ผลของเคอคูมินต่อการสูญเสียหน้าที่ของเอนโดทีเลียม ที่เกิดจากการเหนี่ยวนำด้วยเบาหวานในหนูแรท: บทบาทของโปรตีนไคเนสซี ไซโคลออกซิจิเนส 2 และทรานสคริปชั่น นิวเคลียแฟกเตอร์ แคปปาบี

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EFFECT OF CURCUMIN ON DIABETES-INDUCED ENDOTHELIAL DYSFUNCTION IN RAT: ROLE OF PKC, COX-2 AND NF-κB

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ศิรดา รังษีสันติวานนท์: ผลของเคอคูมินต่อการสูญเสียหน้าที่ของเอนโดทีเลียมที่เกิดจากการเหนี่ยวนำด้วยเบาหวานในหนูแรท: บทบาทของโปรตีนไคเนสซี ไซโคลออกซิจิเนส-2 และทรานสคริปชั่น นิวเคลียแฟกเตอร์ แคปปาบี. (EFFECT OF CURCUMIN ON DIABETES-INDUCED ENDOTHELIAL DYSFUNCTION IN RAT: ROLE OF PKC, COX-2 AND NF-KB.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ร.ศ. ดร.สุทธิลักษณ์ ปทุมราช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผ.ศ. น.สพ. ดร.นริศ เต็งชัยศรี; 130 หน้า.

ระดับน้ำตาลในเลือดที่สูงเป็นระยะเวลานานในโรคเบาหวาน เป็นสาเหตุให้มีการสร้างสารอนุมูลอิสระ (ROS) เป็นจำนวนมาก และส่งผลต่อการสูญเสียหน้าที่ของเอนโดทีเลียมเซลล์ ขมิ้นชัน (*Curcuma longa*, L.) เป็นพืชที่พบมากในประเทศแถบเอเชีย และมีการนำมาใช้เป็นส่วนผสมของอาหารและยารักษาโรคอย่างแพร่หลายนอกจากนี้ยังพบว่าขมิ้นชันมีฤทธิ์ทางการรักษาหลากหลายสรรพคุณ การศึกษาวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ของสารเคอคูมิน (curcumin) ซึ่งเป็นสารออกฤทธิ์ที่สำคัญของขมิ้นชัน ต่อการทำงานของเซลล์เอนโดทีเลียมในโรคเบาหวาน โดยการออกฤทธิ์ผ่านทางคุณสมบัติด้านการเกิดอนุมูลอิสระ (antioxidant) ด้านการอักเสบ (anti-inflammation) และยับยั้งการทำงานของโปรตีนไคเนสซี (PKC inhibition) ในการทดลองใช้หนูขาวเพศผู้พันธุ์ Wistars แบ่งออกเป็น 5 กลุ่ม คือ 1) กลุ่มเบาหวาน (DM) ทำให้เป็นเบาหวานโดยการฉีดสารสเตรปโตโซโตซิน (STZ) ทางหลอดเลือดดำในขนาด 55 มก. ต่อ กก.นน.ตัว 2) กลุ่มเบาหวานที่ได้รับเคอคูมิน 30 มก. ต่อ กก. นน.ตัว (DM+cur30) 3) กลุ่มเบาหวานที่ได้รับเคอคูมิน 300 ม.ก.ต่อ ก.ก. นน. ตัว (DM+cur300) 4) กลุ่มควบคุม (con) และ 5) กลุ่มควบคุมที่ได้รับเคอคูมิน 300 ม.ก.ต่อ ก.ก. นน.ตัว (con+cur300) โดยหนูได้รับเคอคูมินจากการป้อนด้วยขนาด 30 และ 300 มก. ต่อ กก. นน. ตัว ตามลำดับ ซึ่งได้รับเคอคูมินหลังจากการฉีดสาร STZ 6 สัปดาห์

จากสมมติฐานระดับน้ำตาลในเลือดสูงจะทำให้เกิดอนุมูลอิสระเป็นจำนวนมาก และทำให้เกิดการกระตุ้นการทำงานของ PKC NF-KB และ COX-2 ปริมาณอนุมูลอิสระที่เกิดขึ้นจำนวนมากในเซลล์เอนโดทีเลียมนั้น ส่งผลต่อการขยายตัวของหลอดเลือดแดงขนาดเล็กต่อสารกระตุ้นลดลง หลังจากการฉีดสาร STZ เป็นเวลา 12 สัปดาห์ การศึกษาภาวะการสูญเสียหน้าที่ของเซลล์เอนโดทีเลียมในเบาหวาน ทำโดยวิเคราะห์การตอบสนองของหลอดเลือดแดงขนาดเล็กในลำไส้ต่อสารอะเซทิลโคลีน (ACh) และสารโซเดียมไนโตรพัรซายด์ (SNP) โดยเทคนิคทางอินทราไวทัล ฟลูออเรสเซนท์ ไมโครสโคปี พบว่าเบาหวานทำให้การตอบสนองของหลอดเลือดแดงขนาดเล็กในลำไส้ต่อสารอะเซทิลโคลีนซึ่งมีฤทธิ์ขยายหลอดเลือดที่ทำงานผ่านเซลล์เอนโดทีเลียมลดลง ($P<0.01$) ในขณะที่ไม่มีผลการตอบสนองของหลอดเลือดแดงขนาดเล็กในลำไส้ต่อสารโซเดียมไนโตรพัรซายด์ ซึ่งมีฤทธิ์ขยายหลอดเลือดโดยไม่ผ่านเซลล์เอนโดทีเลียม นอกจากนี้การเสริมเคอคูมินทำให้ตอบสนองของหลอดเลือดแดงขนาดเล็กต่อสารอะเซทิลโคลีนเพิ่มขึ้น ทั้งสองระดับความเข้มข้น (30 และ 300 มก.ต่อ ก.ก.นน.ตัว) ($P<0.01$)

จากนั้นศึกษากลไกการทำงานในระดับโมเลกุลของผลของเคอคูมินต่อการต้านการเกิดอนุมูลอิสระ (antioxidant) โดยใช้สารไฮโดรเอธิดีน ซึ่งมีฤทธิ์จำเพาะต่ออนุภาค $O_2^{\bullet-}$ ภายในเซลล์ พบ $O_2^{\bullet-}$ มีปริมาณมากในหนูเบาหวาน ($P<0.01$) และมีปริมาณ $O_2^{\bullet-}$ ลดลงเมื่อให้เคอคูมินเสริมทั้งสองขนาดความเข้มข้น ($P<0.01$) และพบความสัมพันธ์แบบผกผันระหว่างปริมาณอนุมูลอิสระภายในเซลล์และการขยายตัวของหลอดเลือดแดงอย่างมีนัยสำคัญทางสถิติ ($r^2=0.7$, $P<0.01$) การ วิเคราะห์โดยการหาปริมาณพรอสตาแกลนดินเพื่อดูบทบาทของเคอคูมิน ต่อสารพรอสตาแกลนดินผ่านทางเอนไซมไซโคลออกซิจิเนส-2 ซึ่งมีผลต่อการอักเสบ พบว่าในหนูเบาหวานมีระดับสารพรอสตาแกลนดิน 6-keto-$PGF_{1\alpha}$ น้อยลงแต่สารพรอสตาแกลนดิน TXB_2 มากขึ้น เมื่อให้เคอคูมินเสริมพบ 6-keto-$PGF_{1\alpha}$ มากขึ้นในหนูเบาหวานที่ได้รับเคอคูมิน ($P<0.05$) เมื่อดูอัตราส่วนระหว่างพรอสตาแกลนดิน 6-keto-$PGF_{1\alpha}$ ต่อ TXB_2 ของหลอดเลือดในหนูเบาหวานมีสัดส่วนน้อยกว่าในหนูกลุ่มควบคุม การให้เคอคูมินเสริมทำให้อัตราส่วนนี้มีค่าเพิ่มขึ้น การวิเคราะห์ทาง Immunohistochemistry ของหลอดเลือดแดงขนาดเล็ก พบว่าการแสดงออกของ PKC COX-2 และ NF-KB เพิ่มขึ้นในหนูเบาหวาน การแสดงออกเหล่านี้ถูกยับยั้งโดยการให้เคอคูมินเสริมในหนูเบาหวาน (DM+cur)

