF	=	Polyvinylidene difluoride
R6G	=	Rhodamine-6G
ROS	=	Reactive oxygen species
STZ	=	Streptozotocin
TBA acid	=	Thiobarbituric acid
TRX	=	Thioredoxin
Txnip	=	Thioredoxin interacting protein

CHAPTER I

INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic diseases characterized by chronic hyperglycemia resulting from defects in insulin production, insulin action, or both. The metabolic disease of DM is associated with type 1 and type 2 diabetes mellitus. From the uncontrolled hyperglycemia overtime can lead to oxidative stress that causes serious macro- and microvascular complications. Globally, the number of people with diabetes worldwide is estimated to increase from 171 Million in 2000 to 366 Million by 2030 (1). Diabetes could lead to substantial economic burden because the diabetic patients have participated frequently in an intensive health care system. At this time, diabetes might become one of major health problem threatening in developed and developing countries around the world.

Diabetes-induced hyperglycemia disturbs the vascular homeostasis that characterized by abnormal vascular flow, increased vascular permeability, and non-perfusion of capillaries. Several reports show that hyperglycemia-induced reactive oxygen species (ROS) overproduction continuously during many metabolic processes such as nicotin amide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, the enzymes of the mitochondrial respiratory chain, and uncouple eNOS (2-5).

ROS are strongly reactive chemicals which can react with amino acid residues as well as or lipid peroxidation and rapidly eradicated by anti-oxidants to control cellular redox state. Normally, cells possess protective systems against free radicals including enzymatic and non-enzymatic antioxidants. Enzymatic oxidants such as superoxide dismutase (SOD), catalase, glutathione system, and thioredoxin system provide the first line of defense (6, 7).

In diabetes, ROS overproduction above the physiological levels could disturb cellular redox state leading to oxidative stress (OS) that suppressing the antioxidant capacity of cells. In addition, hyperglycemia could induce thioredoxin-interacting protein (Txnip) expression through p38 MAPK (8). Txnip is an endogenous inhibitor of TRX system activity that binds to redox-active cysteine residues of TRX (9-11). The TRX-Txnip interaction leads to increase intracellular ROS, activate stress signaling pathways such as apoptosis (12, 13) and induce inflammation such as ICAM-I expression, resulting in endothelial dysfunction (10).

Up-regulation of Txnip has been reported to contribute vascular inflammation that enhances vascular cell adhesion molecule expression such as ICAM-1 on endothelial surface. In addition to ROS-dependent TRX-Txnip interaction, ROS could stimulate NF-kB activation by increase vascular cell adhesion molecule expression. These refer to promote leukocyte-endothelium interaction that plays a central role in diabetic vascular inflammation (14). Moreover, plugged leukocyte in microcirculation causes development of microvascular damage in the second degree by leukocyte NADPH oxidase-mediated superoxide radical formation (15-17).

In recently, the attention of the use of natural plant products is increased among clinicians for prevention and treatment in various diseases. The natural plants products can reduce the risk of developing pathology and have side effects less than synthesized medicine. Plants contain numerous bioactive molecules such as polyphenols. Curcumin is the one of polyphenols which exhibits antioxidant, anti-inflammatory, antitumorgenic, and antimicrobial properties.

In antioxidant activities, curcumin can inhibit ROS generation in human red blood cells exposed to high glucose levels (18) and an increase in 8-hydroxydeoxyguanosine (8-OHdG) (19), protect islets against STZ-induced oxidative stress by scavenging free radicals (20), reduce MDA levels in STZ-induced diabetic rats (21, 22), and promote antioxidant system through activation of NF-E2-related factor 2 (Nrf2) (23-26).

By anti-inflammatory activity, curcumin can attenuate pro-inflammatory cytokine levels including TNF-α, IL-1β, and IL-6 by using LPS- or PMA- stimulated macrophages (27) and it can decrease leukocyte-endothelial interaction in postcapillary venules of iris of STZ-induced diabetic rat (21). In addition, curcumin can suppress NF-kB activation by inhibiting nuclear translocation of the p65 NF-κB subunit (28) and preventing phosphorylation and degradation of IκB (29). Altogether, these studies show that curcumin can reduce a risk of diabetic complications.

Based on these reviews, the mechanisms of curcumin on hyperglycemia-promoted ROS overproduction which are involved in NOX2 oxidase activation and up-regulation of Txnip expression in hyperglycemia remain unclear. Therefore, the present study aims to

investigate the mechanisms of curcumin on diabetes-induced leukocyte-endothelial interaction related to NOX2 oxidase activation and up-regulation of Txnip expression in rats.

Research question

1. Can curcumin reduce diabetes-induced leukocyte-endothelium interaction through its actions on NOX2 expressions?
2. Can curcumin reduce diabetes-induced leukocyte-endothelium interaction through its actions on Txnip expressions?

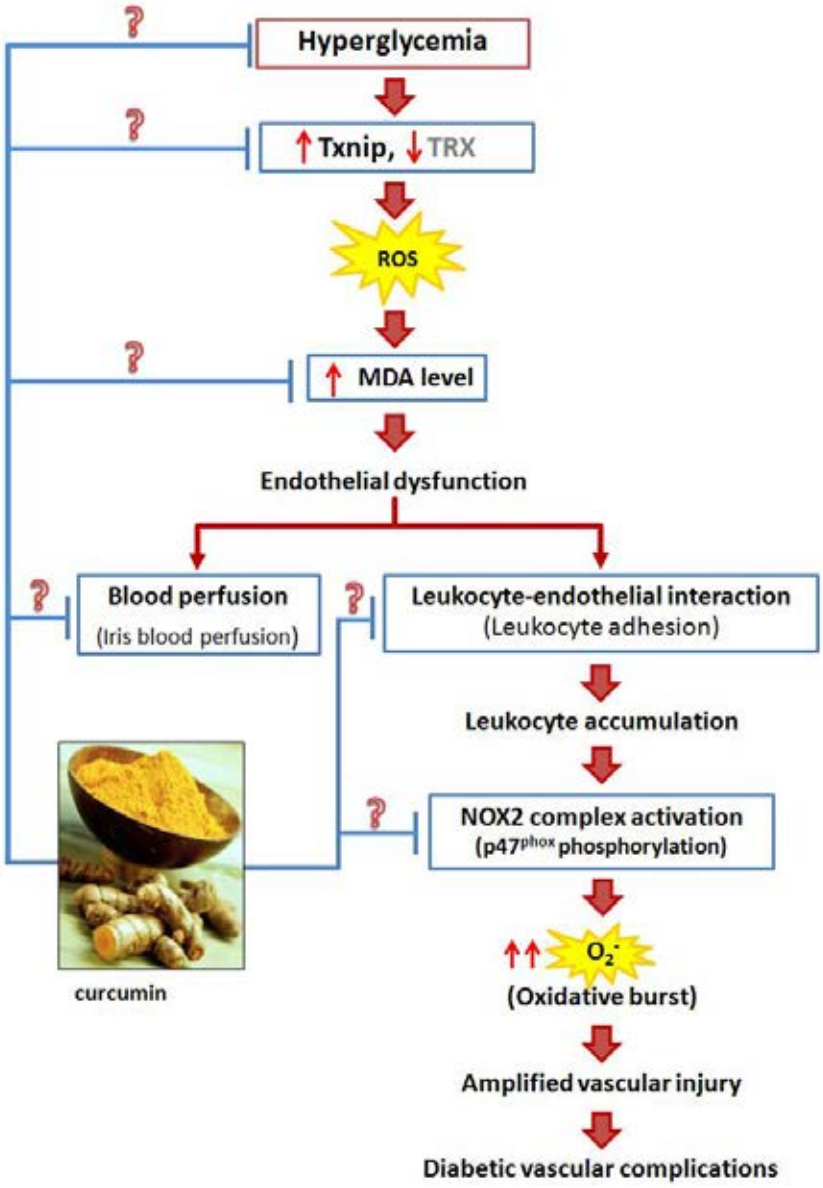
Research objective

1. To study the mechanisms of curcumin on reducing diabetes-induced leukocyte-endothelium interaction in association with its actions on NOX2 expressions.
2. To study the mechanisms of curcumin on reducing diabetes-induced leukocyte-endothelium interaction in association with its actions on Txnip expressions.

Hypothesis

Curcumin can reduce diabetes-induced leukocyte-endothelium interaction through suppressing of NOX2 and Txnip expressions.

Conceptual framework



CHAPTER II

LITERATURE REVIEWS

Diabetes

Diabetes is a group of metabolic disorder disease characterized by chronic hyperglycemia. The metabolic disorder disease refers to glucose from digested food cannot enter into cells that causes glucose build up in the blood stream. This is because pancreas cannot produce enough or no insulin, the cells cannot respond to insulin, or both. The raising blood glucose level for long time could lead to oxidative stress that affects all organs and cell types such as endothelial cells to develop diabetic complications such as retinopathy, nephropathy, and neuropathy.

Classification of diabetes

Type 1 diabetes

Type 1 diabetes is characterized by deficient insulin producing pancreatic β-cells. Type 1 diabetes is called “insulin dependent juvenile or childhood onset” because it is usually diagnosed in children and young adults. The most cause of type 1 diabetes is autoimmune inflammation to destroy β-cells of pancreas leading insufficient production of insulin. Because of the absence of insulin to take glucose into cells that causes lack of body energy source, so alternative sources of body energy such as protein and fat to be found under influence of glucagon. Protein and fat are catabolized to provide energy that

contributes to the weight loss and increase free fatty acid in blood stream. Pateints of type 1 diabetes require insulin for their survival. This type accounts for 10-15 % of the total diabetic population (30).

Type 2 diabetes

Type 2 diabetes is characterized by insulin resistance, which a decreased ability of cells to respond to insulin, and relative β-cell dysfunction. Type 2 diabetes is frequently associated with genetic factors, older age, overweight, obesity, and life style such as lack of exercise. This cause in a condition called hyperinsulinemia leads to protein and fat are catabolized to use instead of glucose to provide energy. The type 2 diabetic patients with insulin insistence are associated with abdominal obesity and promote weight gain. In addition, the weight gain and lacking of exercise could promote more insulin resistance as a result a vicious cycle occurs. The patients of type 2 diabetes have to manage their lifes by increasing exercise, dietary modification such as low-fat and low-carbohydrate diets, or controlling blood glucose level by medications or insulin. This type accounts for 85-90% of the total diabetic population (30).

Vascular complications in diabetes

Hyperglycemia induces vascular complications including macrovascular complications (stroke, heart disease, and peripheral vascular disease) and microvascular complications (retinopathy, nephropathy, and neuropathy) (31, 32).

The vascular consists of endothelial cells that are lined by a single layer of vessels. Endothelial cells play a major role in control of vascular homeostasis as a dynamic and heterogenous autocrine/paracrine organ. A key regulator of endothelial function is endothelium-derived nitric oxide (NO). NO induces vasodilation to prevent platelet aggragation and leukocyte adhesion. Hyperglycemia-induced free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) impair endothelial dysfunction resulting in reducing bioactivity of NO resulting in altered vascular tone, releasing chemokine/cytokine, and increasing adhesion molecule expression as well as decreasing anticoagulant properties (14, 33). The result of endothelial dysfunction might initiate vascular inflammation in the development of vascular complications (17, 21).

Mechanisms of vascular complications

Hyperglycemia-induced oxidative stress

Free radicals are highly reactive molecules due to having one or more unpaired electron (e^-) and rapidly eradicated by antioxidant (34). They can be generated in many sources including the mitochondrial electron transport chain, nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), xanthine oxidase, uncoupled endothelial nitric oxide synthase, and cytochrome p450 (2-5).

Under physiological condition, ROS are the one of free radicals including superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and nitric oxide (NO) as shown in Figure 2.1. They act as beneficial secondary messenger in host defense mechanism and in cellular signaling systems involved transcription factor regulation and intracellular redox activation. In pathological condition such as diabetes, the excessive concentrations of ROS can directly oxidize biological molecules including lipid, protein, and DNA leading imbalance between ROS overproduction and intracellular antioxidant system that called oxidative stress (12).

ROS molecule	Main sources	Enzymatic defense systems	Product(s)
Superoxide ($O_2^{\bullet -}$)	'Leakage' of electrons from the electron transport chain Activated phagocytes Xanthine oxidase Flavoenzymes	Superoxide dismutase (SOD) Superoxide reductase (in some bacteria)	$H_2O_2 + O_2$ H_2O_2
Hydrogen peroxide (H_2O_2)	From $O_2^{\bullet -}$ via superoxide dismutase (SOD) NADPH-oxidase (neutrophils) Glucose oxidase Xanthine oxidase	Glutathione peroxidase Catalases Peroxiredoxins (Prx)	$H_2O + GSSG$ $H_2O + O_2$ H_2O
Hydroxyl radical ($^{\bullet}OH$)	From $O_2^{\bullet -}$ and H_2O_2 via transition metals (Fe or Cu)		
Nitric oxide (NO)	Nitric oxide synthases	Glutathione/TrxR	GSNO

Figure 2.1 The major ROS molecules and their metabolisms (35)

Hyperglycemia-induced thioredoxin-interacting protein (Txnip) expression

In addition to ROS overproduction, the time-dependent high concentration of glucose could induce Txnip expression via inducing MondoA:MLX protein or carbohydrate responsive element binding protein (ChREBP) transcription factor to bind Txnip promoter (36-39). Moreover, hyperglycemia could activate p38MAPK to stimulate forkhead box protein O1 (FOXO1) transcription factor to bind Txnip promoter (8, 36, 40).

Txnip, also termed Vitamin D up-regulated protein 1, is an endogenous inhibitor of thioredoxin (TRX) activity (5-6). TRX as a member of thiol-disulfide oxidoreductases family is an important protein to involve several cellular processes such as a role in reduction-oxidation (redox) signaling in TRX system (35, 41-44). TRX system consists of TRX, TRX reductase, and NADPH (Fig. 3). This system can reduce oxidized cysteine groups on protein through interaction with active site of TRX (Cys-Gly-Pro-Cys) to form disulfide bond. The oxidized TRX form (TRX-S_2) is reversed to the reduced TRX form (TRX-$(SH)_2$) by TRX reductase through electron transfer from NADPH as well as recycle- and reuse- mechanism (45) (Figure 2.2).

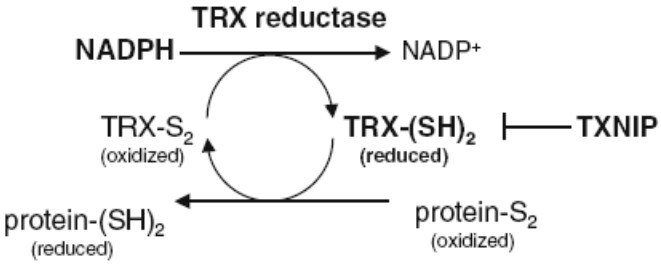


Figure 2.2 The TRX system-$(SH)_2$ reduces protein disulfides by its oxidoreductase activity generating TRX-S_2, and the oxidized form is regenerated by TRX reductase and NADPH (13).

In addition to ROS scavenger, the TRX system can nuclear translocate to enhance the binding of transcription factor (46, 47) and play regulatory roles in variety of cellular function through protein-protein interaction (48-51). TRX expression is widespread roles in a various cells, including endothelial cells to protect endothelial cells from oxidative stress and nitrosative stress (41-44).

Txnip acts as a competitive inhibitor to react with active site (Cys-X-X-Cys) of TRX to decrease TRX activity leading to cellular redox imbalance resulting in increased free radicals (Figure 2.3). Moreover, Txnip could remove protein from TRX which its function is

inhibited by TRX, such as Apoptosis signal-regulating kinase 1 (ASK-1) which is involved in regulation of apoptosis and inflammation. Normally, TRX reacts with ASK-1 to inhibit ASK-1 kinase activity that has anti-apoptosis (49, 52) and anti-inflammation functions (14, 51). In hyperglycemia, Txnip binds to TRX thereby releasing ASK1 from inactive form to active form leading to initiate apoptosis and inflammation processes.

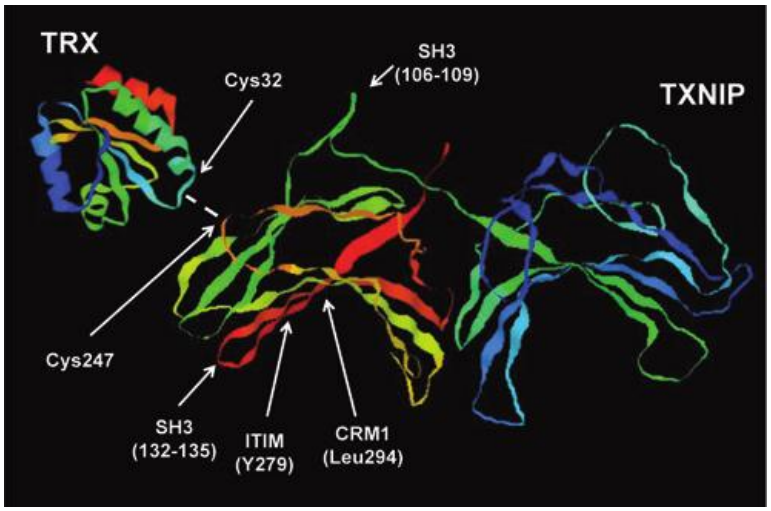


Figure 2.3 TXNIP, via Cys247, forms a disulfide bond with TRX catalytic site Cys32 to inhibit TRX activity and function (11).

Txnip plays a critical role in endothelial dysfunction by vascular inflammation, apoptosis, and inhibition nitric oxide production (10, 12, 13, 53, 54). Txnip-induced activated ASK-1 stimulates adhesion molecule expression such as ICAM-1 (14, 50, 51). The increased ICAM-1 expression plays a major role in promoting endothelial dysfunction (10, 55, 56). ICAM-1 is a ligand for lymphocyte function-associated antigen 1 (LFA-1) which is a receptor found on leukocyte surface.

In addition, Txnip-induced ROS could activate pro-inflammatory cytokine such as transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)

to induce ICAM-1 expression (57, 58). NF-κB is an important transcription factor which involve in endothelial dysfunction and pathogenesis of various vascular disorders.

These refer to promote adhesion molecule expression leading to leukocyte-endothelium interaction.

Vascular inflammation

The highly vascular adhesion molecule expression causes vascular inflammation characterized by leukocyte adhesion to endothelial cells. The leukocyte adhesion occurs mostly in postcapillary venules which has minimized hemodynamic shear forces. Leukocyte adhesion to endothelium process has several steps, outlined below as chemoattraction, capture, rolling, and tight adhesion (Figure 2.4).

1. Chemoattraction: Hyperglycemia-induced ROS overproduction stimulates endothelial cells to release the pro-inflammatory cytokines such as NF-κB, TNF-α, and IL-1 (59-61). Circulating leukocytes are localized close to the endothelial cells by these pro-inflammatory cytokines.
2. Capture: During the vascular inflammatory response, activated endothelial cells express the primary adhesion molecules for leukocyte capture such as P-selectin to initiate rolling process (62).
3. Rolling: After leukocyte are captured, P-selectin also is the most important to involve in rolling. In addition, the activated endothelial cells express L-selectin and E-selectin to support the rolling process. Importantly, E-selectin shows a slower rolling than P-selectin and L-selectin that can be possibly the initiation of tight adhesion.

This report demonstrated that E-selectin deficiency has reduced the tight adhesion leukocytes in response to vascular inflammation (63).

4. Tight adhesion: In this step, leukocytes express adhesion molecules known as integrin such as ICAM-1. ICAM-1 binds to LFA-1, which is an adhesion molecule on leukocyte surface, to decrease leukocyte rolling velocity before undergoing adhesion (64).

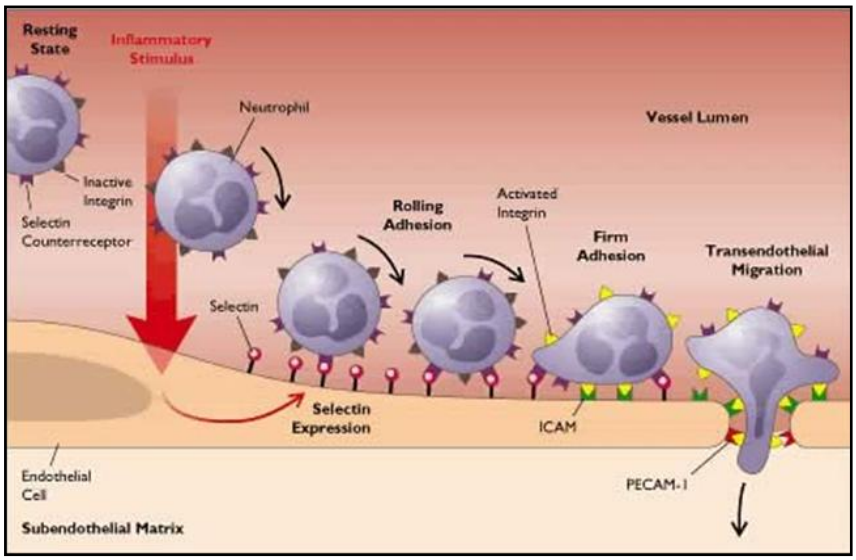


Figure 2.4 Leukocyte adhesion to endothelium process
(www.ladinfo.com)

NOX2 NADPH oxidase

NOX2 NADPH oxidase was first characterized in phagocytes, which plays an essential role in host defense. The NADPH oxidase is an enzyme that generates superoxide (O_2^-) as shown in this equation:

$$2O_2 + NADPH \xrightarrow{\text{NADPH oxidase}} 2O_2^{-} + NADP^{+} + H^{+}$$

NOX2 NADPH oxidase is the prototype NOX family of NADPH oxidase. It functions in membrane of phagocytic cells, especially phagocytic cells such as neutrophil and macrophage to protect against microorganism (65). In addition, it also is found in non-phagocytic cells such as endothelial cells (66-68), vascular smooth muscle cells (69), cardiac fibroblast (70), and cardiomyocytes (67). NOX family of NADPH oxidase has many subunit homologues: NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2 (Figure 2.5). NOX2 complex consists of catalytic subunit $gp91^{phox}$ (also known as NOX2) interaction with $p22^{phox}$, the cytoplasmic subunit ($p47^{phox}$, $p67^{phox}$, and $p40^{phox}$), and small G-protein Rac. NOX2 has six transmembrane domains and its COOH terminus-binding NADPH and NH_2 terminus are facing the cytoplasm.

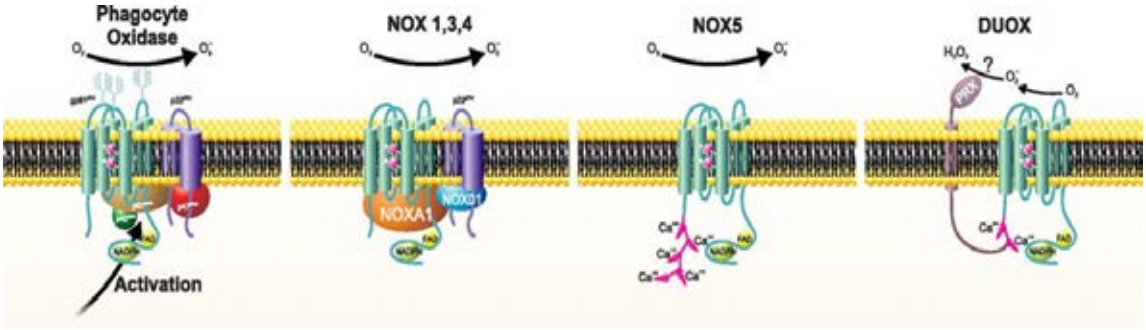


Figure 2.5 Activation of NADPH oxidase isoforms generates O_2^{-} by catalyzed electron transfer from NADPH to molecular oxygen. Despite their similar structure and enzymatic function, NOX family enzymes differ in their mechanism of activation (71)

In the resting, NOX2 and $p22^{phox}$ are found in the membrane of intracellular vesicles. Upon activation of phagocytic NADPH oxidase (oxidative burst) such as pro-inflammatory cytokines, there is an exchange of small G-protein Rac from GDP to GTP leading to $p47^{phox}$ activation. Phosphorylation of the cytoplasmic subunit $p47^{phox}$ promotes to conformational change to interact with $p22^{phox}$, and brings with other cytoplasmic subunits ($p67^{phox}$ into contact with NOX2 and $p40^{phox}$ to the complex) to activate NOX2 enzyme complex in plasma membrane. The active enzyme complex transports electron from NADPH across membrane to generate O_2^- from O_2 (Figure 2.6) (71).

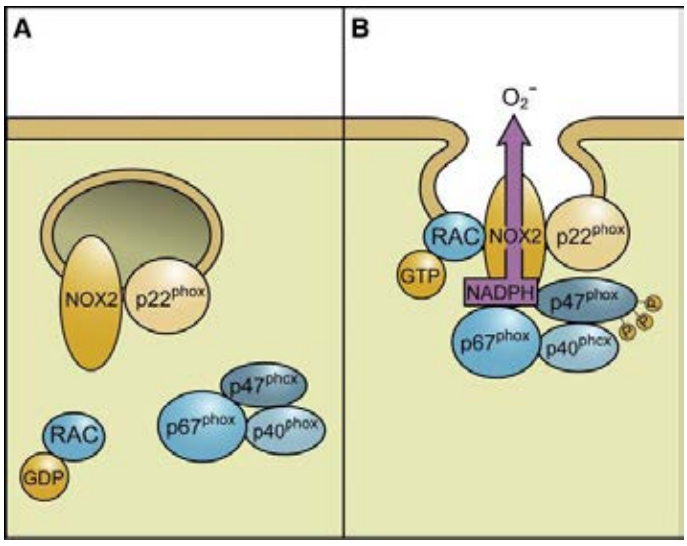


Figure 2.6 In resting condition (A), NOX2 and p22phox are found in the membrane of intracellular vesicles. Upon activation (B), there is an exchange of GDP for GTP on Rac leading to its activation. Phosphorylation of the cytosolic p47phox subunit leads to conformational changes allowing interaction with p22phox. The movement of p47phox brings with it the other cytoplasmic subunits, p67phox and p40phox, to form the active NOX2 enzyme complex (71).

The $p47^{phox}$ is a key protein in the assembly of NADPH complex for NOX2 enzyme activation (Figure 2.6) resulting in increased ROS production (71). NOX2 NADPH oxidase-induced ROS overproduction mediates retinal vascular inflammation in acute and chronic

models of retinal vascular disease (17). However, NADPH inhibitor has the potential to protect against diabetic nephropathy via reducing oxidative stress (72, 73).

Curcumin

Curcumin Longa Linn. is a tropical plant in southern and southeastern asia which is a member of the ginger family (Zingiberaceae). Curcuminoids (yellow powder) is turmeric extract which is the polyphenolic compounds. It composes of curcumin (77 %), demethoxycurcumin (17 %), and bisdemethoxycurcumin (3%) (Figure 2.7). Curcumin has a molecular formula of $C_{21}H_2O_6$ and molecular weight 368.37 g/mol. It exhibits antioxidant, anti-inflammation, anti-tumorgenic, and antimicrobial properties. Chemical structure of curcumin contains phenolic group which can normalize free radical molecule to stable molecule by donating H-atom (74).

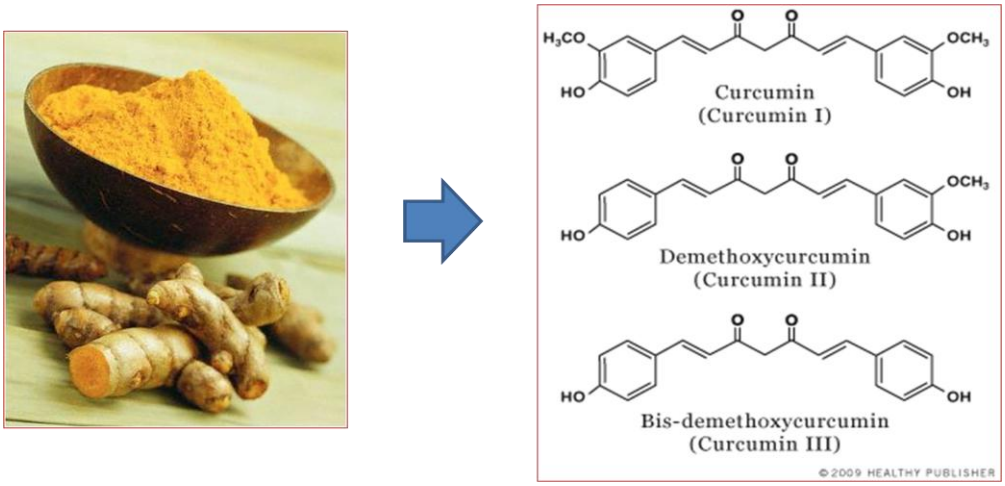


Figure 2.7 *Curcuma longa* Linn and chemical structure of curcuminoids

Numerous studies have shown direct antioxidant activity of curcumin which is a free-radical scavenger (74, 75) and indirect antioxidant activity of curcumin which can activate cytoprotective transcription factor such as NF-E2 related factor 2 (Nrf2) transcription factor as shown in Figure 2.8 (23-26). Curcumin can inhibit ROS generation in human red blood cell exposed to high glucose levels (18), and prevent increasing of 8OH-dG (19), protect islets against STZ-induced oxidative stress (20). In anti-inflammation, curcumin can attenuate pro-inflammatory cytokine levels such as TNF-α, IL-1β, and IL-6 by LPS- or PMA-stimulated macrophages (27) and decrease leukocyte-endothelium interaction in iris of STZ-induced diabetic rats (21, 22). Moreover, curcumin can suppress NF-κB activation by preventing nuclear translocation of the p65 NF-κB subunit (28) and inhibit phosphorylation and degradation of IκB (29).

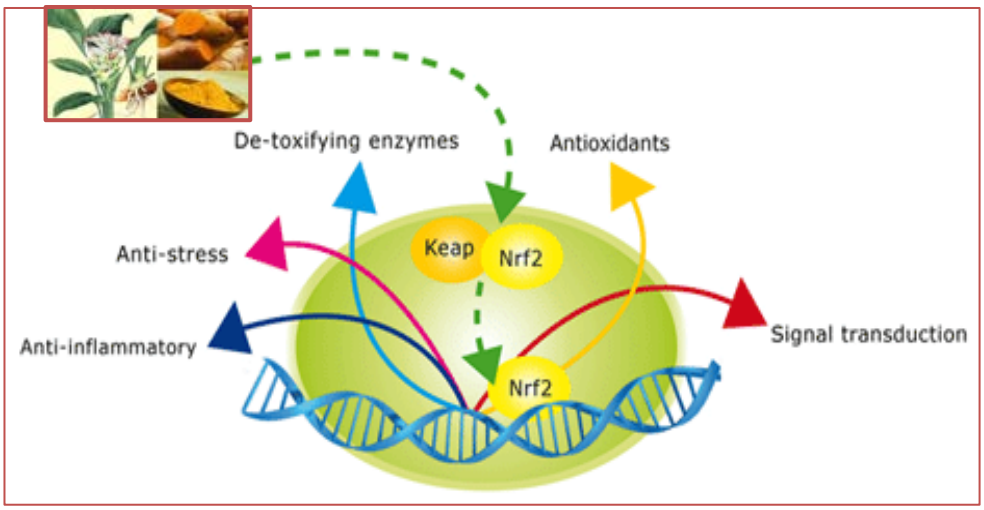


Figure 2.8 Role of curcumin activate NF-E2 related factor 2 (Nrf2) transcription factor to induce cellular defense mechanisms

However, given orally curcumin at a dose of 300 mg/kg to Wistar rats of both sexes has no pathological, behavioral abnormalities or lethality (76, 77). In human clinical trials also demonstrate that curcumin has no toxicity when administered at dose of 1-8 g/day (78) and 10 g/day (79).

CHAPTER III

MATERIALS AND METHODS

Animal preparation

Male Wistar rats (220-250 g) were obtained from National Laboratory Animal Center, Salaya Campus, Mahidol University, Thailand. The rats were allowed to rest for a week after arrival at the Animal center, Faculty of Medicine, Chulalongkorn University. All rats were housed under optimal conditions (constant room temperature 25±2 °C, 12/12 hour light/dark cycles and standard rat chow and water *ad libitum* with the guideline for experimental animals suggested by the National Research Council of Thailand (1999) and approved by the Institutional Animal Care and Use Committee of Chulalongkorn University.

Diabetic induction

All rats were ramdomly divided into diabetic and non-diabetic groups The diabetic group was induced by streptozotocin (STZ, Sigma Co., USA) which was freshly prepared by dissolving in citrate buffer pH 4.5 (Sigma Co., USA) and immediately injected into tail vein of fasted rats (6-8 hours) at dose 55 mg/kgBW (21, 22). The diabetic condition was defined as a glucose concentration more than 200 mg/dL (The Export Committee on the Diagnosis and Classification of DM, 2004) and verify at 48 hours after STZ injection by using a glucometer (ACCU-CHECK, Advance, Roche Diagnostics, Germany). In the control groups, they will receive the same volume of citrate buffer. The diabetic rats were characterized by polyuria,

polydipsea and elevated blood glucose levels, whereas their body weights were reduced compared to nondiabetic group.

Experimental design

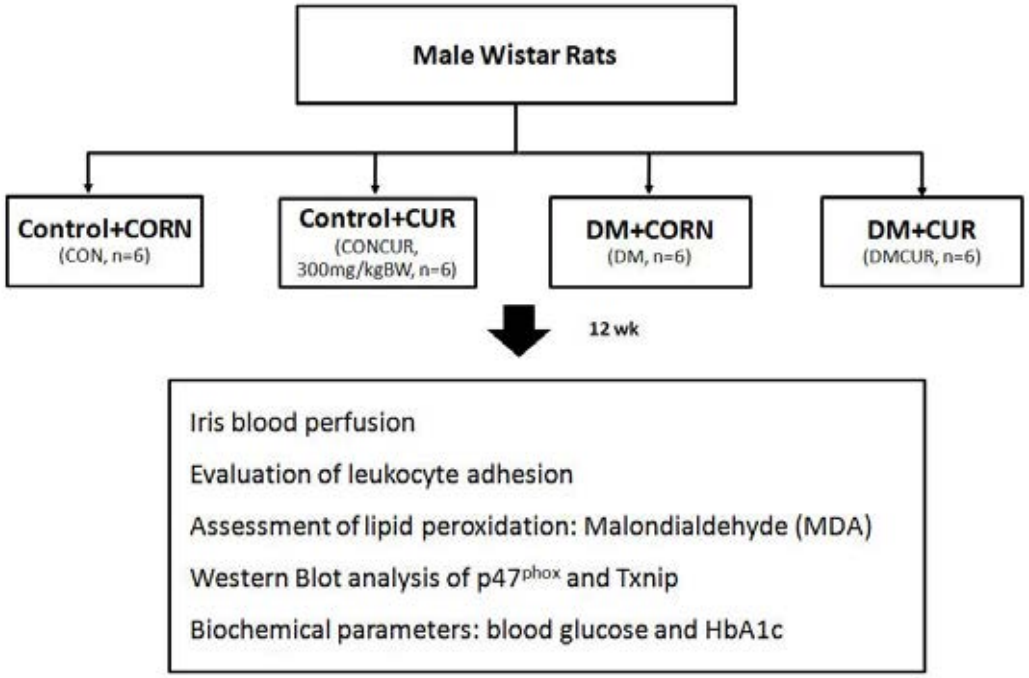


Figure 3.1 The experimental design was conducted in order to test the hypothesis based on the present conceptual framework

The rats were divided into four groups.