โดยสรุปการศึกษาครั้งนี้แสดงให้เห็นว่าการเสริมเคอคูมินในหนูเบาหวานสามารถทำให้ภาวะการสูญเสียหน้าที่ของเซลล์เอนโดทีเลียมในเบาหวานดีขึ้น ซึ่งผลของเคอคูมินนี้อาจผ่านทางการกำจัดอนุมูลอิสระ (antioxidant) หรือยับยั้ง COX-2 และ NF-KB (anti-inflammation) และยับยั้งการทำงานของโปรตีนไคเนสซี (PKC inhibition) ดังนั้นการให้เคอคูมินเสริมจะมีประโยชน์สำหรับผู้ป่วยเบาหวานต่อการทำงานของหลอดเลือดขนาดเล็กให้ดีขึ้น และป้องกันการเกิดภาวะแทรกซ้อนของหัวใจและหลอดเลือดได้

ลายมือชื่อนิสิต ศิรดา รังษีสันติวานนท์

สาขา สรีรวิทยา ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก

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SIRADA RUNGSEESANTIVANON: EFFECT OF CURCUMIN ON DIABETES-INDUCED ENDOTHELIAL DYSFUNCTION IN RAT: ROLE OF PKC, COX-2 AND NF-κB. THESIS ADVISOR: ASSOC. PROF. SUTHILUK PATUMRAJ, Ph.D., THESIS CO-ADVISOR: ASSIST. PROF. NARIS THENGCHAISRI, D.V.M., Ph.D., 130 pp.

Chronic hyperglycemia in diabetes mellitus leads to the overproduction of free radicals and contributes to the development of endothelial dysfunction. Turmeric (*Curcuma longa* L.), an Asian flavoring and coloring agent, is known for its wide spectrum of therapeutic effects. In the present study, we proposed that curcumin can improve diabetes-induced endothelial dysfunction through its antioxidant, anti-inflammatory and PKC inhibitory actions. Male Wistar rats were divided into five groups; 1) diabetes (DM, induced by a single injection of streptozotocin (STZ, 55 mg/kg, *iv.*), 2) DM-supplemented with curcumin 30 mg/kg (DM+cur30), 3) DM-supplemented with curcumin 300 mg/kg (DM+cur300), 4) control (con) and 5) control supplemented with curcumin 300 mg/kg (con+cur300). Daily curcumin oral feedings were started six weeks after the STZ injection. Based on the hypothesis, hyperglycemia causes an increase in free radicals (ROS) which in turn enhances PKC, NF-κB, and COX-2 activations. The increased in ROS inside the arteriolar endothelial cells consequently reduce vasodilatation of microvessels to the endothelium-derived vasodilators. Twelve weeks after STZ injection, the endothelial dysfunction was determined by the functional responses of mesenteric arterioles to vasodilators, acethylcholine (ACh) and sodium nitropusside (SNP) using a real time intravital fluorescence videomicroscopy. The dilatory response to ACh significantly decreased in DM arterioles compared to control arterioles ($P<0.01$). SNP-induced arteriolar dilatation was not different among groups ACh-induced arteriolar dilatation were significantly improved by both low and high doses (30 and 300 mg/kg, respectively) of curcumin supplementation ($P<0.01$). Further, we aimed to examine the underlying molecular mechanisms of the curcumin on protecting endothelial cell against diabetes-induced endothelial dysfunction. An oxygen radical-sensitive fluorescent probe, hydroethidine, was used to detect intracellular superoxide anion ($O_2^{\bullet-}$) production. $O_2^{\bullet-}$ production was markedly increased in DM arterioles ($P<0.01$), but it was significantly reduced in DM rats supplemented with either low or high doses of curcumin ($P<0.01$). It is strongly supported by an invert correlation between the increment of superoxide production and a reduction of endothelial vascular response ($r^2=0.7$, $P<0.01$). Next, prostanoids determination demonstrated the role of curcumin in COX-2-derived prostaglandins-mediated inflammation. DM rats demonstrated higher TXB_2 (stable metabolite of TXA_2) and lower 6-keto-$PGF_{1\alpha}$ (stable metabolite of PGI_2) levels compared to control rats, whereas curcumin supplementation significantly increased 6-keto-$PGF_{1\alpha}$ level ($P<0.05$). The ratio of 6-keto-$PGF_{1\alpha}$/ TXB_2 decreased in DM rats compared to control. Interestingly, curcumin supplementation caused a shift in this ratio back to control rats. It is strongly supported by immunohistochemical analysis, the arteries of DM rats showed marked expression of PKC, COX-2 and NF-κB. In addition, these signals were reduced by curcumin supplementation at the dose of 30 and 300 mg/kg.

In conclusion, the present study indicates that curcumin supplementation significantly improve diabetes-induced endothelial dysfunction related to its potential to superoxide scavenging, COX-2 and NF-κB suppression, and PKC inhibitory effects. It is possible that curcumin supplementation may be beneficial for diabetic patients by improving microvascular function and preventing DM-related cardiovascular complications.

Field of Study: Physiology Student's Signature Sirada Rungseesantivanon

Academic Year: 2009 Advisor's Signature

Co-Advisor's Signature Naris Thengchaisri

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LIST OF ABBREVIATIONS

ACh	=	Acetylcholine
AngII	=	Angiotensin II
AGEs	=	Advanced glycation end-products
AP-1	=	Activatior protein-1
AR	=	Aldose reductase
ATP	=	Adenosine triphosphate
BH_4	=	Tetrahydrobiopterin
COX-2	=	Cyclooxygenase-2
DM	=	Diabetes Mellitus
DAG	=	Diacyl glycerol
EB	=	Ethidium bromide
EDCF	=	Endothelium-derived constrictor factor
EDRF	=	Endothelium-derived relaxing factor
ET-1	=	Endothelin-1
FITC-dextran	=	Fluorescein isothiocyanate labeled dextran
GAD	=	Glutamic acid decarboxylase
GAPDH	=	Glyceraldehyde-3-phosphate dehydrogenase
GDM	=	Gestational diabetes mellitus
GLUT1	=	Glucose transporter 1
GLUT2	=	Glucose transporter 2
GLUT4	=	Glucose transporter 4 (insulin dependent)
G-3-P	=	Glycerolaldehyde-3-phosphate
HbA1c	=	Glycosylated hemoglobin A1c
HE	=	Hydroethidine

LIST OF ABBREVIATIONS

HMECs	=	Human microvascular endothelial cells
H_2O_2	=	Hydrogen peroxide
ICAM-1	=	Intracellular adhesion molecules-1
I-κB	=	Inhibitor protein NF-κB
mABP	=	mean Arterial blood pressure
MNU	=	*N*-methyl-*N*-nitrosourea
NADH	=	Nicotinamide adenine dinucleotide
NADPH	=	Nicotinamide adenine dinucleotide phosphate
NE	=	Norepinephrine
NF-κB	=	Nuclear transcription factor- kappa B
NO	=	Nitric oxide
NOS	=	Nitric oxide synthase
NOX	=	NADPH oxidase
$O_2^{\bullet-}$	=	Superoxide anion
$ONOO^-$	=	Peroxynitrite
PAI-1	=	Plasminogen activator inhibitor-1
PARP	=	Poly ADP-ribose polymerase
PA	=	Phosphatidic acid
PC	=	Phosphatidyl choline
PEPCK	=	Phosphoenol pyruvate carboxykinase
PGE_2	=	Prostaglandin E_2
PGI_2	=	Prostacyclin
PGHS	=	Prostaglandin H synthase
PKA	=	Protein kinase A
PKB	=	Protein kinase B

LIST OF ABBREVIATIONS

PKC	=	Protein kinase C
PLA_2	=	Prospholipase A_2
PLC	=	Phospholipase C
PMA	=	Phorbol myristic acid
RNS	=	Reactive nitrogen species
ROS	=	Reactive oxygen species
SNP	=	Sodium nitropusside
SOD	=	Superoxide anion dismutase
STZ	=	Streptozotocin
TGF-β	=	Transformation growth factor- β
TXA_2	=	Thromboxane A_2
TXB_2	=	Thromboxane B_2
UDP	=	Uridine diphosphate
VCAM-1	=	Vascular adhesion molecule-1
VEGF	=	Vascular endothelial growth factor
XDH	=	Xanthine dehydrogenase
XO	=	Xanthine oxidase

CHAPTER I

INTRODUCTION

Diabetes mellitus (DM) is one of the most common metabolic diseases throughout the world today. All forms of diabetes are characterized by chronic hyperglycemia and developed to diabetic complications, in particular, macro- and microangiopathies. These pathophysiological complications are often responsible for a decreased quality of life in diabetic patient (UK Prospective Diabetes Study (UKPDS) Group, 1998). Several hypotheses have been proposed to explain the adverse effect of hyperglycemia on vascular cells. These include the polyol pathway (Williamson *et al*., 1993), the nonenzymatic glycation (Mullarkey *et al.*, 1990), the diacyl glycerol (DAG)-protein kinase C (PKC) pathway (Ishii *et al*., 1998) and redox potential alterations (Nishikawa *et al.*, 2000).

Among various possible mechanisms, both PKC activation and oxidative stress have increasingly received attention in recent year (Hink *et al*., 2001; Cosentino *et al*., 2003; Pricci *et al*., 2003; Yakubu *et al*., 2004). Because PKC is a critical intracellular signal molecule that can regulate many vascular functions, activation PKC may underline various deleterious alterations in vascular functions in diabetes. Experimental evidence indicates that the relationships between PKC and reactive oxygen species (ROS). Hink, *et al*. (2001) found that *in vitro* PKC inhibition with PKC inhibitor reduced vascular superoxide in diabetic vessels, whereas it had no effect on superoxide ($O_2^{\bullet-}$) levels in normal vessels. In agreement with these findings, Cosentino, *et al*., (2003) have shown that high glucose via PKC signaling induce oxidative stress in human aortic endothelial cells. A recent study also demonstrated that free radicals or PKC contribute to glucose-induced impairment of vasorelaxation (Yakubu *et al.*, 2004). This alteration was restored by antioxidant as well as PKC inhibitor. However, the role of PKC as well as the molecular mechanism linking oxidative stress remains not fully understood.

In the present study, we emphasize the role of prostaglandin-mediated endothelium-dependent vasoconstriction in diabetic rats. Beside decreased formation of the endothelium-derived relaxing factors (primarily nitric oxide) in diabetic vascular diseases, accumulating evidence demonstrated an increased in formation of vasoconstrictor, most of which are prostaglandins (Akamine *et al*., 2006). It has been shown by several studies that PKC activation plays a mediating role in the overproduction of vasoconstrictor prostaglandins. Cosentino, *et al*. (2003) also reported that high glucose caused PKC-dependent up-regulation of inducible cyclooxygenase (COX-2). Its upregulation was associated with a shift in the balance of vasodilatory and vasoconstricting prostanoids produced by the endothelial cells in favor of the latter.

Moreover, high glucose induced PKC signaling promote oxidative stress, resulting in reduced nitric oxide (NO) availability and altered prostaglandin profile (Beckman *et al.*, 2002; Pricci *et al*., 2003; Yakubu *et al*., 2004). Recently, it has been found that PKC inhibitor also ameliorates vascular complications in diabetes (Hink *et al*., 2001; Beckman *et al*., 2002; Cosentino *et al*., 2003; Pricci *et al*., 2003; Yakubu *et al*., 2004). These studies suggested that oxidant and PKC contribute to glucose-induced attenuation of vasorelaxation and may mediate other mechanisms responsible for diabetes-induced endothelial dysfunction.

In our previous study, we demonstrated that vitamin C supplementation and PKC inhibition improve endothelial dysfunction of mesenteric arterioles in streptozotocin-induced diabetic rats (Rungseesantivanon *et al.*, 2006). Our findings are associated with the above-mentioned the role of free radicals and PKC.

In animal models of diabetes, antioxidants defense capacity is diminished in certain tissues (Wohaieb and Godin, 1987). In addition, human and animal studies have addressed a restored function of vascular endothelium with antioxidant administrations in diabetes (Timimi *et al*., 1998; Jariyapongskul *et al*., 2002; Sridulyakul *et al*., 2006). However, a critical evaluation of clinical trials suggests a difference in the specific ROS to various vascular diseases, thereby limiting the effectiveness of specific antioxidants (Taniyama and Griendling, 2003).

Various herbal extracts are known to possess antioxidant properties. Curcumin, a yellow pigment from root of *Curcuma longa* L., is a major component of turmeric and commonly used as a spice and food-coloring agent. Curcumin has a wide range of activities which have been well documented by previous studies (Masuda *et al.*, 2001; Masuda *et al*., 2002; Taniyama and Griendling, 2003; Bengmark, 2006). Numerous studies have indicated beneficial role of curcumin in terms of antioxidant, anti-inflammatory and antitumourgenic properties (Masuda *et al.*, 2001; Masuda *et al.*, 2002; Balasubramanyam *et a*l., 2003; Taniyama and Griendling, 2003; Bengmark, 2006). Due to curcumin has been shown to affect the activity of enzymes such as COX-2. In line with a potential role of PKC in hyperglycemia-induced responses, previous studies have demonstrated that treatment with calphostin C, a PKC inhibitor, completely attenuated NF-κB activation by high glucose (Yerneni *et al*., 1999). Furthermore, NF-κB is known as one major intracellular target of hyperglycemia and oxidative stress. It is possible that NF-κB contributes to abnormal vascular function in diabetes (Srivastava, 2002).

Although aforementioned studies demonstrate the partially beneficial effects of curcumin, there were no studies on the efficacy of curcumin in restoration of the endothelial dysfunction involve PKC and free radical production in diabetes remain elusive. Therefore, the present study was designed to determine the effects of curcumin on vascular function of diabetes-induced rats, and the possible mechanism(s) of curcumin action on free radical, PKC and prostaglandin was (were) also tested.

Research questions

Could curcumin restore endothelial dysfunction in diabetes-induced model through its antioxidant, anti-inflammatory or inhibitory effect on PKC?

Hypothesis

Curcumin ameliorate endothelial dysfunction in diabetes-induced rat model through antioxidant, anti-inflammatory or inhibitory effect on PKC.

Research objectives

1. To determine the effects of curcumin on restoration of endothelial dysfunction in diabetes-induced rat model.
2. To study the possible mechanisms underlying the beneficial effect of curcumin on free radicals and PKC activation involve in diabetes-induced rat model.

CHAPTER II
LITERATURE REVIEWS

Diabetes Mellitus

Diabetes mellitus is a common metabolic disease with a high and growing prevalence affecting 4% of the population worldwide, 171 million people in the year 2000 and an expected 366 million in 2030 (Wild *et al.*, 2004). Diabetes is a condition primarily defined by the level of hyperglycemia. It is associated with reduced life expectancy; morbidity due to diabetes related micro- and macrovascular complications and diminished quality of life.

Since 1965 the World Health Organization (WHO) and the National Diabetes Data Group (NDDG) produced diagnostic criteria and classification system for diabetes mellitus. These were published as the guidelines for the definition, diagnosis and classification of diabetes mellitus. In 1997, an International Expert Committee, working under the sponsorship of American Diabetes Association (ADA) was convened to reexamine the classification and diagnostic criteria of diabetes, which were based on the 1979 publication of the National Diabetes Data Group and subsequent WHO study Group. In addition, in November 2005 a joint WHO and International Diabetes Federation (IDF) Technical Advisory Group met in Geneva to review and update the current WHO guidelines (World Health Organization, 2006).

1. Definition of diabetes mellitus

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both. The effects of diabetes mellitus include long-term damage, dysfunction, and failure of various organs. Several pathogenesis processes are involved in the development of diabetes. These include process which destroys the beta cells of pancreases with consequent insulin deficiency, and others that result in resistance to insulin action. The abnormalities of carbohydrate, fat and protein metabolism are due to deficient action of insulin on target tissues resulting from insensitivity or lack of insulin (World Health Organization, 1999).