1. Control-treated with corn oil group (80) (CON, n=6)
2. Control-treated with curcumin group (CONCUR, curcumin 300mg/kgBW (Cayman, USA) dissolved in corn oil, n=6)
3. Diabetic-untreated group (DM, n=6)
4. Diabetic-treated with curcumin group (DM+CUR, n=6).

The dairy gavage feeding of curcumin dissolved in corn oil and corn oil in each group was started on 10th day after the STZ injection.

Experiments

On the 12th week after STZ injection, the rat was anesthesized with pentobarbital sodium (60 mg/kgBW i.p.), kept the rats warm at 37 $^{\circ}$C using warming pad and a trecheotomy was performed. A jugular vein and carotid artery were cannulated with polyethylene tube for injection of fluorescence tracers and for recording of systolic and diastolic blood pressure, respectively. The blood pressure was measured by using Statham pressure transducer connected to the Polygraph system (Nihon Koden, Japan). Mean arterial blood pressure was calculated from diastolic pressure + 1/3(systolic-diastolic).

1. Iris blood perfusion measurement

The right iris blood perfusion was measured using the Laser Doppler Blood Perfusion Monitoring (Perimed AB, Sweden) with the optic needle probe (0.1 millimeter). The needle probe was fixed perpendiculary above iris about 1 mm. Eight different measurement point of iris around pupil was performed at each time and the mean of iris blood perfusion was determined for each rat. (Figure 3.2 and 3.3)

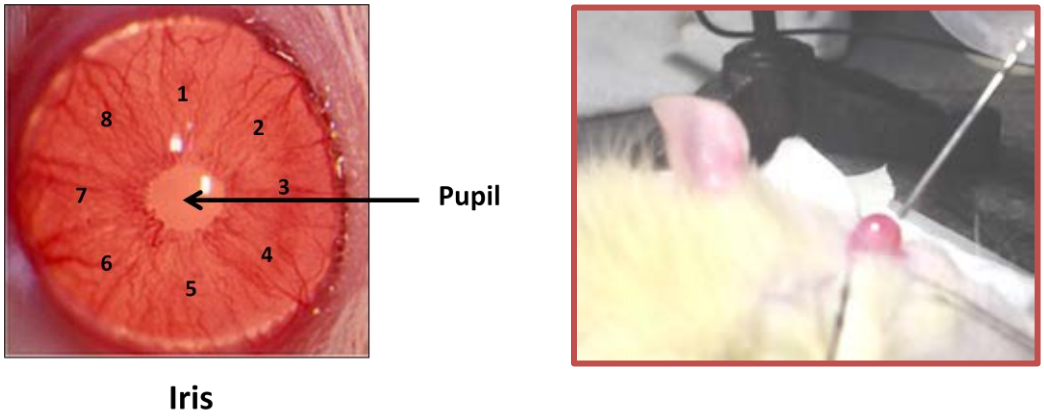


Figure 3.2 The method of measuring the regional iris blood perfusion

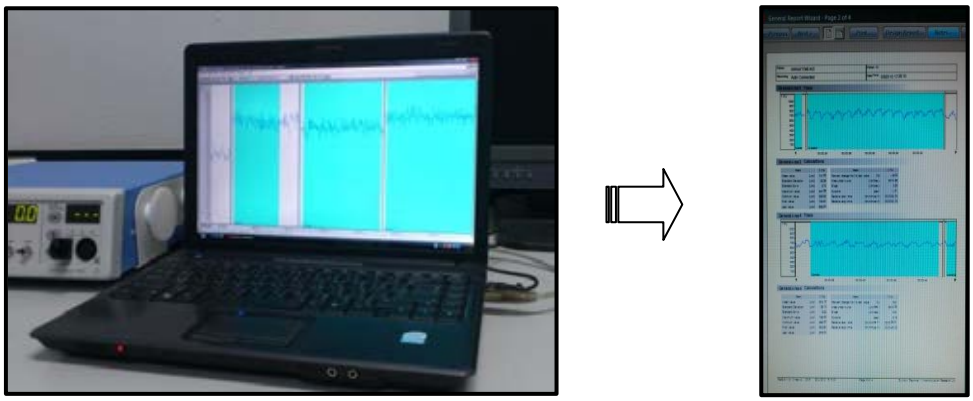


Figure 3.3 A photograph of iris blood perfusion calculation by PeriSoft for Windows 2.5

Principle of Laser Doppler Blood Perfusion Monitoring (LDF)

LDF is an established technique for the real-time measurement of the total microcirculatory blood perfusion including the perfusion in capillaries, arterioles, venules, and shunting vessel.

LDF works by illuminating the interested tissue under observation with scattered beam laser light carried by a fiber-optic probe. Laser light is partly absorbed within studied tissue, partly hit moving blood cells and is reflected back to the probe and is converted into an electronic signal as the result demonstrated on the screen (Figure 3.4).

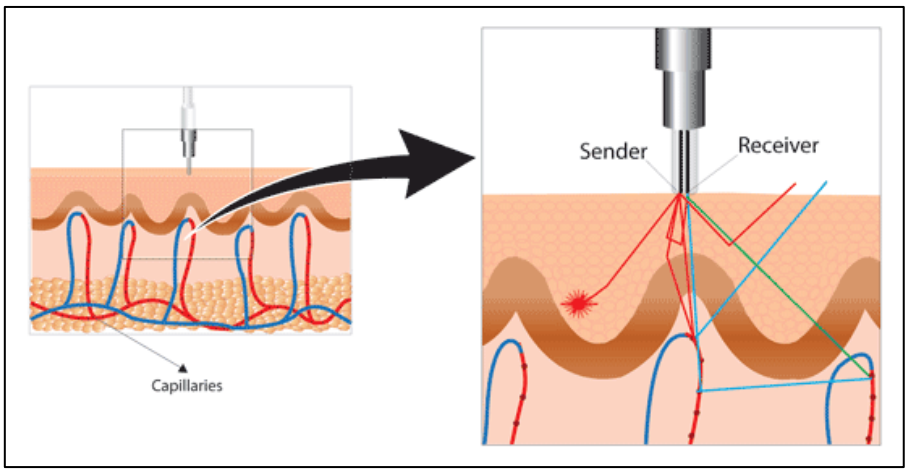


Figure 3.4 Schematic diagram of Laser Doppler probe, skin surface and skin microcirculation (http://www.perimed-instruments.com)

2. **Leukocyte imaging**

The iris blood vessel was used to observe instead of retina because the diabetic retinopathy is significantly related to the pressure of diabetic retinopathy in varying degree of severity (81). During the experiment, the real time image of iris blood vessel was recorded by an epi-illumination fluorescence videomicroscopy system (Optiphot 2, Nikon, Japan) equipped with a 100 W mercury lamp, CCD camera (Hamamatsu C2400, Japan), a

video recorder (VC-S5, Sharp, Japan) with a videotimer (VTG-33, For-A, Japan) and a 20x objective lens (CF Plan Fluor, Nikon, Japan) (Figure 3.5).

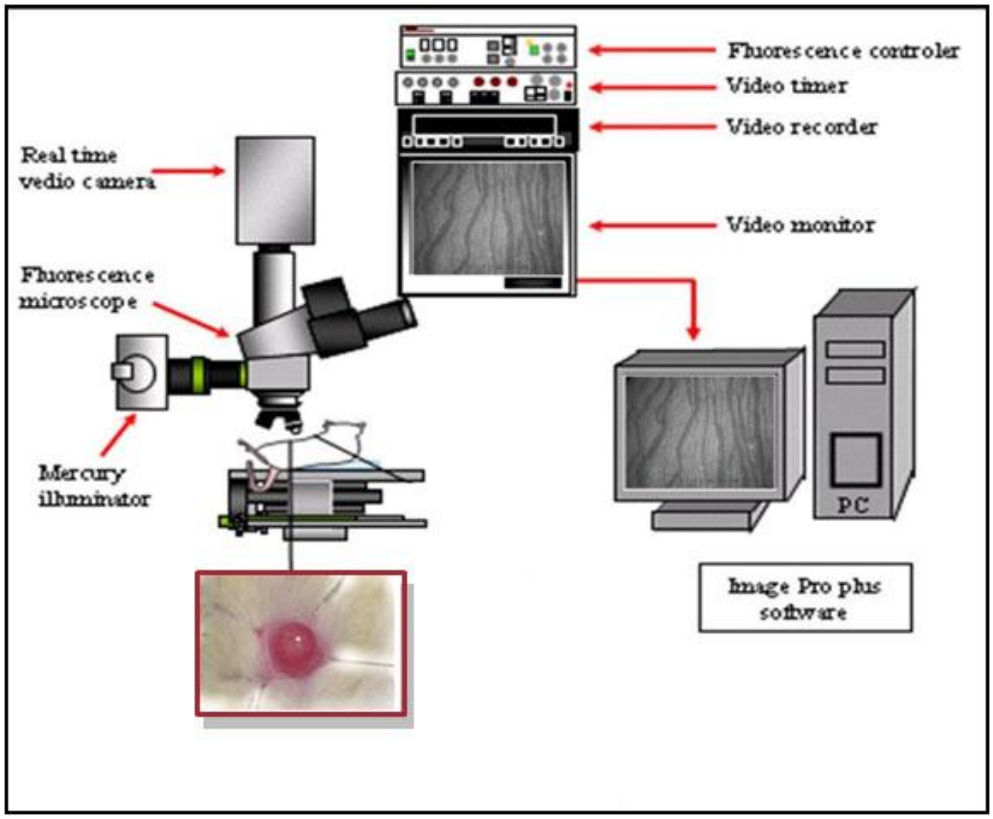


Figure 3.5 Schematic of setup for intravital fluorescence microscope of the iris microvasculature in the rat

Principle of intravital fluorescence microscopy technique

The fluorescence microscopy technique has become an essential tool in biology and the biomedical sciences that uses fluorescence to generate an image. A sample is illuminated with light of a wavelength which which is absorbed by the fluorophores, and then to emit light of longer wavelengths. The emitted light can be separated with emission filters designed for that specific wavelength. Therefore, the filters are chosen to match the

spectral excitation and emission characteristics of the fluorophore used to label the specimen. (Figure 3.6)

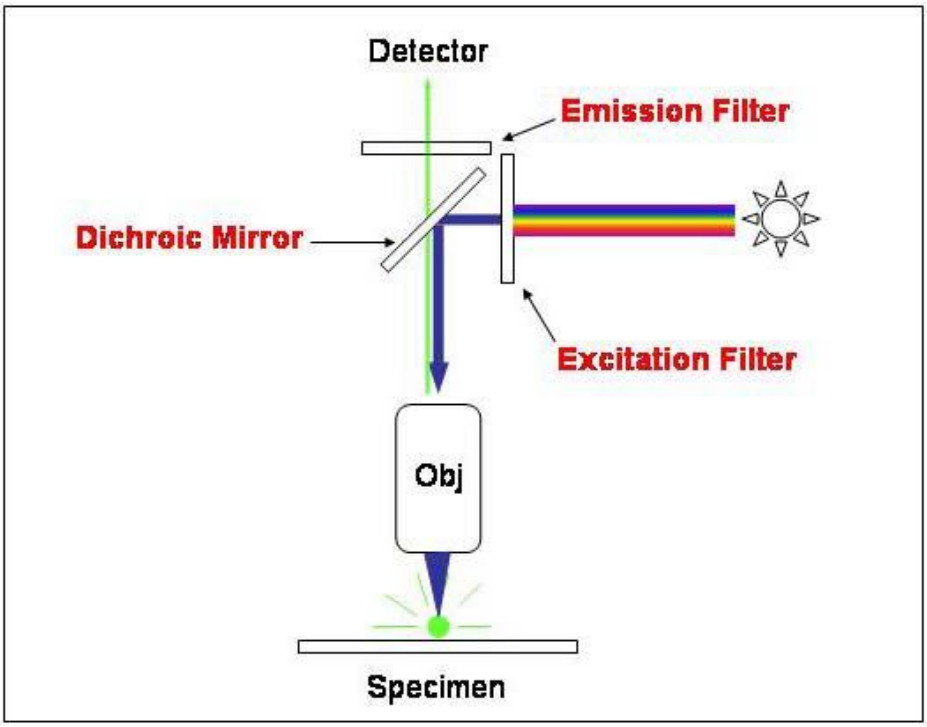


Figure 3.6 Schematic of a fluorescence microscope
(http://serc.carleton.edu/microbelife/research_methods/microscopy/fluromic.html)

Evaluation of leukocyte adhesion

Leukocyte adhesion in postcapillary venules of iris was determined by rhodamine-6G (R6G) which can label mitochondria especially in leukocyte (Sigma, USA) at 0.15 mg/kgBW as follow the method described by Jariyapongskul et al (82). The emission wavelength of R6G lies between 530 and 540 nm. Rhodamine-6G was injected intravenously for visualizing the leukocyte adhesion. Five observation views were chosen

and recorded for further determine leukocyte adhesion (83). The leukocytes which remained stationary to postcapillary venules for ≥ 30 seconds were considered to be leukocyte adhesion (21, 84). The number of leukocyte adhesion in each view was manually counted and reported by the mean number of cells per field of view (82).

3. Biochemical parameters

The parameters for metabolic changes were blood glucose and HbA1c. All these were determined at the end of the experiments by collected blood sample from abdominal aorta under anesthesia. Blood glucose and HbA1c were measured using enzymatic method and turbidimetric immunoinhibition method, respectively (Bangkok RIA Laboratory Co., Bangkok, Thailand).

4. Protein assay

After the collecting blood, the eyeball was collected immediately by enucleating from the orbital cavity. Lens and eye fluid were excised from the eye ball, the eyeball without lens and fluids called fundus. The fundus was washed in phosphate buffer (0.1 M, pH 7.4). Then, the fundus was chopped with fine scissors and was directly in lysis buffer containing 1X RIPA buffer (250 µl of lysis solution per 25 mg of tissue, Cell Signaling, Beverly, MA), 1X phosphatase inhibitor cocktails (1:100, Sigma Co., USA), and 1X protease inhibitor (1:100, Sigma Co., USA) for 30 minutes (85). After that, the chopped fundus was sonicated for 3 times for 10 seconds each, and was centrifuged at 15,000 g for 10 minutes

to spin down cellular debris. The supernatant was collected as the whole cell lysate to determine protein concentration (86).

The bicinchoninic acid assay (BCA assay) (Pierce, Rockford IL) is commonly used for determining the total concentration of protein in a solution (0.5 µg/mL to 1.5 mg/mL), similar to Lowry protein assay, Bradford protein assay or biuret reagent. The BCA assay primarily relies on two reactions. Firstly, the peptide bonds in protein reduce Cu^{2+} ions from the cupric sulfate to Cu^{+} (a temperature dependent reaction). The amount of Cu^{2+} reduced is proportional to the amount of protein present in the solution. Next, two molecules of bicinchoninic acid chelate with each Cu^{+} ion, forming a purple-colored product that strongly absorbs light at a wavelength of 562nm (87).

After protein quantification, the samples were used to determine MDA level and Txnip and p47phox expressions.

5. Free radicals by-products: Malondialdehyde (MDA)

Lipid peroxidation is one of mechanisms of cellular injury in many biological systems that polyunsaturated lipids are oxidized to form additional radical species such as malondialdehyde (MDA) as well as toxic by-products. MDA is a volatile, low-molecular-weight ($C_3H_4O_2$; formula weight = 72.07), short-chain, 1,3-dicarbonyl compound and a moderately weak acid (pK a= 4.46).

Thiobarbituric acid (TBA) assay is a commonly used method to determined malondialdehyde. This method is based on the reaction of TBA and MDA. The lipid material is simply heated with TBA under acidic condition, and the formation of a pink color is

measure at or close to 532 nm. The pink-colored is form by reaction of one molecule of MDA with two molecules of TBA (Figure 3.7) (21).

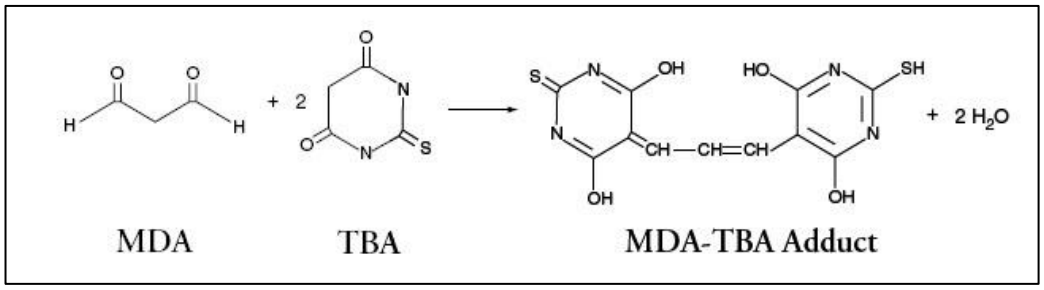


Figure 3.7 Formation of the fluorescent red 1:2 adduct between MDA and TBA

6. Western Blot analysis for NOX2 and Txnip expression

The protein expressions of Txnip and p47phox were further examined by Western Blot which is a commonly analytical technique to detect specific proteins. Western Blot was performed with 80 µg of fundus protein extraction per lane which was separated according to their size or molecular weight by 10% SDS-PAGE gel (Bio-Rad Laboratories) for 2 hours at 100 volt. Then the protein of SDS-PAGE gel was transferred to polyvinylidene difluoride (PVDF) membrane for 5 hours at 4˚C at 400 mA. After the protein was transferred to PVDF membrane, it can be used for probing (Figure 3.8).