2. Diagnostic criteria for diabetes mellitus

The criteria for the diagnosis of diabetes are shown in Table 2.1 (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2004).

Table 2.1. Criteria for the diagnosis of diabetes mellitus.

1. Symptoms of diabetes plus casual plasma glucose concentration ≥200 mg/dL (11.1 mM). Casual is defined as any time of day without regard to time since last meal. The classic symptoms of diabetes include polyuria, polydipsia, and unexplained weight loss. or
2. FPG ≥126 mg/dL (7.0 mM). Fasting is defined as no caloric intake for at least 8 hour. or
3. 2-hour postload glucose glucose ≥200 mg/dL (11.1 mM) during an oral glucose tolerance test (OGTT). The test should be performed as described by WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.

In the absence of unequivocal hyperglycemia, these criteria should be confirmed by repeat testing on a different day. The third measure (OGTT) is not recommended for routine clinical use.

3. Classification of diabetes mellitus

The first widely accepted classification of diabetes mellitus was published by WHO in 1980 (WHO Expert Committee on Diabetes Mellitus, 1980) and in modified from, in 1985 (World Health Organization, 1985). The 1985 classification was accepted and is used internationally. It represented a compromise between clinical stages and aetiological types of diabetes mellitus.

The current Expert Committee has considered the data and rationale for what was accepted in 1979. They proposed changes to the NDDG/WHO classification in Table 2.2 (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2004).

Table 2.2. Etiological classification of diabetes mellitus.

I. Type 1 diabetes (beta-cell destruction, usually leading to absolute insulin deficiency)
   A. Immune mediated
   B. Idiopathic

II. Type 2 diabetes (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance)

III. Other specific types
   A. Genetic defects of beta-cell function
   B. Genetic defects in insulin action
   C. Diseases of the exocrine pancreas
   D. Endocrinopathies
   E. Drug- or chemical-induced
   F. Infection
   G. Uncommon forms of immune-mediated diabetes
   H. Other genetic syndromes sometimes associated diabetes

IV. Gestational diabetes mellitus (GDM)

V. Impaired glucose tolerance (IGT) and impaired fasting glucose (IFG)

4. Description of etiological type 1 and 2 diabetes mellitus

Type1 diabetes mellitus

This form of diabetes previously referred to as insulin-dependent diabetes, or juvenile-onset diabetes, results from a cellular-mediated autoimmune destruction of the beta-cells of the pancrease. Due to the pathophysiology, insulin therapy is indicated at the onset of this type. There is also evidence that type 1 diabetes is associated with markers of the immune destruction of beta-cell include islet cell autoantibodies (ICAs), autoantibodies to insulin (IAAs), autoantibodies to glutamic acid decarboxylase (GAD_{65}) and autoantibodies to tyrosine phosphatase IA-2 and IA-2β (Athkinson *et al*., 1986; Kaufman *et al*., 1992; Schott *et al*., 1994; Lan *et al.*, 1996; Lu *et al*., 1996). The disease has strong

HLA associations, with linkage to the DQA and DQB genes (Huang *et al*., 1996). These *HLA-DR/DQ* alleles can be either predisposing or protective. At this latter stage of the disease, there is little or no insulin secretion, as manifested by low or undetectable levels of plasma C-peptide. Some forms of type 1 diabetes have no known etiologies. Some patients have permanent insulinopenia and are prone to ketoacidosis, but have no evidence of autoimmunity (idiopathic diabetes) (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003).

Type2 diabetes mellitus

Type 2 diabetes previously referred to as non-insulin-dependent diabetes, or adult-onset diabetes, results from insulin resistance and relative insulin deficiency. Type 2 diabetes frequently goes undiagnosed for many years because the hyperglycemia develops gradually and at earlier stages is often not severe enough for the patient to notice any of the symptoms of diabetes (Harris, 1989). Nevertheless, such patients are at increased risk of developing macro- and microvascular complications (Anderson and Svaardsudd, 1995). Unlike type 1 diabetes, there is no evidence that autoimmune mechanisms are involved. It is well established that obesity and insulin resistance are generally present. The two defects that characterize are 1) a derangement in beta-cell secretion of insulin and 2) a decreased response of peripheral tissues to respond to insulin (insulin resistance) (Cotran, 1999). However, the cellular mechanisms that contribute to insulin resistance are not fully understood.

5. Diabetic animal models

Numerous animal models of diabetes have been developed to mimic human diabetes mellitus. Early studies used pancreatectomised dogs to confirm the central role of the pancreas in glucose homeostasis. Apart from animal experiment used in studying the pathogenesis of the disease and its complications, all new treatment for diabetes is investigated in animals. Diabetes can be induced by surgical or pharmacological models. The classic surgical model of diabetes is total pancreatectomy in the dog. This model was used by Banting and Best in their studies leading to the discovery of insulin and the mechanism of diabetes (Gelati *et al*., 1979).

5.1 Animal models of type 1 diabetes mellitus

Non-surgical methods of inducing hyperglycemia via a number of agents that selectively destroy pancreatic beta-cells. Table 2.3 (Rees and Alcolado, 2005) lists a variety of agents known to produced diabetes in experimental animals and streptozotocin is discussed further below.

Table 2.3. Substances with a diabetogenic effect in experimental animals

Alloxan
Streptozotocin
Vacor
Dithizone
8-hydroxyquinolone

Streptozotocin (STZ) is synthesized by *Streptomycetes achromogenes* and widely used to induce experimental diabetes in animals. In 1963, Rakieten *et al*. (Rakieten et al., 1963) reported that STZ is diabetogenic. Figure 2.1 shows a schematic diagram of the triphasic blood glucose responses induced by STZ, when injected (Lenzen, 2008). The responses are accompanied by corresponding inverse changes in plasma insulin and sequential ultrastructural changes resulting in necrotic beta cell death.

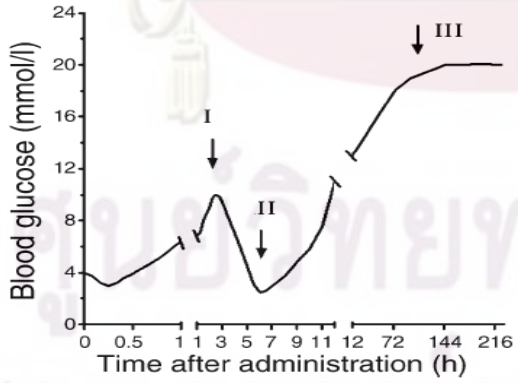


Figure 2. 1. Phasic blood glucose response to a diabetogenic dose of streptozotocin (triphasic; I-III) (Adapted from Lenzen, 2008).

The first phase starts with an increase in the blood glucose concentration, 1 hour after administration of STZ, and a decrease in plasma insulin. This first hyperglycemic phase, which usually lasts 2-4 hours, is caused by inhibition of insulin secretion leading to hypoinsulinemia. During this phase the beta cells show the following morphological characteristics: intracellular vacuolization, dilation of the rough endoplasmic reticulum, decreased Golgi area, reduced secretory granules and insulin content and swollen mitochondria.

The second phase, a hypoglycemic phase, typically occurs 4-8 hours after the injection of STZ and lasts several hours. It may be fatal in particular when liver glycogen stores are depleted through starvation.

The third phase is the permanent diabetic hyperglycemic phase. Morphologically, complete degranulation and loss of beta cell integrity is seen within 12-48 hour. Non-beta cells remain intact, demonstrating the beta cell-selective character of the toxic action. Cell debris originating from the dying beta cells is removed by non-activated scavenger macrophages.

Thus, injection of STZ principally causes an insulin-dependent type1-like diabetes syndrome. These morphological features of beta cell destruction are characteristic of necrotic cell death (Lenzen, 2008).

5.2 Beta cell selectivity of streptozotocin

STZ is a nitrosourea analogue in which the *N*-methyl-*N*-nitrosourea (MNU) moiety is linked to the carbon-2 of a hexose (Figure 2.2). The toxic action of STZ and chemically related alkylating compounds requires their uptake into the cells. Nitrosoureas are usually lipophilic and tissue uptake through the plasma membrane is rapid; however, as a result of the hexose substitution, STZ is less lipophilic. STZ is selectively accumulated in pancreatic beta-cells via the low-affinity GLUT2 glucose transporter in the plasma membrane (Karunanayake *et al*., 1976).