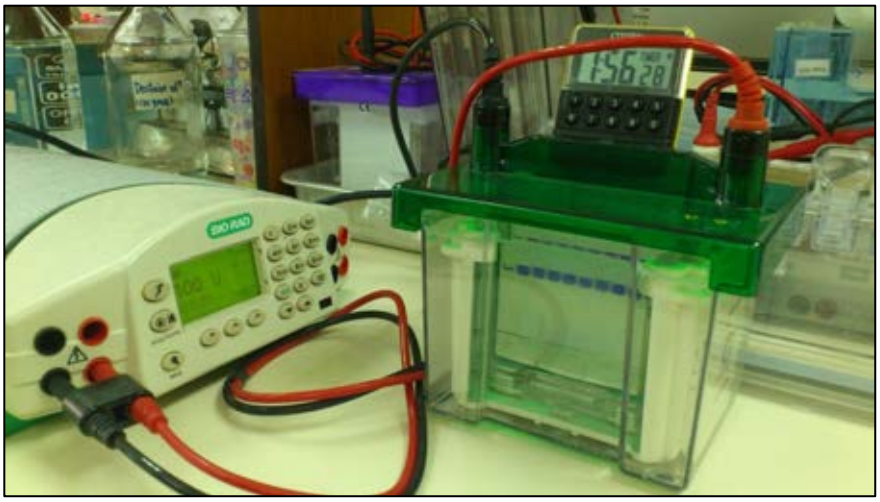


Figure 3.8 Schematic of electrophoresis for separating protein sample

For reducing background of nonspecific binding protein, the PVDF membrane was incubated with blocking solution (3% BSA (Sigma Co., USA) in TTBS) for overnight at 4°C. After blocking, the PVDF membrane was incubated with either anti-mouse Txnip antibody (1μg/ml, MBL, IL) in TTBS with 3% BSA for 2 hours at room temperature, or anti-mouse p47phox antibody (1:200, Santa Cruz, CA) in TTBS with 3% BSA for 2 hours at room temperature, or anti-mouse β-actin antibody (1:200, Santa Cruz, CA) in TTBS with 3% BSA for 1 hour at room temperature with gentle shaking. After primary antibody incubation, the membrane was washed 2 times for 5 minutes and 2 times for 10 minutes in TTBS, respectively.

Then horseradish-peroxidase conjugated goat anti-mouse secondary antibody at 1:2000 dilution (Santa Cruz, CA) in TTBS with 3% BSA for 1 hour at room temperature with gentle shaking. After secondary antibody incubation, the membrane was washed 2 times for 5 minutes and 2 times for 10 minutes in TTBS, respectively.

For chemiluminescence detection, the PVDF membrane was incubated with chemiluminescence substrate solution (GE, USA) and the bands were detected using ChemiDoc™ system (Bio-Rad Laboratories) with Qauntity One program version4.6.9 (Figure 3.9). Then, densitometry was performed using Image J analysis software (NIH) and the results of Txnip and p47phox expressions were quantified as a ratio to β-actin expression.

Figure 3.9 Schematic of Chemiluminescence detection

Statistical analysis

All data was presented as means ± SEM (standard error of mean). For comparison among groups of rats, one way analysis of variance (one-way ANOVA) was used and followed by Turkey post hoc test. $P < 0.001$ and 0.05 was considered statistically significant. For correlation between interesting parameters, Pearson Correlation Coefficient test was used $P < 0.05$. The data was analyzed using the SPSS program (version 16.0) for windows.

CHAPTER IV

RESULTS

This chapter of results was separated into seven major parts. Mainly, these results were addressed on the effects of curcumin supplementation on diabetes-induced endothelial dysfunction. Particularly, it described the possible mechanisms of curcumin on improving diabetes-induced leukocyte-endothelium interaction involving oxidative stress, Txnip, and p47phox expressions.

Part 1 **The effects of curcumin on physiological characteristics**

- Body weight (BW)
- Mean arterial pressure (MAP)

Part 2 **The effects of curcumin on biochemical parameters**

- Blood glucose (BG)
- Plasma glycosylated hemoglobin (HbA1c)

Part 3 **The effects of curcumin on hemodynamic changes**

- Iris blood perfusion (IBP)

Part 4 **The effects of curcumin on leukocyte-endothelium interaction**

Part 5 **The effects of curcumin on free radical by products**

- Fundus lipidperoxidation: Malondialdehyde level (MDA)

Part 6 **The mechanisms of curcumin on leukocyte-endothelium interaction: Roles of Txnip** and p47phox expression

Part 7 **Study relationships on MDA level and Txnip expression, leukocyte-endothelium interaction and Txnip expression, and leukocyte-endothelium interaction and p47phox expression**

Part 1 The effects of curcumin on physiological characteristics

Male Wistar rats weighing 220-250g were used in this study. Diabetes was induced by intravenous injection (tail vein) of streptozotocin (STZ; 55mg/kgBW, Sigma, St. Louis, USA). The diabetic signs including polydipsia, polyuria, polyphagia, and hyperglycemia were observed within 48 hours and showed persistent hyperglycemia throughout the experimental period. In the present study, the criteria used for diabetic condition was the blood glucose level that had to be higher than 200 mg/dL (The Export Committee on the Diagnosis and Classification of Diabetes Mellitus, 2004).

As shown in Table.4.1, the body weights (BW) of 12-week DM, DMCUR rats were significantly decreased (35.93% and 39.15%) as compared to CON and CONCUR groups ($P < 0.001$). Mean arterial blood pressure (MAP) was calculated for each group by using the equation: MAP = diastolic pressure + 1/3 (systolic pressure – diastolic pressure) as shown previously in Chapter III. MAPs of 12-week DM and DMCUR groups were not significantly different as compared to CON and CONCUR groups (Table 4.1).

Table 4.1 Body welght (BW, g) and mean arterial blood pressure (MAP, mmHg) were shown for each group (n=6), CON (control), CONCUR (control treated with curcumin), DM (diabetes), and DMCUR (diabetes treated with curcumin)

Group	BW (g)	MAP (mmHg)
12-wk CON (n=6)	424.00 ± 28.28	124.77±9.60
12-wk CONCUR (n=6)	480.67 ± 17.26^{ns}	132.58±5.50^{ns}
12-wk DM (n=6)	271.67 ± 13.22**	113.56±6.98^{ns}
12-wk DMCUR (n=6)	258.00 ± 17.64^{**,NS}	112.80±4.99^{ns,NS}

Values are mean±SEM (n=6 for each group).

ns not significant difference compared to CON group

NS not significant difference compared to DM group

** P < 0.001 significant as compared with control

Part 2 The effects of curcumin on biochemical parameters

Blood glucose levels (BG) of DM group (410.33±16.77 mg/dL) and DMCUR group (390.33±11.59 mg/dL) were significantly elevated as compared to CON group (172.83±11.08 mg/DL) and CONCUR group (192.50±13.05 mg/dL) ($P < 0.001$).

Plasma glycosylated hemoglobin levels of DM group (9.3±0.21%) and DMCUR group (9.47±0.47%) were significantly elevated as compared to CON group (4±0.05%) and CONCUR group (4.08±0.05%) ($P < 0.001$) (Table 4.2).

Table 4.2 Blood glucose (BG, mg/dl) and plasma glycosylated hemoglobin (% HbA1c) were shown for each group, CON (control), CONCUR (control treated with curcumin), DM (diabetes), and DMCUR (diabetes treated with curcumin)

Group	BG (mg/dL)	Hemoglobin A1c (%)
12-wk CON (n=6)	172.83 ± 11.08	4.00 ± 0.06
12-wk CONCUR (n=6)	192.50 ± 13.05^{ns}	4.08 ± 0.05^{ns}
12-wk DM (n=6)	410.33 ± 16.77**	9.47 ± 0.47**
12-wk DMCUR (n=6)	390.33 ± 11.59^{**,NS}	9.30 ± 0.21^{**,NS}

Values are mean±SEM (n=6 for each group).

ns not significant difference compared to CON group

NS not significant difference compared to DM group

** P < 0.001 significant as compared with control

Part 3 The effects of curcumin on hemodynamic changes

By using laser Doppler Flowmetry, the regional iris blood perfusion of each rat was evaluated from eight different points measured around the pupil as described previously (Figure 3.2, Chapter III). Mean regional iris blood perfusion in each group was summarized in Figure 4.1. Mean iris blood perfusion (IBP) of DM group (246±26.99 AU) was significantly reduced as compared to CON group (870.27±39.61 AU) and CONCUR group (921.76±24.77 AU) ($P < 0.001$). IBP in DMCUR group (311.28±31.13 AU) was not significantly different as compared to DM group.

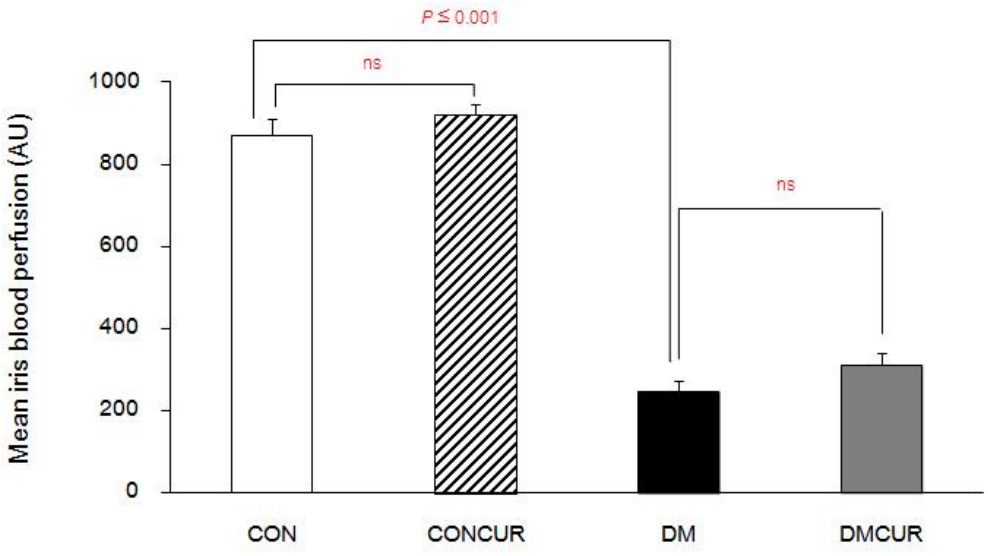


Figure 4.1 The effects of curcumin on mean regional iris blood perfusion in each group
Data are means±SEM (n=6 for each group)

Part 4 The effects of curcumin on leukocyte-endothelium interaction

The leukocyte was counted as adherent one that remained stationary for equal or longer than 30 seconds. The leukocyte adhesion was counted per field of view totally of postcapillary venule (diameter 20-30 μm) as described previously.

In the present video microscopic visualization showed clear image of leukocyte adhering to the endothelium of postcapillary venule in five different monitored views of iris of each rat (Figure 4.2). Five different monitored views of leukocyte adhesion were manually counted and the mean of leukocyte adhesion per field of views as performed in Figure 4.3.

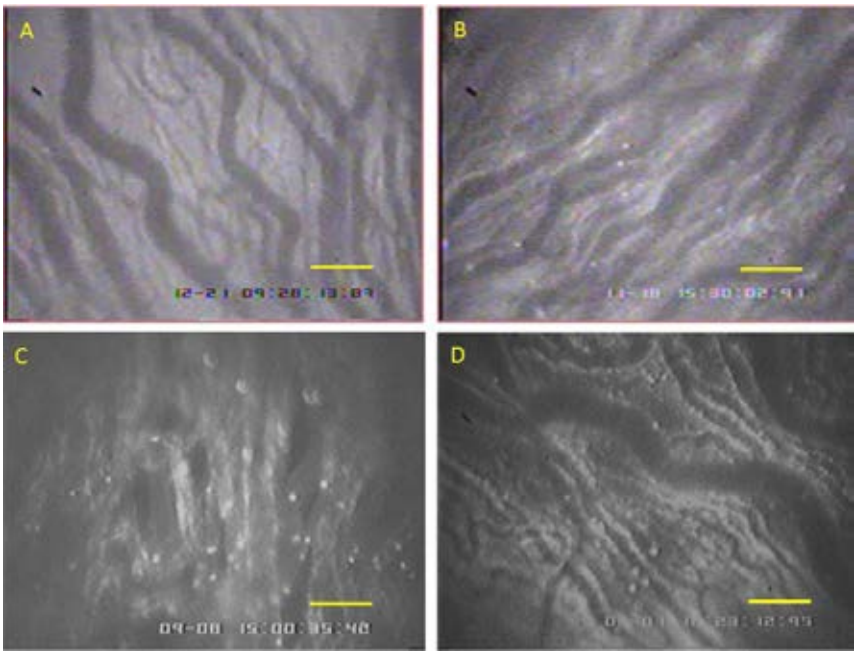


Figure 4.2 Intravital microscopic demonstration of leukocyte adhesion in the postcapillary venule of iris in CON group (A), CONCUR group (B), DM group (C), and DMCUR group (D). White dots represent leukocyte stained by Rhodamine 6-G i.v. injection. (Scale bar represent 100 μm)

The number of leukocyte adhesion was significantly increased in DM group (33.71±0.68 cells/frame) as compared to CON group (3.64±0.68 cells/frame) and CONCUR group (5.37±0.74 cells/frame) ($P < 0.001$). Interestingly, the number of leukocyte adhesion of DMCUR group (14.94±3.85 cells/frame) was significantly reduced as compared to DM group ($P < 0.05$).

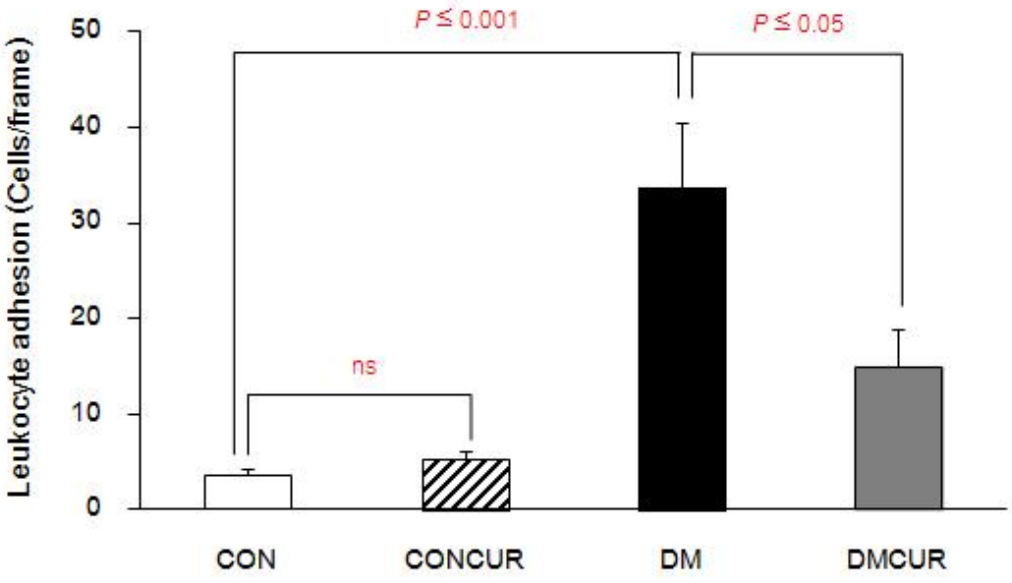


Figure 4.3 The effects of curcumin on leukocyte-endothelium interaction

Data are means±SEM (n=6 for each group).

Part 5 The effects of curcumin on free radical by products

In this study fundus malondialdehyde level (MDA), product of lipidperoxidation was used as indicator of oxygen free radicals in fundus. MDA level was significantly elevated in DM group (11.69±0.22 nmole/protein100µg) as compared to CON group (5.17±1.05 nmole/protein100µg) and CONCUR group (4.63±0.57 nmole/protein100µg) ($P < 0.001$). Interestingly, MDA level of DMCUR group (8.36±0.63 nmole/protein100µg) was significantly reduced as compared to DM group ($P < 0.05$) (Figure 4.4).

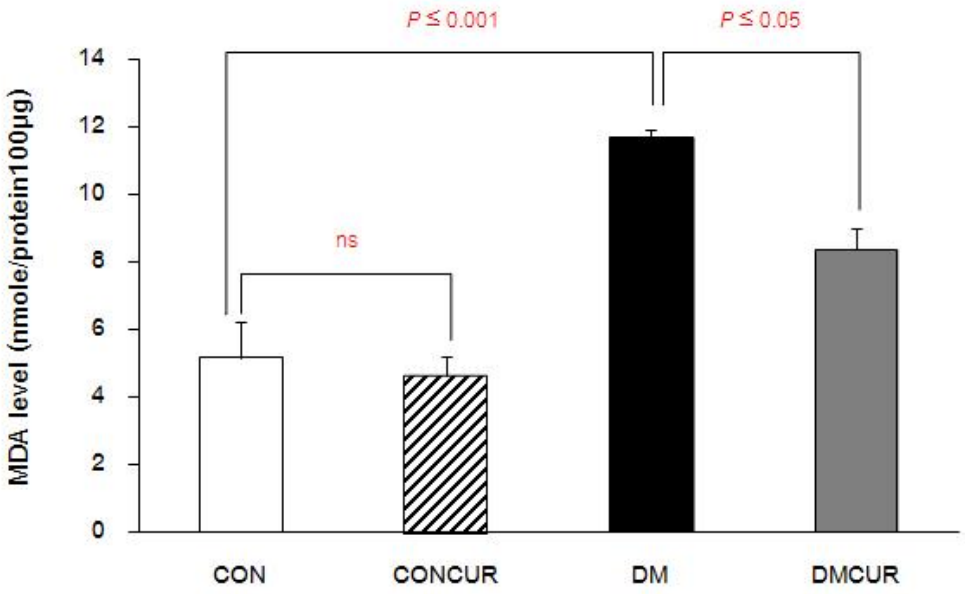


Figure 4.4 The effects of curcumin on fundus MDA level

Data are means±SEM (n=5 for each group).