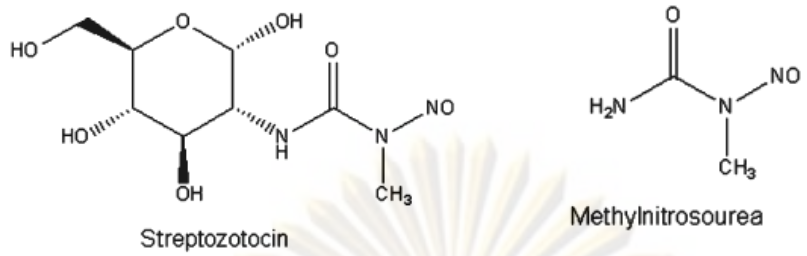


Figure 2.2. Chemical formulas of streptozotocin and methylnitrosourea. (Lenzen. 2008)

5.3 Beta cell toxicity of streptozotocin

It is generally assumed that the toxicity of STZ is dependent on DNA alkylation activity of its methylnitrosourea moiety (Murata *et al*., 1999), especially at the O^6 position of guanine (Lenzen, 2008). The transfer of the methyl group from STZ to the DNA molecule causes damage results in the fragmentation of the DNA (Yamamoto *et al.*, 1981). In the attempt to repair DNA, poly (ADP-ribose) polymerase (PARP) is overstimulated. This diminishes cellular NAD^+, and subsequently ATP, stores (Yamamoto *et al*., 1981; Sandler and Swenne, 1983). The depletion of the cellular energy stores ultimately results in beta-cell necrosis. Although STZ also methylates proteins (Wilson *et al.*, 1988), DNA methylation is ultimately responsible for beta-cell death, but it is likely that protein methylation contributes to the functional defects of the beta-cells after exposure to STZ.

An alternative hypothesis proposes that part of the diabetogenic effect of STZ may relate not to its alkylating ability but to its potential to act as intracellular nitric oxide (NO) donor (Turk *et al.*, 1993). Both STZ and MNU contain a nitroso group (Figure 2) and can liberate NO. In fact, STZ has been shown to increase the activity of guanylyl cyclase and the formation of cGMP, which are characteristic effects of NO. However, the alkylating agent methyl methanesulphonate is the most toxic compound, it is not a NO donor (Delaney *et al*., 1995), indicating that NO is not an indispensable prerequisite for the toxic action of the family of alkylating agents that STZ belongs to. Finally, some minor generation of ROS, including superoxide and hydroxyl radicals originating from hydrogen peroxide dismutation during hypoxanthine metabolism (Nukatsuka *et al*., 1990), may accompany the effect of STZ and accelerate the process of beta-cell destruction but ROS do not play a crucial role (Lenzen, 2008).

5.4 Inhibition of insulin secretion by streptozotocin

The effects of STZ on glucose and insulin homeostasis reflect the toxin-induced abnormalities in beta cell function. Initially, insulin biosynthesis, glucose-induced insulin secretion and glucose metabolism are all affected (Nukatsuka *et al.*, 1990; Bedoya *et al*., 1996). At later stages of functional beta cell impairment and lead to the deterioration of both glucose transport and metabolism (Wang and Gleichmann, 1998). STZ-induced depletion of NAD^+ may result in the inhibition of insulin biosynthesis and secretion (Yamamoto *et al*., 1981; Strandell *et al.*, 1988). Later, inhibition of glucose-induced and amino acid-induced insulin secretion (Eizirik *et al.*, 1988) as a result of mitochondrial enzyme dysfunction (Rasschaert *et al*., 1992). This impairment is more marked for nutrient- than for non-nutrient-induced insulin secretion. This interpretation has been confirmed through studies which have shown that pre-treatment of isolated pancreatic islets with the poly(ADP-ribose) polymerase (PARP) inhibitor nicotinamide prevents early inhibition of beta cell function during the first day after STZ exposure, while long-term inhibition of insulin secretion 6 days after STZ exposure was not counteracted by nicotinamide (Strandell *et al*., 1989).

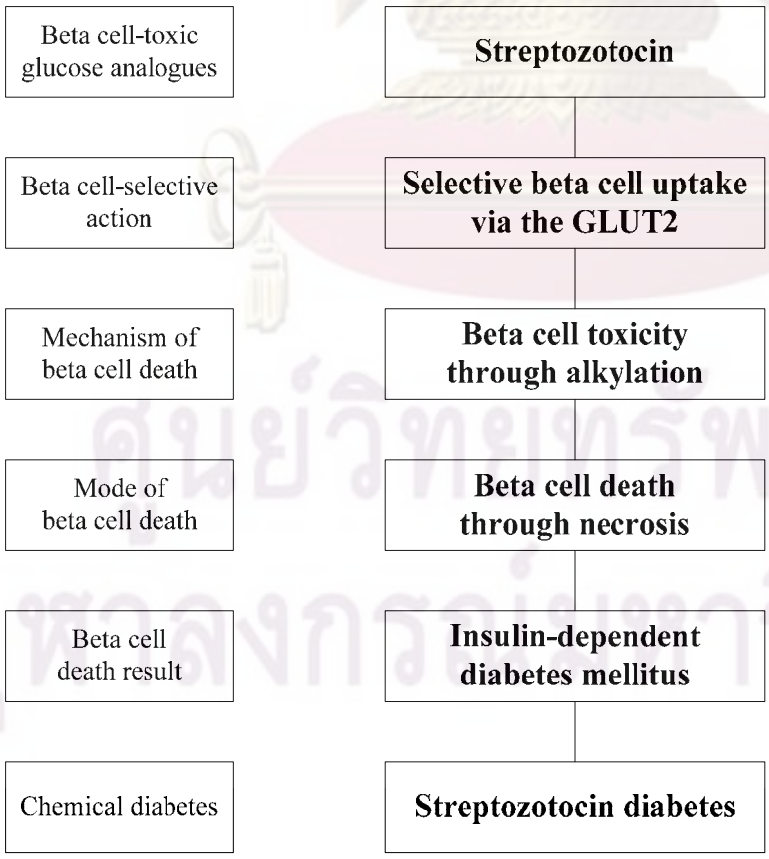


Figure 2.3. Schematic representation of the toxic effects of the glucose analogues streptozotocin in beta cells, which produce chemical diabetes.

STZ induce insulin deficiency. While the mechanisms of beta cell-selective action through uptake via the GLUT2 glucose transporter and beta cell deaths via necrosis are DNA alkylation mediate the toxic action of these glucose analogues (Figure 2.3). Due to its chemical properties, in particular the greater stability (Table 2.4), STZ is the agent of choice for reproducible induction of diabetic metabolic state in experimental animals.

Table 2.4. The chemical properties of streptozotocin.

	Streptozotocin
Chemical name	2-deoxy-2-([(methylnitrosoamino)carbonyl]amino)-D-glucopryranose
Chemical structure	Cytotoxic methylnitrosourea moiety (*N*-methyl-*N*-nitrosourea) attached to the glucose (2-deoxyglucose) molecule; glucosamine derivative
Chemical properties	Hydrophilic, beta cell-toxic glucose analogue
	Relatively stable at pH 7.4 and 37° C (at least for up to 1 hour)[a]
Chemical reactivities	DNA alkylating agent
	Protein alkylating agent
Mode of toxicity	DNA alkylation
[a]For injection, a stable solution in citrate buffer (pH 4.5) is most suitable	

Diabetic complications

Diabetes is a national as well as global epidemic in terms of incidence, healthcare costs, and overall complications. As reported by WHO, diabetes caused 19 thousand deaths in 2002 and made it the fourth leading cause of death in Thailand (World Health Organization, 2006). Diabetic vascular disease represents a major cause of mortality and morbidity in diabetic patients. Both microvascular and macrovascular complications are the burden of the disease are not only in terms of individual health and well-being but also in having an impact on the economic status of their families and the country.

Molecular mechanisms of diabetic complications

Hyperglycemia is the initiating cause of the diabetic tissue damage. The most cells are able to reduce the transport of glucose inside the cell when they are exposed to hyperglycemia, so that their internal glucose concentration stays constant. In contrast, the few cell types damaged by hyperglycemia are those that cannot do this efficiently (Kaiser *et al.*, 1993). Thus, diabetes selectively damages cells, like endothelial cells and mesangial

cells lead to high glucose inside the cell. The general features of hyperglycemia-induced tissue damage are shown in Figure 2.4 (Brownlee, 2005). Hyperglycemia induces repeated acute changes in intracellular metabolism as well as cumulative long-term changes in the structure and function of macromolecules.

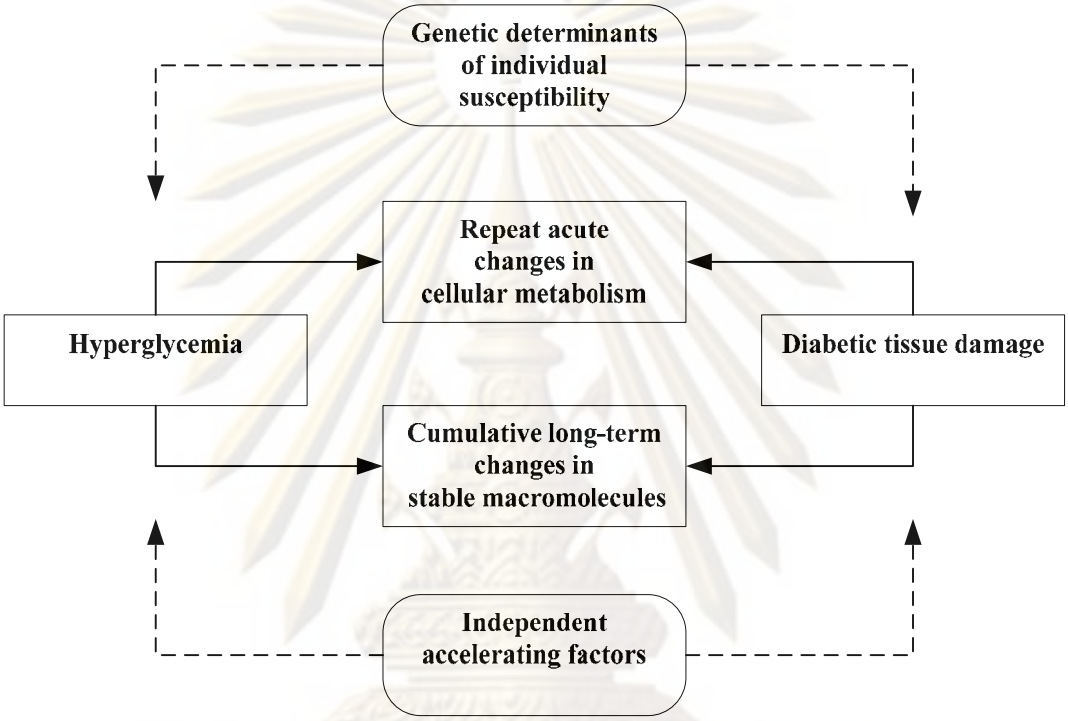


Figure 2.4. General features of hyperglycemia-induced tissue damage.

Hyperglycemia is one of the major causes of vascular dysfunction. Because glucose and its metabolites are utilized by numerous intracellular pathways, the adverse effects of hyperglycemia involve multiple pathways. Over the several years, four theories have attempted to major pathways implicated in the pathogenesis of hyperglycemia-induced vascular dysfunction. These theoretical pathways are: aldose reductase-polyol pathway flux; advanced glycation end-product (AGE) formation; hexosamine pathway flux and activation of protein kinase C (PKC). They are reviewed in the following.

1. Increase polyol pathway flux

The first discovered pathway was increased polyol pathway flux, described in the 1966 (Gabbay *et al*., 1966). The polyol pathway focuses on the enzyme aldose reductase (AR). AR is a cytosolic, monomeric oxidoreductase that catalyses the NADPH-dependent reduction of a variety of carbonyl compounds, including glucose. Its crystal structure has a single domain folded into an eight-stranded parallel α/β-barrel motif, with the substrate-binding site located in a cleft at the carboxy-terminal end of the β-barrel (Wilson *et al*., 1992). AR has a low affinity (high K_m) for glucose. At the normal glucose conditions found in non-diabetics, metabolism of glucose by this pathway is a very small percentage of total glucose use. AR normally has the function of reducing toxic aldehydes in the cell to inactive alcohols. When the glucose concentration in the cell becomes too high, AR also reduces the glucose to sorbitol, which is later oxidized to fructose. In this reducing process, AR consumes the cofactor NADPH (Lee and Chung, 1999). However, NADPH is also the essential cofactor for regenerating a critical intracellular antioxidant, reduced glutathione. By reducing the amount of reducing glutathione, the polyol pathway increases susceptibility to intracellular oxidative stress (Brownlee, 2005).

Another the potential effects of hyperglycemia-induced increased in polyol pathway flux include sorbitol-induced osmotic stress, decreased Na-K ATPase activity (Xia *et al*., 1995), and an increase in cytosolic NADH/NAD^+ (Williamson *et al*., 1993).

2. Increased intracellular formation of advanced glycation end-products

Ten years later, in the late 1970s, the second discovery pathway is the intracellular production of advanced glycation end-products (AGEs). AGEs can arise from intracellular auto-oxidation of glucose to glyoxal (Wells-Knecht *et al*., 1995), decomposition of the Amadori product (glucose-derived 1-amino-1-deoxyfructose lysine adducts) to 3-deoxyglucosone perhaps accelerated by an amadoriase) and fragmentation of glyceraldehydes-3-phosphate and dihydroxyacetone phosphate to methylglyoxal (Thornalley, 1990). These reactive intracellular dicarbonyls, glyoxal, methylglyoxal and 3-deoxyglucoson, react with amino groups of intracellular and extracellular proteins to form AGEs. Methylglyoxal and glyoxal are detoxified by the glyoxalase system (Thornalley, 1990). All three AGEs precursors are also substrates for other reductase (Suzuki *et al*., 1998).

Production of intracellular AGEs precursors damage target cells by three mechanisms. First, the modification of intracellular proteins involved in the regulation of gene transcription (Shinohara *et al.*, 1998). Second, AGE precursors can diffuse out of the cell and modify extracellular matrix molecules nearby (McLellan *et al*., 1994), which changes signaling between the matrix and the cell and causes cellular dysfunction (Charonis *et al*., 1990). Third, these AGE precursors diffuse out of the cell and modify circulating proteins in the blood such as albumin. These modified proteins can then bind to AGE receptors and activate them, thereby causing the production of inflammatory cytokines and growth factors (Kirstein *et al*., 1992; Neeper *et al*., 1992; Abordo and Thornalley, 1997), which in turn cause vascular pathology.

AGE formation alters the functional properties of several important matrix molecules. On type I collagen, intermolecular crosslinking by AGEs induces an expansion of the molecular packing (Tanaka *et al*., 1988). AGEs decrease elasticity in large vessel from diabetic rats and increase fluid filtration across the carotid artery (Huijberts *et al*., 1993). AGE formation on type IV collagen from basement membrane inhibits lateral association of these molecules into a normal network-like structure (Tsilibary *et al*., 1988).

3. Protein kinase C activation

In the late 1980s and early 1990s, the third pathway of hyperglycemia-induced damage was discovered: hyperglycemia-induced activation of protein kinase C (PKC). The PKC family comprises at least eleven isoforms. Intracellular hyperglycemia increases the synthesis of a molecule called diacylglycerol (DAG). It seems to achieve this primarily by increasing *de novo* DAG synthesis from the glycolytic intermediate dihydroxyacetone phosphate, through reduction of the latter to glycerol-3-phosphate and stepwise acylation (Koya and King, 1998). Increased *de novo* synthesis of DAG activates PKC both in cultured vascular cells (Xia *et al.*, 1994) and in diabetic animals (Koya and King, 1998). DAG is a critical activating cofactor for the classic isoforms of PKC-α,-β,-δ. Among the PKC family, several isoenzymes have been shown to contribute to insulin signaling, such as PKC-θ and PKC-ε and insulin-stimulated D-glucose uptake (PKC-ς and PKC-λ), while others (most prominently PKC-β) are involved in the development of diabetes-link complications (Spitaler and Graier, 2002). Activation of PKC has a number of pathogenic consequences by affecting expression of endothelial nitric oxide synthetase (eNOS), endothelin-1(ET-1), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β) and plasminogen actvator inhibitor-1 (PAI-1) and by activation NF-κB and NADPH oxidase which are shown in Figure 2.5.