Part 6 The mechanisms of curcumin on leukocyte-endothelium interaction: Roles of Txnip and p47phox expression

The levels of Txnip and p47phox of each group was examined on fundus extracts (as described previously) by Western Blot analysis. The results of Txnip and p47phox expressions were quantified as a ratio to β-actin expression.

6.1 Results of Txnip expression

The level of Txnip was significantly elevated in DM group (0.83±0.14) and DMCUR group (0.71±0.1) as compared to CON group (0.45±0.06) ($P < 0.05$) (Figure 4.5).

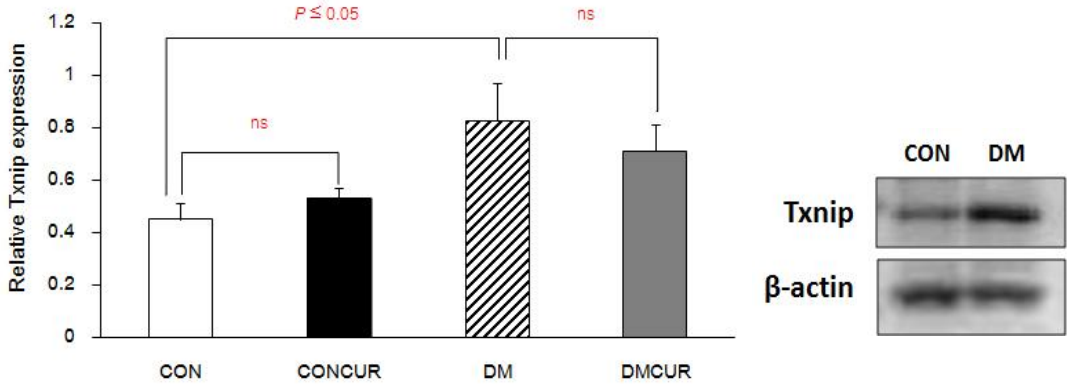


Figure 4.5 The mechanisms of curcumin on Txnip expression

Data are means±SEM (CON and DMCUR groups; n=5, CONCUR and DM groups; n=6)

6.2 Results of p47phox expression

The level of p47phox was significantly elevated in DM group (0.87±0.14) as compared to CON group (0.39±0.03) and CONCUR group (0.52±0.05) ($P < 0.05$). Interestingly, the level of p47phox of DMCUR group (0.51±0.05) was significantly reduced as compared to DM group, decreased Txnip expression approximately 1.71-fold ($P < 0.05$) (Figure 4.6).

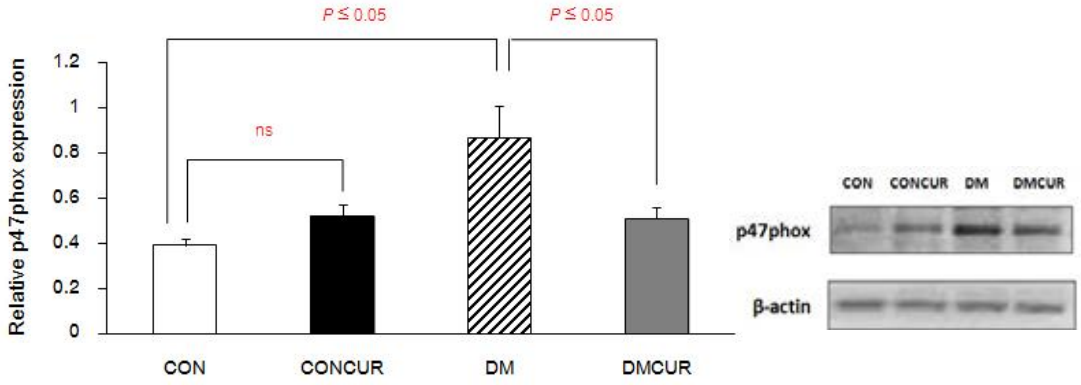


Figure 4.6 The mechanisms of curcumin on p47phox expression

Data are means±SEM (CON and DMCUR groups; n=5, CONCUR and DM groups; n=6)

Part 7 Study of relationships between mean blood glucose vs mean Txnip expression, and mean leukocyte-endothelium interaction vs mean p47phox expression

Correlation analysis was performed by using Pearson correlation test to examine the relationship between means blood glucose and means Txnip expression of all four groups. The relationship between means leukocyte-endothelium interaction and means p47phox expression were determined by the same method as well.

7.1 Correlation between means blood glucose and means Txnip expression

The mean blood glucose and mean Txnip expression were plotted and the results showed that these two parameters were correlated and could fit in the linear equation of: $y = 0.0013x + 0.246$, $r = 0.966$, $P = 0.034$ (Figure 4.7).

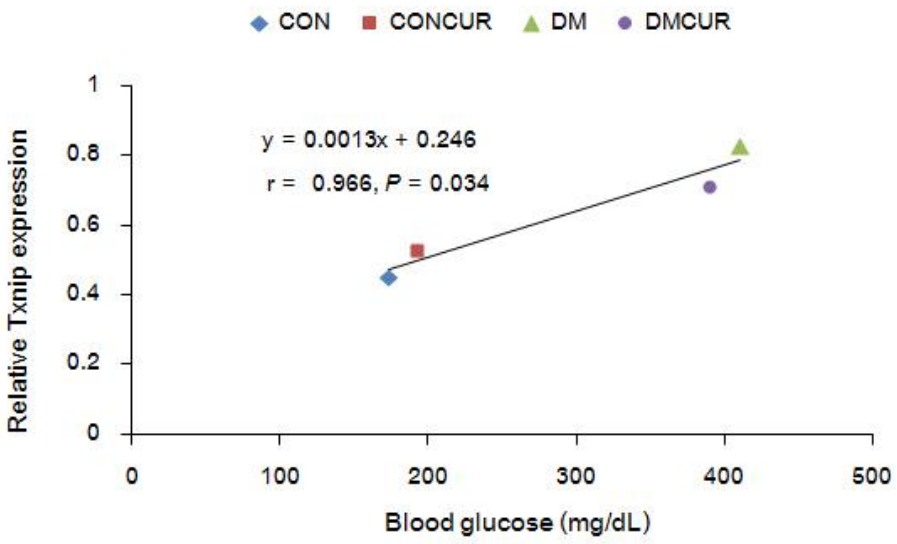


Figure 4.7 Correlation of mean blood glucose and mean Txnip expression

7.2 Correlation between means MDA level and means Txnip expression

The mean MDA level and mean Txnip expression were plotted and the results showed that these two parameters were correlated and could fit in the linear equation of: y = 0.0505x + 0.2533, r = 0.958, *P* = 0.042 (Figure 4.8).

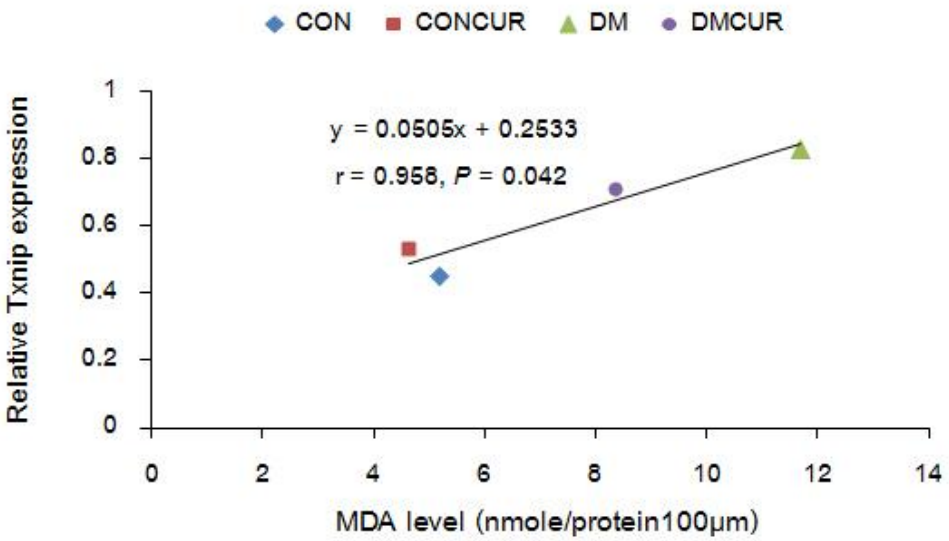


Figure 4.8 Correlation of means MDA levels and means Txnip expression

7.3 Correlation of mean leukocyte adhesion and mean p47phox expression

The mean leukocyte-endothelium interaction and mean p47phox expression were plotted and the results showed that these two parameters were correlated and could fit in the linear equation of: y = 0.0143x + 0.3666, r = 0.952, *P* = 0.048 (Figure 4.9).

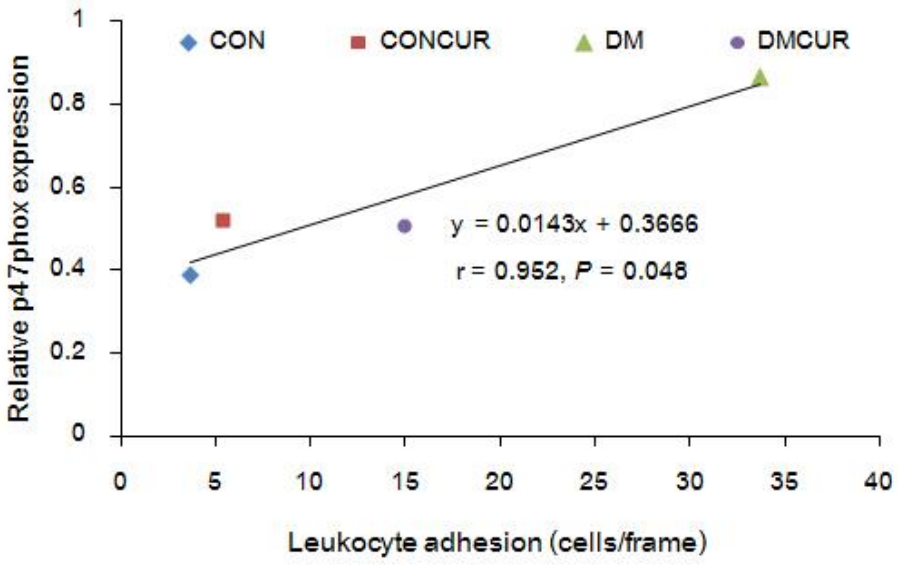


Figure 4.9 Correlation of means leukocyte-endothelium interaction and means p47phox expression

CHAPTER V

DISCUSSION

In the present study, the experiments were conducted to investigate the mechanisms of curcumin on leukocyte-endothelium interaction in diabetic rats. By using streptozotocin (STZ) to induce diabetes, rats were become chronic diabetes without insulin supplementation. It has been demonstrated that the rat model of STZ-induced diabetes will show the endothelial cell dysfunction at about 6 weeks after the STZ-injection (88-90). STZ has greatly specific action on the pancreatic via GLUT2 transporter, which is β-cell's glucose transporter (91). When STZ gets into the β-cells, it will extremely damage β-cells by reactive oxygen species (ROS). STZ caused much greater damage to the β-cells because β-cells probably have very low level of antioxidant enzyme expression and activity (92, 93).

In present study, hyperglycemia occurs within 48 hours after intravenous STZ injection at dose 55 mg/kgBW and persists throughout the experiment. Blood glucose (BG) was significantly elevated in DM group as compared to CON group ($P < 0.001$). In addition, BG was confirmed with HbA1c test, HbA1c in DM group was significantly increased as compared to CON group ($P < 0.001$) (Table 2). Also weight loss was found in DM group significantly decreased as compared to CON group ($P < 0.001$) (Table 1).

In Table 1, it also showed that body weights of DM and DMCUR were significantly less than those CON and CONCUR. Numerous reports who performed with this STZ model, indicated that β-cells are destroyed that causes lacking of insulin to take glucose into cells.

Finally, hyperglycemia develops and body lack of energy, so alternative energy from protein or fat in the body is converted into glucose under influence of glucagon. Protein and fat are catabolized to provide the energy that contributes to the weight loss (90).

Role of curcumin in anti-diabetic action in diabetic model

This experiment aimed to investigate the effect of curcumin on diabetic action in diabetic model. From results of BG and HbA1c in DMCUR group demonstrated that curcumin did not affect to reduce level of blood glucose and HbA1c. These results were consistent with the results of body weight in DMCUR group because hyperglycemia still occurred in diabetic rats that contributed to negative energy balance. The results of BG were consistent with HbA1c and body weight in diabetic rats as compared to non-diabetic rats that agreed with several reports (19, 94, 95).

However, there is controversy about the effect of curcumin on these results. Some studies have reported curcumin can ameliorate the severity of hyperglycemia and suppress body weight loss in type 2 diabetes to improve insulin sensitivity while concomitantly with anti-inflammatory effect (96) and the results did not collaborate with the previous studies in efficacy of curcumin in reduction of hyperglycemia in type 1 diabetes (21, 97, 98).

Role of curcumin on iris blood perfusion in diabetic model

Diabetes-induced hyperglycemia impairs endothelial function that causes vascular homeostasis alterations such as abnormal vascular blood perfusion. In this experiment, blood perfusion in iris was chosen to represent the alteration of vascular homeostasis. Iris blood perfusion (IBP) was assessed from regional iris around pupil. The result (Figure 4.1)

showed that mean of IBP in DM group was significantly decreased as compared to CON group ($P < 0.001$). The mean of IBP in DMCUR group had trend to increase up to 4.78% as compared to DM group, though the result was not significantly difference (Figure 4.1). Therefore, curcumin supplementation could not prevent diabetes reduced IBP as which supported by this study (99).

The possible mechanism that explained how diabetes induced decrease in IBP might be involved in nitric oxide (NO). Nitric oxide, which is constitutively produced by normal vascular endothelium, is a major factor in regulating vascular flow resistance. NO is an important constitutive vasodilator derived by endothelium. Therefore, IBP is decreased that implies the decrease in NO in diabetes. Hyperglycemia-induced reactive oxygen species (ROS) overproduction which can react with BH4 to BH2 to inactivate eNOS function called “eNOS uncoupling” leading decreased NO production (100). In addition, altered IBP may consequently contribute to the development of morphogical and functional vascular abnormalities including capillary basement membrane thickening (101), platelet aggregation (102), and leukocyte adhesion (21, 55) in diabetic eyes.

The result in DMCUR group is consistent with the previous study (22). However, there are several studies reported that curcumin could increase cerebral blood flow in diabetic rats (103) and improve endothelium-dependent relaxation in mesentery of diabetic rats (97). Interestingly, the effect of curcumin to improve IBP in diabetes might be involved in antioxidant property. However, the antioxidant potential of curcumin dependents on dose of curcumin and pathological condition such as concentration of cellular oxidants (74, 104).

Role of curcumin on leukocyte-endothelium interaction in diabetic model

Hyperglycemia-induced ROS overproduction can stimulate adhesion molecule expression leading vascular inflammation that is characterized by leukocyte adhesion. The leukocyte adhesion was determined by using intravital fluorescence microscope. The results shown in Figure 4.3 demonstrated that the number of leukocyte adhesion in DM group was significantly increased up to 89% as compared with CON group ($P < 0.001$) (Figure 4.3). Several studies have indicated that hyperglycemia-induced ROS overproduction leads to decrease in NO bioavailability resulting in enhanced adhesion molecule expression such as ICAM-1 and VCAM-1 (14, 50, 51, 55).

In this experiment, we have shown that low dose of curcumin supplementation (300mg/kgBW) could decrease diabetes-induced leukocyte-endothelium interaction significantly ($P <0.05$) (Figure 4.3). This might be contributed by pharmacological effects of curcumin including its potential on anti-inflammation and anti-oxidant. The result showed that the number of leukocyte adhesion in DMCUR group was significantly decreased down to 55.68% as compared to DM group. This result was supported by the previous study which indicated that the anti-inflammatory effect of curcumin contributed to prevent leukocyte adhesion (21) by reducing pro-inflammatory cytokines such as IL-1 and IL-6 via blockade of Akt, p38MAPK, and NF-κB pathways (28, 29, 105).

Role of curcumin in antioxidant action in diabetic model

Hyperglycemia induced oxidative stress is known for its major role in causing diabetic complications. Reactive oxygen species (ROS) could cause cellular damage

through its oxidative reaction of macromolecules including protein, lipid, and DNA. For instant, lipid peroxidation product, malondialdehyde (MDA), which is commonly used as an indicator for ROS content, is produced by ROS reacted with lipid molecules. In this experiment, the MDA level in DM group was significantly increased up to 126% as compared to CON group ($P < 0.001$) and MDA level in DMCUR group was significantly decreased down to 28% as compared to DM group ($P < 0.05$) (Figure 4.4). These results of MDA implied the potential of curcumin as an effective anti-oxidant and confirmed with the studies of Patumraj et al 2006 (21). From several studies, they indicated that curcumin has both direct and indirect anti-oxidative mechanisms. The direct action of curcumin can be described through it can scavenge free radicals by redox activity. The indirect anti-oxidant property can enhance cellular antioxidants such as Heme oxygenase-1 (HO-1), Thioredoxin-1 (TRX-1), Glutathione peroxidase (GPx), and Glutathione (GSH) through activating Nrf2 transcription factor which is cytoprotective gene (24-26).

The mechanism of curcumin on reducing leukocyte adhesion via Txnip expression

It is known that reactive oxygen species (ROS) are generated as a normal by-product of aerobic metabolism. However, all mammalian cells have antioxidant defense systems including enzymatic and non-enzymatic mechanisms. The oxidation of the sulfydryl groups on sulfur-containing amino acid residues cysteine and methionine are especially sensitive enzymatic-repairing samples including the thioredoxin and glutaredoxin systems (50).