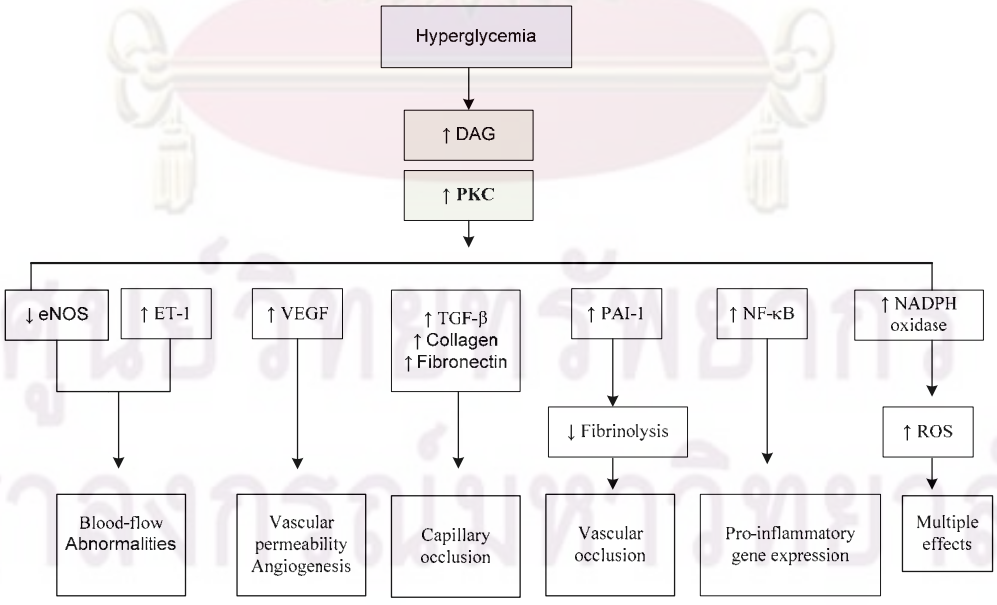


Figure 2.5. Consequences of hyperglycemia-induced activation of PKC.

4. Increased hexosamine pathway activity

In the late 1990s, the fourth pathway was discovered: increased hexosamine pathway flux and consequent overmodification of protein by *N*-acetylglucosamine. When glucose is high inside a cell, most of that glucose is metabolized through glycolysis, going first to glucose-6 phosphate, then fructose-6 phosphate, and then on through the rest of the glycolytic pathway. However, some of that fructose-6-phosphate gets diverted into a signaling pathway in which an enzyme call glutamine:fructose-6 phosphate amidotransferase (GFAT) converts the fructose-6 phosphate to glucosamine-6 phosphate and finally to UDP (uridine diphosphate) *N*-acetylglucosamine. Intracellular glycosylation by the addition of *N*-acetylglucosamine (GlcNAc) to serine and threonine is catalysed by the enzyme *O*- GlcNAc transferase (OGT). Increased donation of GlcNAc moieties to serine and threonine residues of transcription factor such as Sp1, often at phosphorylation sites, increases the production of transformation growth factor-β1 (TGF-β1) and plasminogen activator inhibitor-1 (PAI-1) (Sayeski and Kudlow, 1996; Wells and Hart, 2003). Both are not good for diabetic vessels (Du *et al*., 2000). Thus, activation of the hexosamine pathway by hyperglycemia may result in many changes in both gene expression and protein, which together contribute to the pathogenesis of diabetic complications

Moreover specific inhibitors of aldose reductase activity, AGE formation, PKC activation and the hexosamine pathway each ameliorate various diabetes-induced abnormalities in cell culture and animal models. Recently there has been common element linking the four mechanisms of hyperglycemia-induced damage. Each of four different pathways reflects a single hyperglycemia-induced process: overproduction of superoxide anions (Du *et al*., 2000; Nishikawa *et al*., 2000). When the electrochemical potential difference generated by the proton gradient across the inner mitochondrial membrane is high, the lifetime of superoxide-generating electron-transport intermediates such as ubiseminquinone is prolonged. There seems to increase hyperglycemia is markedly increased superoxide production.

The overall potential pathway of hyperglycemia-induced mitochondrial superoxide overproduction can be summarized by Figure 2.6. Hyperglycemia induces overproduction of mitochondrial superoxide to decrease in 66% GAPDH activity (Du *et al*., 2000). The effect of hyperglycemia on polyol pathway may reflect the accumulation of glycolytic metabolites, including glucose, upstream of GAPDH (Figure 2.6).

In regard to AGEs, methylglyoxal-derived AGE, the primary intracellular AGE induced by hyperglycemia (Shinohara et al., 1998), is formed by fragmentation of triose phosphates. Thus the effect of hyperglycemia on intracellular AGE formation also probably reflects increased triose phosphate levels resulting from inhibition of GAPDH by mitochondrial overproduction of ROS (Figure 2.6).

Hyperglycemia activates PKC by increasing the *de novo* synthesis of DAG (Koya and King, 1998). Thus the effect of hyperglycemia on PKC activation probably reflects increased dihydroxyacetone phosphate levels resulting from inhibition of GAPDH by ROS (Figure 2.6).

In addition to increase hexosamine pathway flux by providing more fructose-6 phosphate for GFAT (the rate limiting enzyme of the pathway), the effect of hyperglycemia on hexosamine pathway flux probably reflects increased fructose-6 phosphate levels. These effect results from inhibition of GAPDH by ROS (Du *et al.*, 2000) were shown in Figure 2.6.

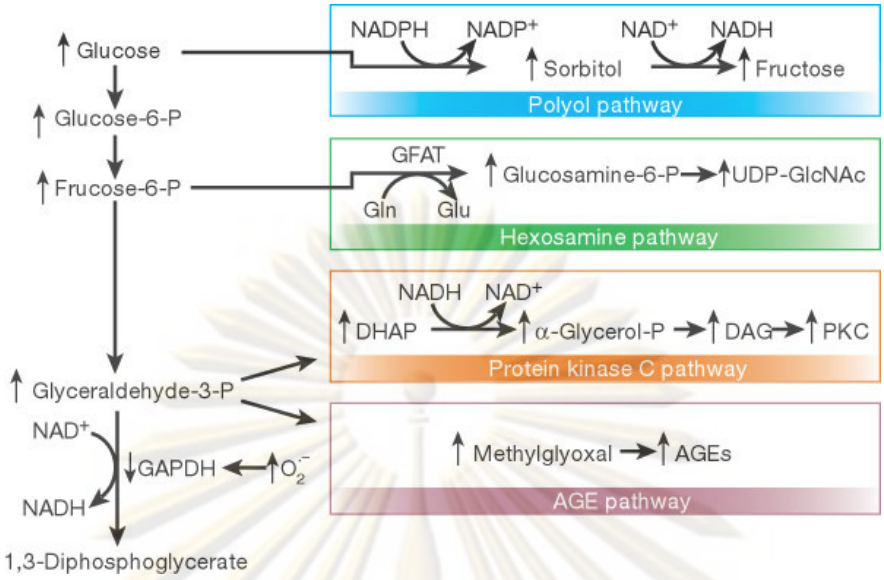


Figure 2.6. Potential pathways by which hyperglycemia-induced mitochondrial superoxide overproduction activates four pathways of hyperglycemic damage. Excess superoxide partially inhibits the glycolytic enzyme (GAPDH), thereby diverting upstream metabolites from glycolysis into pathways of glucose overutilization. This results in increased flux of dihydroxyacetone phosphate (DHAP) to DAG, an activator of PKC, and of triose phosphates to methylglyoxal, the main intracellular AGE precursor. Increased flux of fructose-6 phosphate to UDP-N-acetylglucosamine increases modification of proteins by O-linked N-acetylglucosamine (GlcNAc) and increased glucose flux through the polyol pathway consumes NADPH. (Brownlee, 2001)

Normal endothelial functions

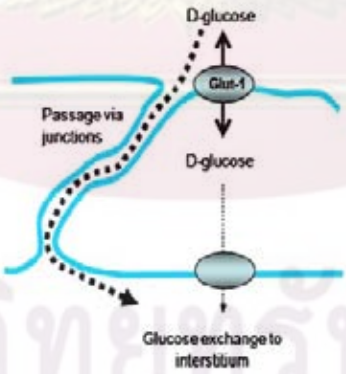
Since the discovery in 1980 that acetylcholine requires the presence of endothelial cells to elicit to relax the underlying vascular smooth muscle (Furchgott and Zawadzki, 1980). The importance of the endothelial cell layer for vascular homeostasis has been increasing studied.