When thioredoxin (Trx) itself was oxidized, it needs to reduce by thioredoxin reductase to restore its activity (13). As previously mention in Chapter II, this redox process of restoring the activity of thioredoxin is modulated by an endogenous inhibitor, thioredoxin interacting protein (Txnip). This small, 38–amino acid protein, Txnip, was also found to be upregulated by high glucose using a microarray-based approach (106). Moreover, it also showed that hyperglycemia induces Txnip expression by stimulate carbohydrate response element binding protein and forkhead box O1 (FOXO1) transcriptional factor (8, 37).

As shown in our data (Figure 4.5), the results showed that Txnip expression in DM eye tissue was significantly increased up to 84.44% as compared to CON group (P = 0.045). Concomitantly with other studies, the results confirmed that hyperglycemia induces Txnip expression. Therefore, Txnip expression may have a critical role in the development of diabetic complications (7, 58, 106, 107). Txnip expression in DMCUR group was not significantly different as compared to DM group and CON group. Although the curcumin had no any effect to inhibit Txnip expression, txnip expression in DMCUR group had tended to reduce (14.46%) as compared to DM group. It might explain that curcumin with this low dose may not be able to decrease BG, and it had no other effects on inhibiting Txnip expression.

The mechanism of curcumin on reducing leukocyte adhesion via p47phox expression (NOX2 enzyme)

As shown in Figure 4.6, it indicated that p47phox expression in DM eye tissue was significantly increased up to 55.17% as compared to the CON eye (P = 0.005). The result of

diabetes-induced increased p47phox expression is supported by several studies (17, 108, 109). As we have reviewed previously (Chapter II), NOX2, known as gp91phox, is a prototype of NADPH oxidase. NOX2 enzyme plays an essential role in host defense of phagocytes to generate superoxide (O_2^-). Activation of NOX2 requires translocation of cytosolic factors, p47phox, in order get to the NOX2/$p22^{phox}$ complex (Figure 2.6 in Chapter II). The present study, we followed the change of NOX2 by evaluating this cytosolic phosphorylation of $p47^{phox}$, the "organizer subunit". Interestingly, the recent report showed that in hyperglycemia, pro-inflammatory cytokines can stimulate leukocyte to generate O_2^-, especially leukocyte plugging in vascular (16). In addition, the report in renal inflammation showed that hyperglycemia-induced PKC activation causes increased p47phox expression (108). Moreover, the study in cardiomyocytes exposed to high glucose indicated that NADPH oxidase-derived ROS-induced apoptosis is mediated via the JNK-dependent activation of NF-**κ**B (110).

As shown in Figure 4.6, our findings also indicated that p47phox expression in DMCUR group was significantly decreased down to 41.38% when compared to DM group (P = 0.041). This result was consistent with our finding that curcumin could decrease leukocyte-endothelial cell interaction shown in Figure 4.3 (Chapter III).

At this point, it may imply that when curcumin decreases adhering leukocytes. Therefore, it will inhibit the consequence of phagocytic/leukocyte action of NOX2-generated O_2^-.

Relationship on leukocyte adhesion and p47phox expression

The increased p47phox expression has been found in diabetic inflammation. As shown in Figure 4.9, it also indicated that increased leukocyte adhesion (x-axis) was correlated to the increased in p47phox expression (y-axis) significantly. As which the correlation between leukocyte adhesion and p47phox expression can be characterized by the linear regression; $y = 0.0143x + 0.3666$, $r = 0.952$, $P = 0.048$. Several studies had reported that increased leukocyte adhesion could induce p47phox expression. Importantly, the increased p47phox also contributed to vascular inflammation and initiation of vascular injury in the second degree (16, 87).

Therefore, we propose that curcumin supplementation should be benefit for preventing further NADPH oxidase-derived ROS-induced eye tissue damage.

Nowadays there are many studies paid attention on compounds that suppress NOX2 activity; because the NOX family of enzymes is one of the primary sources of ROS in the vasculature in particular diabetes mediated endothelial dysfunction (111).

Therefore, our finding should be a good evidence to imply that daily curcumin supplementation at low dose combined with promising NOX inhibitor agent might offer therapeutic benefits to ameliorate diabetic vascular complications in the near future.

Relationship on blood glucose, MDA level, and Txnip expression

One of the objectives of this study was to determine the mechanism of curcumin on reducing diabetes-induced leukocyte-endothelium interaction in associated with its actions

on Txnip expression. The increased Txnip expression has been noted to occur in diabetes and play the role in progression of diabetic complications.

In this study, both two changes of BG and Txnip expression were indicated. Our further analysis of the correlation between each parameter showed that there is a significant correlation between BG and Txnip expression which can be characterized by the linear regression (Figure 4.7); $y = 0.0013x + 0.246$, $r = 0.966$, $P = 0.034$. In addition, the correlation was also shown for Txnip expression and MDA level with fit by linear regression; $y = 0.0505x + 0.2533$, $r = 0.958$, $P = 0.042$ (Figure 4.8).

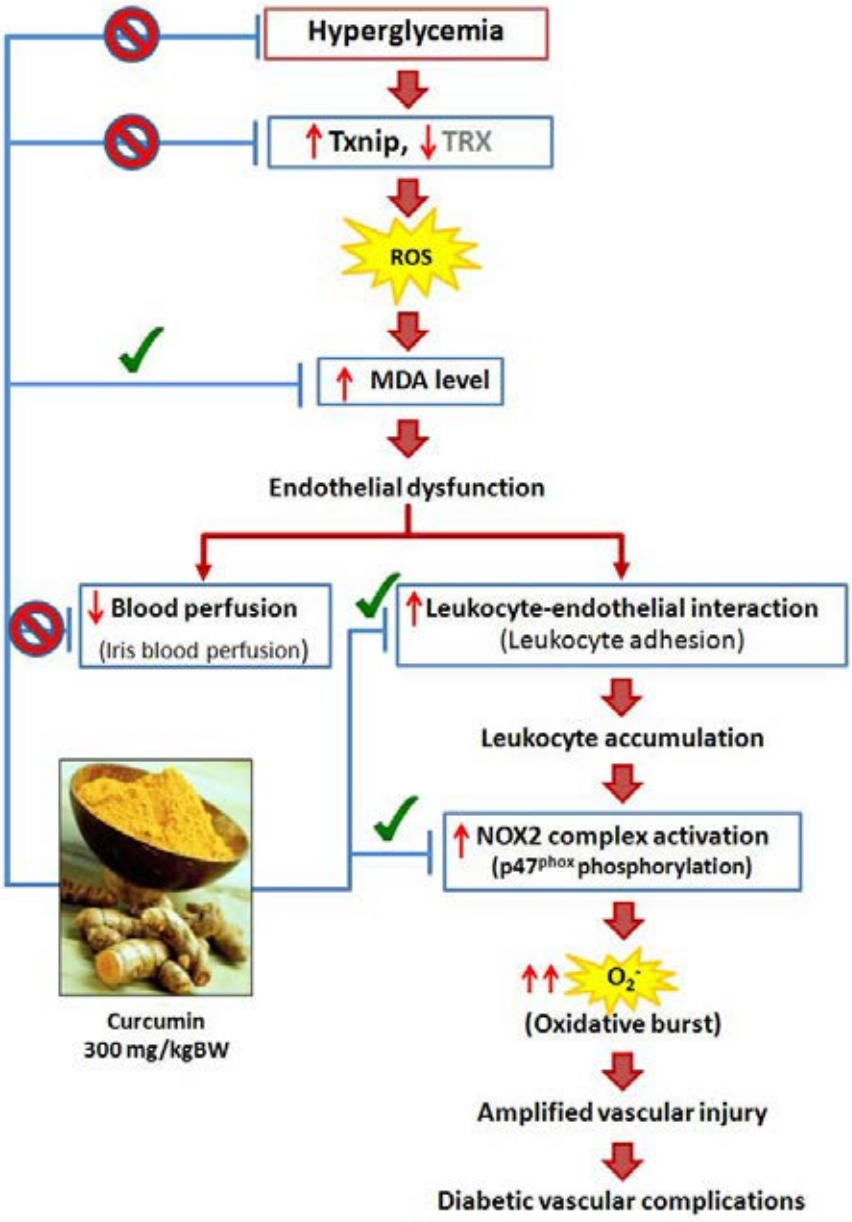


Figure 6.1 The proposed mechanism from the present study is that curcumin (dose 300 mg/kgBW) could ameliorate diabetic vascular inflammation by decreasing ROS overproduction, reducing leukocyte-endothelium interaction, and also inhibiting NOX2 activation. However, 300 mg/kgBW curcumin may not significantly reduce blood glucose and Txnip expression, and could not protect iris blood perfusion to decrease in diabetes.

CHAPTER VI

CONCLUSION

The new findings in this present study are demonstrated that the effect of curcumin on reducing diabetes-induced leukocyte-endothelium interaction through antioxidant and anti-inflammatory actions as follows:

1) The low dose of curcumin had no effect to decrease hyperglycemia, protein glycation, and metabolic derangement in diabetic rats.
2) The antioxidant of curcumin could maintain cellular redox balance via reducing MDA level which is by-product of lipid peroxidation.
3) The low dose of curcumin had tended to restore iris blood flow in diabetic rats, though iris blood flow in DMCUR group was not significantly different when compared to DM group.
4) The anti-inflammation and antioxidant of curcumin on reducing leukocyte-endothelium interaction were not associated with Txnip expression.
5) The anti-inflammation of curcumin could reduce the leukocyte-endothelium interaction in association with its inhibitory effect on NOX2 enzyme.

REFERENCES

(1) Hossain P., Kawar B., and El Nahas M. Obesity and diabetes in the developing world--a growing challenge. N Engl J Med 356 (2007): 213-215.

(2) Fatehi-Hassanabad Z., Chan C.B., and Furman B.L. Reactive oxygen species and endothelial function in diabetes. Eur J Pharmacol 636 (2010): 8-17.

(3) Perez-Matute P., Zulet M.A., and Martinez J.A. Reactive species and diabetes: counteracting oxidative stress to improve health. Curr Opin Pharmacol 9 (2009): 771-779.

(4) Soldatos G., and Cooper M.E. Diabetic nephropathy: important pathophysiologic mechanisms. Diabetes Res Clin Pract 82 Suppl 1 (2008): S75-79.

(5) Turrens J.F. Mitochondrial formation of reactive oxygen species. J Physiol 552 (2003): 335-344.

(6) Forstermann U. Oxidative stress in vascular disease: causes, defense mechanisms and potential therapies. Nat Clin Pract Cardiovasc Med 5 (2008): 338-349.

(7) Chen L., Zhang J., Zhang Y., Wang Y., and Wang B. Improvement of inflammatory responses associated with NF-kappa B pathway in kidneys from diabetic rats. Inflamm Res 57 (2008): 199-204.

(8) Li X., Rong Y., Zhang M., et al. Up-regulation of thioredoxin interacting protein (Txnip) by p38 MAPK and FOXO1 contributes to the impaired thioredoxin activity and increased ROS in glucose-treated endothelial cells. Biochem Biophys Res Commun 381 (2009): 660-665.

(9) Nishiyama A., Matsui M., Iwata S., et al. Identification of thioredoxin-binding protein-2/vitamin D(3) up-regulated protein 1 as a negative regulator of thioredoxin function and expression. J Biol Chem 274 (1999): 21645-21650.

(10) Junn E., Han S.H., Im J.Y., et al. Vitamin D3 up-regulated protein 1 mediates oxidative stress via suppressing the thioredoxin function. J Immunol 164 (2000): 6287-6295.

(11) Spindel O.N., World C., and Berk B.C. Thioredoxin interacting protein: redox dependent and independent regulatory mechanisms. Antioxid Redox Signal 16 (2012): 587-596.

(12) Jones D.P. Radical-free biology of oxidative stress. Am J Physiol Cell Physiol 295 (2008): C849-868.

(13) World C.J., Yamawaki H., and Berk B.C. Thioredoxin in the cardiovascular system. J Mol Med (Berl) 84 (2006): 997-1003.

(14) Harrison C.B., Drummond G.R., Sobey C.G., and Selemidis S. Evidence that nitric oxide inhibits vascular inflammation and superoxide production via a p47phox-dependent mechanism in mice. Clin Exp Pharmacol Physiol 37 (2009): 429-434.

(15) Szabo M.E., Droy-Lefaix M.T., Doly M., and Braquet P. Free radical-mediated effects in reperfusion injury: a histologic study with superoxide dismutase and EGB 761 in rat retina. Ophthalmic Res 23 (1991): 225-234.

(16) Omori K., Ohira T., Uchida Y., et al. Priming of neutrophil oxidative burst in diabetes requires preassembly of the NADPH oxidase. J Leukoc Biol 84 (2008): 292-301.

(17) Al-Shabrawey M., Rojas M., Sanders T., et al. Role of NADPH oxidase in retinal vascular inflammation. Invest Ophthalmol Vis Sci 49 (2008): 3239-3244.

(18) Jain S.K., Rains J., and Jones K. Effect of curcumin on protein glycosylation, lipid peroxidation, and oxygen radical generation in human red blood cells exposed to high glucose levels. Free Radic Biol Med 41 (2006): 92-96.

(19) Kowluru R.A., and Kanwar M. Effects of curcumin on retinal oxidative stress and inflammation in diabetes. Nutr Metab (Lond) 4 (2007): 8.

(20) Meghana K., Sanjeev G., and Ramesh B. Curcumin prevents streptozotocin-induced islet damage by scavenging free radicals: a prophylactic and protective role. Eur J Pharmacol 577 (2007): 183-191.

(21) Patumraj S., Wongeakin N., Sridulyakul P., et al. Combined effects of curcumin and vitamin C to protect endothelial dysfunction in the iris tissue of STZ-induced diabetic rats. Clin Hemorheol Microcirc 35 (2006): 481-489.

(22) Wongeakin N., Sridulyakul P., Jariyapongskul A., et al. Effects of curcumin and tetrahydrocurcumin on stz-induced diabetic rats. Journal of Vascular Research 43 (2006): 32-32.

(23) Kanitkar M., and Bhonde R.R. Curcumin treatment enhances islet recovery by induction of heat shock response proteins, Hsp70 and heme oxygenase-1, during cryopreservation. Life Sci 82 (2008): 182-189.

(24) Mandal M.N., Patlolla J.M., Zheng L., et al. Curcumin protects retinal cells from light- and oxidant stress-induced cell death. Free Radic Biol Med 46 (2009): 672-679.

(25) Jung K.A., and Kwak M.K. The Nrf2 system as a potential target for the development of indirect antioxidants. Molecules 15 (2010): 7266-7291.

(26) Molina-Jijon E., Tapia E., Zazueta C., et al. Curcumin prevents Cr(VI)-induced renal oxidant damage by a mitochondrial pathway. Free Radic Biol Med 51 (2011): 1543-1557.

(27) Gao X., Kuo J., Jiang H., et al. Immunomodulatory activity of curcumin: suppression of lymphocyte proliferation, development of cell-mediated cytotoxicity, and cytokine production in vitro. Biochem Pharmacol 68 (2004): 51-61.

(28) Binion D.G., Heidemann J., Li M.S., et al. Vascular cell adhesion molecule-1 expression in human intestinal microvascular endothelial cells is regulated by PI 3-kinase/Akt/MAPK/NF-kappaB: inhibitory role of curcumin. Am J Physiol Gastrointest Liver Physiol 297 (2009): G259-268.

(29) Deeb D., Jiang H., Gao X., et al. Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1-6-heptadine-3,5-dione; C21H20O6] sensitizes human prostate cancer cells to tumor necrosis factor-related apoptosis-inducing ligand/Apo2L-induced apoptosis by suppressing nuclear factor-kappaB via inhibition of the prosurvival Akt signaling pathway. J Pharmacol Exp Ther 321 (2007): 616-625.

(30) American Diabetes Association 1995; Available from: http://www.diabetes.org/.

(31) Cade W.T. Diabetes-related microvascular and macrovascular diseases in the physical therapy setting. Phys Ther 88 (2008): 1322-1335.

(32) Orasanu G., and Plutzky J. The pathologic continuum of diabetic vascular disease. J Am Coll Cardiol 53 (2009): S35-42.

(33) Bakker W., Eringa E.C., Sipkema P., and van Hinsbergh V.W. Endothelial dysfunction and diabetes: roles of hyperglycemia, impaired insulin signaling and obesity. Cell Tissue Res 335 (2009): 165-189.

(34) Halliwell B., and Gutteridge J.M. The definition and measurement of antioxidants in biological systems. Free Radic Biol Med 18 (1995): 125-126.

(35) Nordberg J., and Arner E.S. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. Free Radic Biol Med 31 (2001): 1287-1312.

(36) Yu F.X., Chai T.F., He H., Hagen T., and Luo Y. Thioredoxin-interacting protein (Txnip) gene expression: sensing oxidative phosphorylation status and glycolytic rate. J Biol Chem 285 (2010): 25822-25830.

(37) Cha-Molstad H., Saxena G., Chen J., and Shalev A. Glucose-stimulated expression of Txnip is mediated by carbohydrate response element-binding protein, p300, and histone H4 acetylation in pancreatic beta cells. J Biol Chem 284 (2009): 16898-16905.

(38) Minn A.H., Hafele C., and Shalev A. Thioredoxin-interacting protein is stimulated by glucose through a carbohydrate response element and induces beta-cell apoptosis. Endocrinology 146 (2005): 2397-2405.

(39) Stoltzman C.A., Peterson C.W., Breen K.T., et al. Glucose sensing by MondoA:Mlx complexes: a role for hexokinases and direct regulation of thioredoxin-interacting protein expression. Proc Natl Acad Sci U S A 105 (2008): 6912-6917.

(40) Papadia S., Soriano F.X., Leveille F., et al. Synaptic NMDA receptor activity boosts intrinsic antioxidant defenses. Nat Neurosci 11 (2008): 476-487.

(41) Nakamura H., Matsuda M., Furuke K., et al. Adult T cell leukemia-derived factor/human thioredoxin protects endothelial F-2 cell injury caused by activated neutrophils or hydrogen peroxide. Immunol Lett 42 (1994): 75-80.

(42) Zhang J., Li Y.D., Patel J.M., and Block E.R. Thioredoxin overexpression prevents NO-induced reduction of NO synthase activity in lung endothelial cells. Am J Physiol 275 (1998): L288-293.

(43) Ejima K., Nanri H., Toki N., Kashimura M., and Ikeda M. Localization of thioredoxin reductase and thioredoxin in normal human placenta and their protective effect against oxidative stress. Placenta 20 (1999): 95-101.

(44) Okuda M., Inoue N., Azumi H., et al. Expression of glutaredoxin in human coronary arteries: its potential role in antioxidant protection against atherosclerosis. Arterioscler Thromb Vasc Biol 21 (2001): 1483-1487.

(45) Holmgren A. Thioredoxin. Annu Rev Biochem 54 (1985): 237-271.

(46) Hayashi T., Ueno Y., and Okamoto T. Oxidoreductive regulation of nuclear factor kappa B. Involvement of a cellular reducing catalyst thioredoxin. J Biol Chem 268 (1993): 11380-11388.

(47) Welsh S.J., Bellamy W.T., Briehl M.M., and Powis G. The redox protein thioredoxin-1 (Trx-1) increases hypoxia-inducible factor 1alpha protein expression: Trx-1 overexpression results in increased vascular endothelial growth factor production and enhanced tumor angiogenesis. Cancer Res 62 (2002): 5089-5095.

(48) Ichijo H., Nishida E., Irie K., et al. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science 275 (1997): 90-94.

(49) Saitoh M., Nishitoh H., Fujii M., et al. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. EMBO J 17 (1998): 2596-2606.

(50) Yamawaki H., and Berk B.C. Thioredoxin: a multifunctional antioxidant enzyme in kidney, heart and vessels. Curr Opin Nephrol Hypertens 14 (2005): 149-153.

(51) Yamawaki H., Pan S., Lee R.T., and Berk B.C. Fluid shear stress inhibits vascular inflammation by decreasing thioredoxin-interacting protein in endothelial cells. J Clin Invest 115 (2005): 733-738.

(52) Liu Y., and Min W. Thioredoxin promotes ASK1 ubiquitination and degradation to inhibit ASK1-mediated apoptosis in a redox activity-independent manner. Circ Res 90 (2002): 1259-1266.

(53) Xiang G., Seki T., Schuster M.D., et al. Catalytic degradation of vitamin D up-regulated protein 1 mRNA enhances cardiomyocyte survival and prevents left ventricular remodeling after myocardial ischemia. J Biol Chem 280 (2005): 39394-39402.

(54) Schulze P.C., Liu H., Choe E., et al. Nitric oxide-dependent suppression of thioredoxin-interacting protein expression enhances thioredoxin activity. Arterioscler Thromb Vasc Biol 26 (2006): 2666-2672.

(55) Joussen A.M., Poulaki V., Le M.L., et al. A central role for inflammation in the pathogenesis of diabetic retinopathy. FASEB J 18 (2004): 1450-1452.

(56) Kamiuchi K., Hasegawa G., Obayashi H., et al. Intercellular adhesion molecule-1 (ICAM-1) polymorphism is associated with diabetic retinopathy in Type 2 diabetes mellitus. Diabet Med 19 (2002): 371-376.

(57) Perrone L., Devi T.S., Hosoya K., Terasaki T., and Singh L.P. Thioredoxin interacting protein (TXNIP) induces inflammation through chromatin modification in retinal capillary endothelial cells under diabetic conditions. J Cell Physiol 221 (2009): 262-272.

(58) Perrone L., Devi T.S., Hosoya K.I., Terasaki T., and Singh L.P. Inhibition of TXNIP expression in vivo blocks early pathologies of diabetic retinopathy. Cell Death Dis 1 (2011): e65.

(59) Wang A.L., Yu A.C., He Q.H., Zhu X., and Tso M.O. AGEs mediated expression and secretion of TNF alpha in rat retinal microglia. Exp Eye Res 84 (2007): 905-913.

(60) Busik J.V., Mohr S., and Grant M.B. Hyperglycemia-induced reactive oxygen species toxicity to endothelial cells is dependent on paracrine mediators. Diabetes 57 (2008): 1952-1965.

(61) Ponugoti B., Dong G., and Graves D.T. Role of forkhead transcription factors in diabetes-induced oxidative stress. Exp Diabetes Res (2012): 939751.

(62) St Hill C.A., Alexander S.R., and Walcheck B. Indirect capture augments leukocyte accumulation on P-selectin in flowing whole blood. J Leukoc Biol 73 (2003): 464-471.

(63) Green C.E., Pearson D.N., Camphausen R.T., Staunton D.E., and Simon S.I. Shear-dependent capping of L-selectin and P-selectin glycoprotein ligand 1 by E-selectin signals activation of high-avidity beta2-integrin on neutrophils. J Immunol 172 (2004): 7780-7790.

(64) Malik A.B., and Lo S.K. Vascular endothelial adhesion molecules and tissue inflammation. Pharmacol Rev 48 (1996): 213-229.

(65) Lambeth J.D. NOX enzymes and the biology of reactive oxygen. Nat Rev Immunol 4 (2004): 181-189.

(66) Bayraktutan U., Blayney L., and Shah A.M. Molecular characterization and localization of the NAD(P)H oxidase components gp91-phox and p22-phox in endothelial cells. Arterioscler Thromb Vasc Biol 20 (2000): 1903-1911.

(67) Bendall J.K., Cave A.C., Heymes C., Gall N., and Shah A.M. Pivotal role of a gp91(phox)-containing NADPH oxidase in angiotensin II-induced cardiac hypertrophy in mice. Circulation 105 (2002): 293-296.

(68) Murdoch C.E., Alom-Ruiz S.P., Wang M., et al. Role of endothelial Nox2 NADPH oxidase in angiotensin II-induced hypertension and vasomotor dysfunction. Basic Res Cardiol 106 (2011): 527-538.

(69) Griendling K.K., Sorescu D., and Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. Circ Res 86 (2000): 494-501.

(70) Pagano P.J., Chanock S.J., Siwik D.A., Colucci W.S., and Clark J.K. Angiotensin II induces p67phox mRNA expression and NADPH oxidase superoxide generation in rabbit aortic adventitial fibroblasts. Hypertension 32 (1998): 331-337.

(71) Bedard K., and Krause K.H. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev 87 (2007): 245-313.

(72) Thallas-Bonke V., Thorpe S.R., Coughlan M.T., et al. Inhibition of NADPH oxidase prevents advanced glycation end product-mediated damage in diabetic nephropathy through a protein kinase C-alpha-dependent pathway. Diabetes 57 (2008): 460-469.

(73) Nam S.M., Lee M.Y., Koh J.H., et al. Effects of NADPH oxidase inhibitor on diabetic nephropathy in OLETF rats: the role of reducing oxidative stress in its protective property. Diabetes Res Clin Pract 83 (2009): 176-182.

(74) Menon V.P., and Sudheer A.R. Antioxidant and anti-inflammatory properties of curcumin. Adv Exp Med Biol 595 (2007): 105-125.

(75) Aggarwal B.B., and Sung B. Pharmacological basis for the role of curcumin in chronic diseases: an age-old spice with modern targets. Trends Pharmacol Sci 30 (2009): 85-94.

(76) Wahlstrom B., and Blennow G. A study on the fate of curcumin in the rat. Acta Pharmacol Toxicol (Copenh) 43 (1978): 86-92.

(77) Shankar T.N., Shantha N.V., Ramesh H.P., Murthy I.A., and Murthy V.S. Toxicity studies on turmeric (Curcuma longa): acute toxicity studies in rats, guineapigs & monkeys. Indian J Exp Biol 18 (1980): 73-75.

(78) Chainani-Wu N. Safety and anti-inflammatory activity of curcumin: a component of tumeric (Curcuma longa). J Altern Complement Med 9 (2003): 161-168.

(79) Aggarwal B.B., Kumar A., and Bharti A.C. Anticancer potential of curcumin: preclinical and clinical studies. Anticancer Res 23 (2003): 363-398.

(80) Sunagawa M., Shimada S., Hanashiro K., Nakamura M., and Kosugi T. Elevation of intracellular cAMP up-regulated thrombomodulin mRNA in cultured vascular endothelial cells derived from spontaneous type-II diabetes mellitus model rat. Endothelium 13 (2006): 325-333.

(81) Bandello F., Brancato R., Lattanzio R., Galdini M., and Falcomata B. Relation between iridopathy and retinopathy in diabetes. Br J Ophthalmol 78 (1994): 542-545.

(82) Jariyapongskul A., Rungjaroen T., Kasetsuwan N., et al. Long-term effects of oral vitamin C supplementation on the endothelial dysfunction in the iris microvessels of diabetic rats. Microvasc Res 74 (2007): 32-38.

(83) Stuehmer C., Schumann P., Bormann K.H., et al. A new model for chronic in vivo analysis of the periosteal microcirculation. Microvasc Res 77 (2009): 104-108.

(84) Saetzler R.K., Jallo J., Lehr H.A., et al. Intravital fluorescence microscopy: impact of light-induced phototoxicity on adhesion of fluorescently labeled leukocytes. J Histochem Cytochem 45 (1997): 505-513.

(85) Byfield G., Budd S., and Hartnett M.E. The role of supplemental oxygen and JAK/STAT signaling in intravitreous neovascularization in a ROP rat model. Invest Ophthalmol Vis Sci 50 (2009): 3360-3365.

(86) Trichonas G., Manola A., Morizane Y., et al. A novel nonradioactive method to evaluate vascular barrier breakdown and leakage. Invest Ophthalmol Vis Sci 51 1677-1682.

(87) Szabo A., Farkas A., Vamos R., Bajor T., and Hrabak A. The inhibition of retinal inducible nitric oxide synthase overexpression and the attenuation of experimental uveitis by anti-inflammatory drugs in rats. Inflamm Res 53 (2004): 262-267.

(88) De Vriese A.S., Verbeuren T.J., Van de Voorde J., Lameire N.H., and Vanhoutte P.M. Endothelial dysfunction in diabetes. Br J Pharmacol 130 (2000): 963-974.

(89) Poston L., and Taylor P.D. Glaxo/MRS Young Investigator Prize. Endothelium-mediated vascular function in insulin-dependent diabetes mellitus. Clin Sci (Lond) 88 (1995): 245-255.

(90) Steele C., Steele D., and Waine C. Diabetes and the eye. (2008): ed2008.

(91) Schnedl W.J., Ferber S., Johnson J.H., and Newgard C.B. STZ transport and cytotoxicity. Specific enhancement in GLUT2-expressing cells. Diabetes 43 (1994): 1326-1333.

(92) Grankvist K., Marklund S.L., and Taljedal I.B. CuZn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidase in pancreatic islets and other tissues in the mouse. Biochem J 199 (1981): 393-398.

(93) Lenzen S., Drinkgern J., and Tiedge M. Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. Free Radic Biol Med 20 (1996): 463-466.

(94) Suryanarayana P., Saraswat M., Mrudula T., et al. Curcumin and turmeric delay streptozotocin-induced diabetic cataract in rats. Invest Ophthalmol Vis Sci 46 (2005): 2092-2099.

(95) Chanpoo M., Petchpiboonthai H., Panyarachun B., and Anupunpisit V. Effect of curcumin in the amelioration of pancreatic islets in streptozotocin-induced diabetic mice. J Med Assoc Thai 93 (2010) S152-159.

(96) Seo K.I., Choi M.S., Jung U.J., et al. Effect of curcumin supplementation on blood glucose, plasma insulin, and glucose homeostasis related enzyme activities in diabetic db/db mice. Mol Nutr Food Res 52 (2008): 995-1004.

(97) Rungseesantivanon S., Thenchaisri N., Ruangvejvorachai P., and Patumraj S. Curcumin supplementation could improve diabetes-induced endothelial dysfunction associated with decreased vascular superoxide production and PKC inhibition. BMC Complement Altern Med 10 (2010): 57.

(98) Rungseesantivanon S., Thengchaisri N., Ruangvejvorachai P., and Patumraj S. Curcumin improves prostanoid ratio in diabetic mesenteric arteries

associated with cyclooxygenase-2 and NF-kappaB suppression. Diabetes Metab Syndr Obes 3 (2010): 421-429.

(99) Pemp B., and Schmetterer L. Ocular blood flow in diabetes and age-related macular degeneration. Can J Ophthalmol 43 (2008): 295-301.

(100) Sridulyakul P., Chakraphan D., and Patumraj S. Vitamin C supplementation could reverse diabetes-induced endothelial cell dysfunction in mesenteric microcirculation in STZ-rats. Clin Hemorheol Microcirc 34 (2006): 315-321.

(101) Roy S., Cagliero E., and Lorenzi M. Fibronectin overexpression in retinal microvessels of patients with diabetes. Invest Ophthalmol Vis Sci 37 (1996): 258-266.

(102) de la Cruz J.P., Moreno A., Ruiz-Ruiz M.I., Garcia-Campos J., and Sanchez de la Cuesta F. Effect of WEB 2086-BS, an antagonist of platelet-activating factor receptors, on retinal vascularity in diabetic rats. Eur J Pharmacol 360 (1998): 37-42.

(103) Awasthi H., Tota S., Hanif K., Nath C., and Shukla R. Protective effect of curcumin against intracerebral streptozotocin induced impairment in memory and cerebral blood flow. Life Sci 86 (2010): 87-94.

(104) Gupta S.K., Kumar B., Nag T.C., et al. Curcumin prevents experimental diabetic retinopathy in rats through its hypoglycemic, antioxidant, and anti-inflammatory mechanisms. J Ocul Pharmacol Ther 27 (2011): 123-130.

(105) Aoki H., Takada Y., Kondo S., et al. Evidence that curcumin suppresses the growth of malignant gliomas in vitro and in vivo through induction of autophagy: role of Akt and extracellular signal-regulated kinase signaling pathways. Mol Pharmacol 72 (2007): 29-39.

(106) Schulze P.C., Yoshioka J., Takahashi T., et al. Hyperglycemia promotes oxidative stress through inhibition of thioredoxin function by thioredoxin-interacting protein. J Biol Chem 279 (2004): 30369-30374.

(107) Shalev A. Lack of TXNIP protects beta-cells against glucotoxicity. Biochem Soc Trans 36 (2008): 963-965.

(108) Yang J., Lane P.H., Pollock J.S., and Carmines P.K. Protein kinase C-dependent NAD(P)H oxidase activation induced by type 1 diabetes in renal medullary thick ascending limb. Hypertension 55 (2010): 468-473.

(109) Asaba K., Tojo A., Onozato M.L., et al. Effects of NADPH oxidase inhibitor in diabetic nephropathy. Kidney Int 67 (2005): 1890-1898.

(110) Tsai K.H., Wang W.J., Lin C.W., et al. NADPH oxidase-derived superoxide anion-induced apoptosis is mediated via the JNK-dependent activation of NF-kappaB in cardiomyocytes exposed to high glucose. J Cell Physiol 227 (2012): 1347-1357.

(111) Sharma A., Bernatchez P.N., and de Haan J.B. Targeting endothelial dysfunction in vascular complications associated with diabetes. Int J Vasc Med (2012): 750126.

APPENDICES

APPENDIX A

REAGENTS, MATHERIALS AND INSTRUMENTS

A. Reagents

Bovine serum albumin	(Sigma Co., USA)
Citrate buffer	(Sigma Co., USA)
Corn oil	(Sigma Co., USA)
Curcumin	(Cayman, USA)
ECL prime substrate	(GE, USA)
Phosphatase inhibitor	(Sigma Co., USA)
Protease inhibitor	(Sigma Co., USA)
Rhodamin-6G	(Sigma Co., USA)
RIPA buffer	(Cell signaling, USA)
Streptozotocin	(Sigma Co., USA)

B. Instruments

ChemiDoc	(Bio-rad, UK)
Intravital Fluorescence Microscope	(Nikon, Japan)
Microplate Reader	(Bio-rad, UK)
Mini Analytical Protein Electrophoresis Cells	(Bio-rad, UK)
Mini Trans-Blot	(Bio-rad, UK)
Sonicator	(Sonics & Materials INC, USA)

APPENDIX B

REAGENT PREPARATION

1. **8.1% (w/v) Sodium docecyl sulfate (SDS)**
   - Sodium dodecyl sulfate (SDS) 8.1 g
   - Adjust DW up to 100 ml

2. **20% (v/v) of acetic acid solution (pH 3.5)**
   - 37% HCl 200 ml
   - Adjust DW up to 100 ml

3. **0.8% (w/v) Thiobarbituric acid (TBA)**
   - Thiobarbituric acid (TBA) 0.8 g
   - Adjust DW up to 100 ml

4. **1,1,3,3-Tetramethoxypropane (TMP) or malondialdehyde bis solution**
   - TMP 16.4 µl
   - Adjust DW up to 100 ml

   * Prepared stock 10^3 nmole TMP with distilled water, then pipette 0.04, 0.08, 0.12, 0.16, 0.20, and 0.24 mL of this stock TMP solution. These will give the following concentration of standard TMP: 4, 8, 12, 16, 20, and 24 nmole/mL. Prepare stock TMP fresh.

5. 10% resolving gel (for 2 gels)

- Distilled water (DW)	4.9 ml
- 1.5 M Tris-HCl, pH 8.8	2.5 ml
- Acrylamide 40%	2.5 ml
- 10% Sodium dodecyl sulfate (SDS)	100 µl
- 10% ammonium persulfate (APS)	50 µl
- Tetramethylethylenediamine (TEMED)	20 µl

* Allow the gel to polymerize for at least 30 minutes at room temperature before casting the stacking gel.

6. 5% stacking gel (for 2 gels)

- Distilled water (DW)	2.4 ml
- 1.5 M Tris-HCl, pH 8.8	1008 µl
- Acrylamide 40%	500 µl
- 10% Sodium dodecyl sulfate (SDS)	40 µl
- 10% ammonium persulfate (APS)	40 µl
- Tetramethylethylenediamine (TEMED)	8 µl

* Allow the gel to polymerize for at least 30 minutes at room temperature before loading samples.

7. **4X sample buffer (10ml, store at -20°C)**

- 1M Tris-HCl, pH 6.8	2.4 ml
- Bromophenol blue 0.1%	10 µl
- Sodium dodecyl sulfate (SDS)	0.8 g
- Glycerol	4 ml
- β-mercapto ethanol	1 ml
- DW	2.8 ml

* Prepare the solution within a fume-hood.

Dilute samples at least 1:3 with sample buffer and heat at 95°C for 5 minutes.

8. **SDS-PAGE running buffer 1 L (10X, store at 4°C)**

- Tris base	30.3 g
- Glycine	144.2 g
- Sodium dodecyl sulfate (SDS)	10 g
- Adjust DW up to 900 ml	
- Adjust pH 8.3 with HCl	
- Adjust volume up to 1000 ml with DW	

9. **Transfer buffer 1 L (10X, store at 4°C)**

- Tris base	30.3 g
- Glycine	144.2 g

10. **Transfer buffer 1 L (1X for working solution)**

- 10X transfer buffer	100 ml

- DW 700 ml
- Absolute Methanol alcohol (MeOH) 200 ml

11. Tris buffered saline 1 L (TBS, 10X)

- Tris base 24 g
- Nacl 88 g
- Dissolve in DW 900 ml
- Adjust pH 7.6 with HCl

12. TBS-Tween 1 L (TTBS)

- 1X TBS 999 ml
- Tween-20 1 ml

13. Blocking buffer (3% BSA)

- Bovine serum albumin (BSA) 3 g
- TTBS 100 ml

14. Destain solution

- Acetic acid 90 ml
- Absolute Methanol alcohol (MeOH) 430 ml
- DW 480 ml

APPENDIX C

FURTHER INFORMATIONS FOR METHODOLOGY

1. Thiobarbituric (TBA) assay

Solution (ml)	Blank (ml)	Standard (ml)	Sample (ml)
Sample	-	-	0.1
TMP stock standard	-	0.1	-
DW	0.1	-	-
8.1% SDS	0.1	0.1	0.1
20% Acetic acid	1.5	1.5	1.5
0.8% TBA	1.5	1.5	1.5

- Mix well with a vortex mixer.
- Heated all tubes in the water-bath at 95°C for 1 hour.
- After cooling with top water, 1 ml of DW and 5 ml of the mixture of n-butanol and pyridine are added and mix vigorously with the vortex mixer (at least 1 min).
- Then, centrifuge at 3,500 rpm for 10 minutes, the organic layer is taken and its absorbance at 532 nm is measured with spectrophotometer.
- Then, plot the optical densities of standard series to quantify lipid peroxide (nmole)
- The content of lipid peroxide is expressed in terms of nmole/protein100mg

2. **Western Blotting protocol**

SDS-PAGE protocol

1. Load samples and molecular marker into gel.
2. Run gel at 100 Volts until the blue dye front is at the end of the glass plates, but does not migrate off the gel (2 hours).

Protein transfer protocol

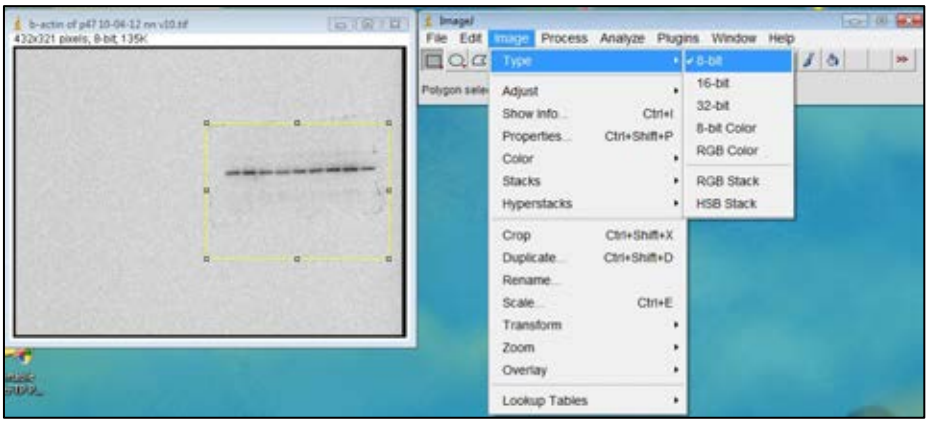
3. Remove the gel from electrophoresis apparatus.
4. Prepare the sandwich on the black side of the cassette holder; lay the first fiber pad, then the chromatography paper on top, then the gel on top of the chromatography paper, then the PVDF membrane, then the chromatography paper, then the next fiber pad.

   * Make sure to smooth out any bubbles using a tube.
5. Transfer for 5 hours at 400 mA in 4°C.
6. Block in 3% BSA in TTBS overnight at 4°C.
7. Incubated with primary antibody for 1-2 hours at room temperature, shaking in 3% BSA in TTBS.
8. Wash 2 times for 10 minutes each and 2 times for 5 minutes each with TTBS.
9. Incubated with secondary antibody for 1 hour at room temperature, shaking in 3% BSA in TTBS.
10. Wash 2 times for 10 minutes each and 2 times for 5 minutes each with TTBS.
11. Add 600 µl of substrate and expose in ChemiDoc imaging system.

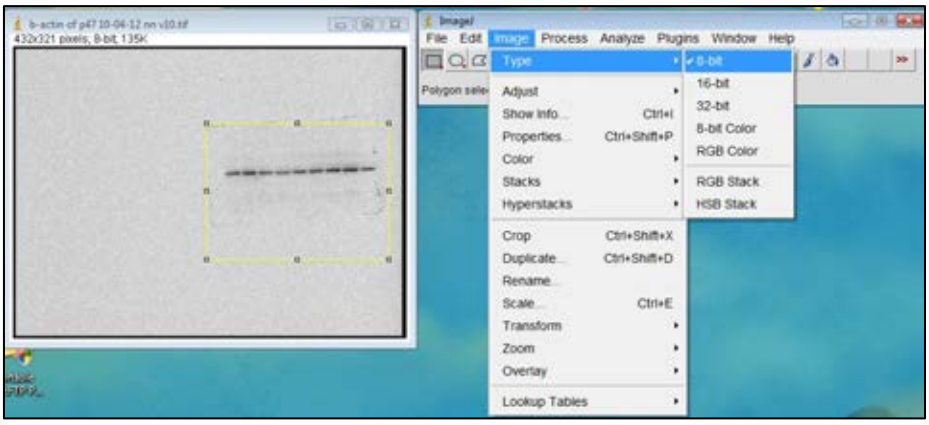
3. **Densitometry of Western blots using Image J software**

1. Open Image J program.

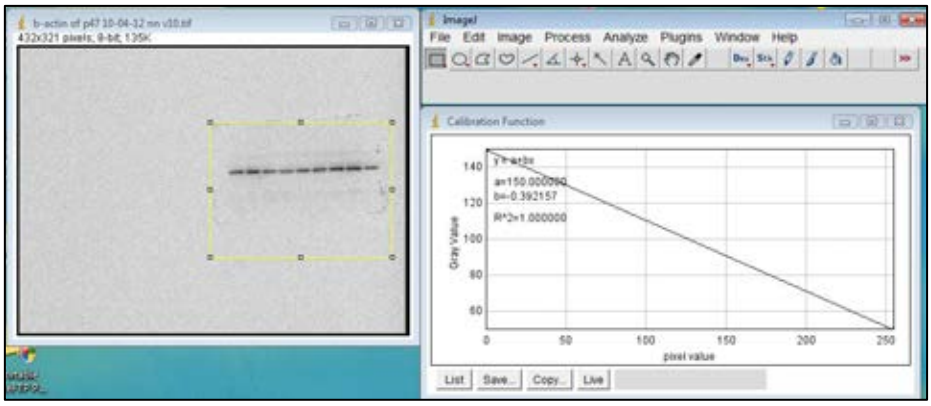
2. Open the file of gel. The file must be in grayscale, select type ⟶8 bit.



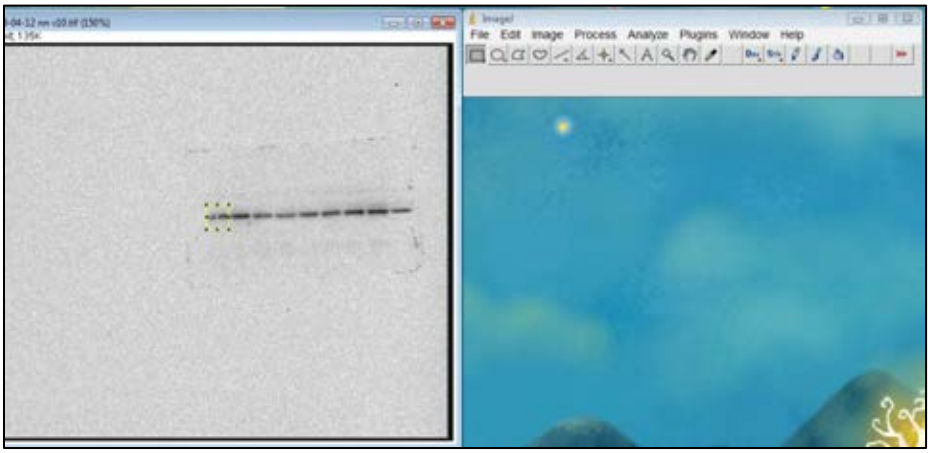
3. This file will be converted from minimum band intensity to 256 (White) and maximum band intensityto 0 (black), select analyze ⟶calibrate function ⟶straight line ⟶ok.



4. If you see the calibration bar, click on the image and draw rectangle around interested band in your first lane.



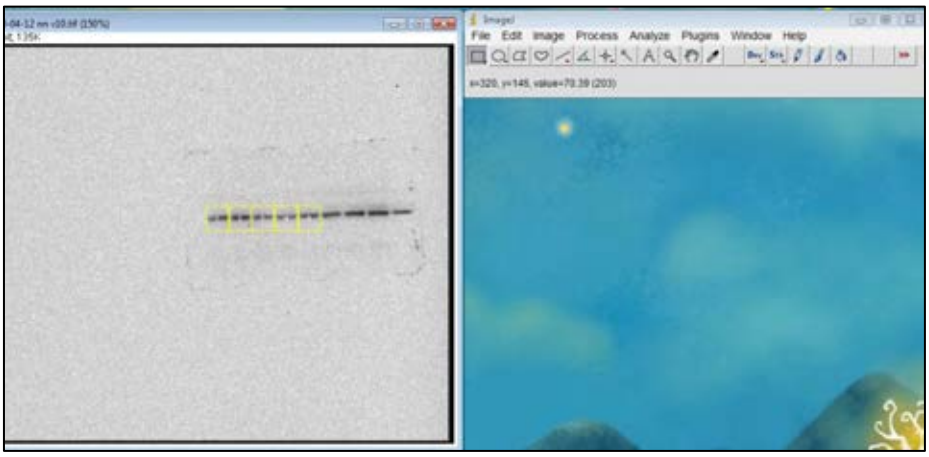
5. Once you have your band in a rectangle, select analyze ⟶ gels ⟶ select first lane (or click control-1 in windows). This will draw a box around the interested band.



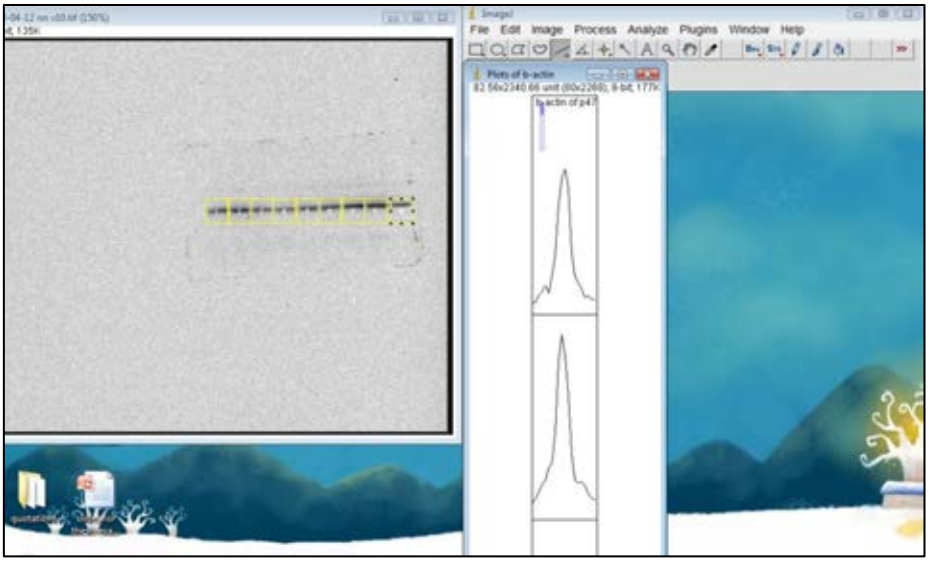
6. After that, take the cursor and move it within the box in the first lane. This will allow you to click-and-hold, and drag the box to the second lane.

7. Then, select the analyze ⟶ gels ⟶ select next lane (or click control-2 in windows). This will select an area in the second lane that is exactly equal to the area selected in

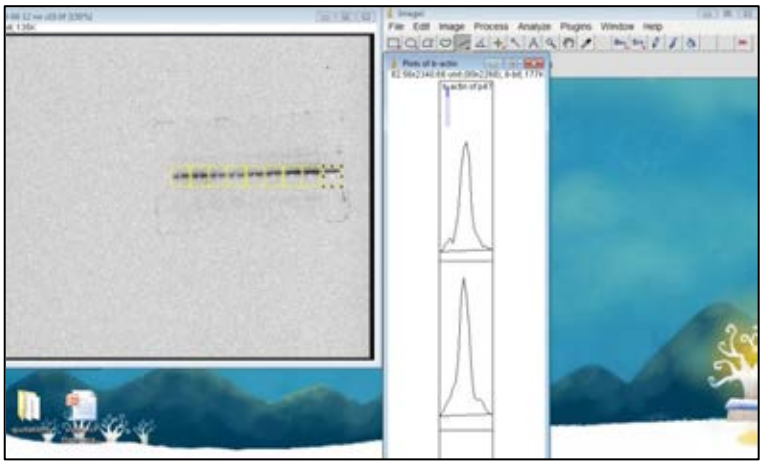
the first lane. However, you can repeat the lane selection process for all of your western blot lanes.



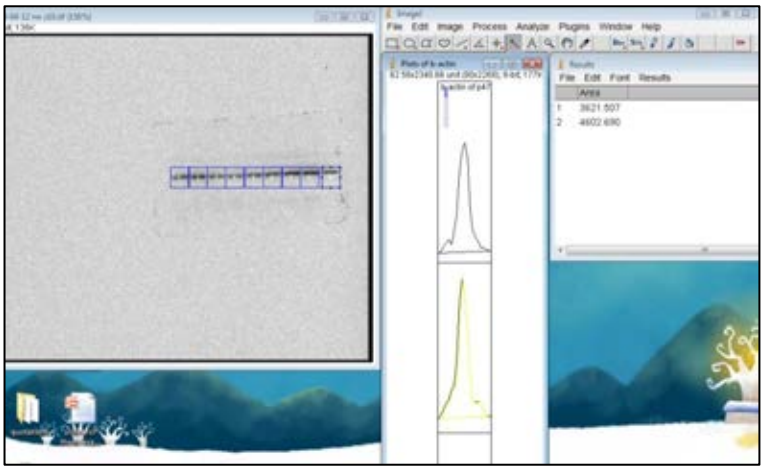
8. After the last lane, you need to select the analyze →gels →plot lanes. A window with will pop open that shows the intensity of each band in plot forms.



9. To measure the band intensity, you want to measure the area under the peak from this plot. To do so, select the straight line tool from the main menu tools. Then, use the straight line tool to mark off the area under the peak for the first plot and repeat this process for all plots. You need to close off all of the peak area that rises above the background level.



10. Then, select the wand tool and go back to the window with the plots of lanes. Click inside each plot with the wand tool. You will see the peak become outlined in yellow. As you continue to select the plots, another window showed results will open.



BIOGRAPHY

NAME	Miss Natchaya Wongeakin
DATE OF BIRTH	January 28, 1983
PLACE OF BIRTH	Bangkok, Thailand
INSTITUTIONS ATTENDED	Kasetsart University, 2000-2003: Bachelor of Science (Zoology) Chulalongkorn University, 2008-2011: Master of Science (Medical Science)

AWARD

1. Best Poster Presentation Award, 13th TSM Annual Meeting, Bangkok, Thailand, December 22, 2010.

PUBLICATION

1. Wongeakin N, Bhattarakosol P, and Patumraj S. Anti-oxidative Effect of Curcumin Prevents Diabetes-induced Decreases in Blood Perfusion and Capillary Vascularities in Iris and Femur Bone. Proceeding of The 9th World Congress for Microcirculation 2010, Moduzzi Editore 2010 - Medimond inc: 91-96.