The vascular endothelium is a single cell layer lining the vascular wall and plays an important role in maintaining the function of vessels. By the nature of its location, the interaction between flowing blood and endothelium sense mechanical stimuli, in particular shear forces. This sensing leads the endothelium to respond by vasoregulation. The vasoregulation is achieved by the endothelial vasoactive factors which regulate vascular tone. In addition vasodilator and vasoconstrictor factors, the endothelium also produces a variety of bioactive factors which regulate coagulation, inflammation and cell proliferation

(Kitamoto and Egashira, 2004). Among the vasodilatory substances produced by endothelium are nitric oxide (NO), prostacyclin (PGI_2), different endothelium-derived hyperpolarizing factors (EDHF) and C-type natriuretic peptide (Moncada and Vane, 1978; Furchgott and Zawadzki, 1980; Furchgott and Vanhoutte, 1989; Feletou and Vanhoutte, 1996). Vasoconstrictors include endothelin (ET-1), angiotensin II (Ang II), thromboxane A_2 (TXA_2) (Moncada and Vane, 1978; Yanagisawa *et al*., 1988; Katusic and Vanhoutte, 1989; Masaki, 1995). Inflammatory modulators include intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), E-selectin and NF-κB. A balanced release of these bioactive substances facilitates vascular homeostasis.

Endothelium in glucose metabolism

Endothelial cells are metabolically active cells. They can use both glucose and fatty acids as nutrients. In endothelial cells, the uptake of D-glucose occurs via GLUT-1 glucose transporter. In contrast to GLUT-4 in muscle cells, GLUT-1 is not affected by insulin signaling. Therefore, glucose uptake in the endothelial cells reflects the glucose level in the blood independently of insulin sensitivity. In addition, glucose exchange from the plasma to interstitial fluid proceeds mainly via intercellular gaps/junctions (Figure 2.7).



Figue 2.7. Delivery of D-glucose in endothelial cells (Bakker, 2009).

Endothelial dysfunction and diabetes

The term “endothelial dysfunction” has been referred to in the scientific literature more than 40,000 (PubMed search, July 2009). Since the term endothelial dysfunction has been used to refer to several functional alterations in the vascular endothelium, such as impaired vasodilatation, angiogenesis and barrier function, inflammatory activation, and increased plasma level of endothelial products. These are summarized in Table 2.5 (Bakker *et al.*, 2009). Some of them are mainly associated with hyperglycemia and microangiopathy, whereas others are induced by more complex metabolic alterations and particularily contribute to macroangiopathy. Several studies have demonstrated that endothelial dysfunction of microvessels is an early manifestation of the diabetic vascular complications (Stehouwer *et al*., 1997; De Vriese *et al*., 2000). In addition, evidence for the initially identification of endothelial dysfunction has been evaluated by impaired vasodilatation to specific stimuli such as acethylcholine or bradykinine (Sikorski *et al*., 1993; Endemann and Schiffrin, 2004).

Table 2.5. Endothelial dysfunctions associated with the vascular complications in diabetes.

Type of endothelial dysfunction
Structural changes in endothelial barrier and matrix
Increased basal membrane thickness
Reduced glycocalyx
Formation of AGEs and products and improper matrix crosslinking
Microalbuminuria
Reduced vasodilator response
Reduced EDRF production
Increased EDCF synthesis
Increase inflammatory activation
Increase expression of cell adhesion molecules and leukocyte adhesion
Increased production of and response to circulating mediators
Altered hemostasis
Elevated plasma levels of von Willebrand factor

Mechanisms of diabetes-induced endothelial dysfunction

In animal models, endothelial dysfunction occurs in association with increased ROS in diabetic condition (Cai and Harrison, 2000). Therefore, oxidative stress has been proposed to one of the major causes of hyperglycemia-induced endothelial dysfunction (Nishikawa *et al*., 2000; Griendling and FitzGerald, 2003; Griendling and FitzGerald, 2003). *In vivo* studies linking ROS and diabetes have been performed in rats treated with STZ (a model of type 1 diabetes) (Hink *et al*., 2001; Onozato *et al*., 2002), genetically diabetic rats (a model of type 2 diabetes) (Kim *et al*., 2002), as well as in human patients with type 2 diabetes (Guzik *et al*., 2002).

1. Reactive oxygen species

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbital (Halliwell and Gutteridge, 1999). Oxygen free radicals or more generally, reactive oxygen species (ROS), as well as reactive nitrogen species (RNS), are products of normal cellular metabolism. ROS and RNS are well recognized for playing a dual role since they can be either harmful or beneficial to living system (Mombouli and Vanhoutte, 1999). Beneficial effects of ROS occur at low/moderate concentrations and involve physiological roles in cellular signaling system. On the other hand, the harmful effect of free radicals cause biological damage is termed oxidative stress and nitrosative stress. These occur in biological systems when there is an overproduction of ROS/RNS on one side and a deficiency of enzymatic and non-enzymatic antioxidants on the other. In other words, oxidative stress results from the metabolic reactions that use oxygen and represents a disturbance in the equilibrium status of pro-oxidant/antioxidant reactions in living organisms. The excess ROS can damage cellular lipids, proteins, or DNA inhibiting their normal function (Valko *et al*., 2007).

One of the most important ROS in the vascular cells is superoxide anion ($O_2^{\bullet-}$), which is formed by the univalent reduction of oxygen (Droge, 2002). This reaction is mediated by several enzymes (equation 1), which are reviewed later. $O_2^{\bullet-}$ is a negatively charged free radical that can itself exert effects on vascular function. It is also pivotal in generating other reactive species (Figure 2.8). Although $O_2^{\bullet-}$ reacts with itself with a rate constant of $8x10^4$ $mol^{-s}.L.s^{-1}$ to form H_2O_2 and O_2 (equation 2). Superoxide dismutase (SOD) enzyme accelerate the removal of $O_2^{\bullet-}$ as a result of their rate constant of $2x10^9$ $mol^{-s}.L.s^{-1}$ for the reaction with $O_2^{\bullet-}$ (Wolin, 2000).

(1) $O_2 + \text{electron} \xrightarrow{\text{oxidase}} O_2^{\bullet-}$

(2) $O_2^{\bullet-} + O_2^{\bullet-} \xrightarrow{\text{SOD}} H_2O_2 + O_2$

(3) $O_2^{\bullet-} + NO \longrightarrow ONOO^-$

Vascular tissue contains a cytosolic copper-zinc form of SOD (CuZn-SOD), a mitochondrial manganese form of SOD (Mn-SOD), and an extracellular CuZn-SOD. The activities of intracellular oxidases typically seen in vascular cell should result in levels of $O_2^{\bullet-}$ in the nanomolar range in the absence of SOD, and the presence of SOD is likely to lower $O_2^{\bullet-}$ concentrations into the picomolar range. However, these low levels of $O_2^{\bullet-}$ can be a source, through reactions associated with equation 2, of concentrations of H_2O_2 in the high picomolar to low nanomolar rage that interact with signal systems. Because $O_2^{\bullet-}$ reacts with NO with a rate constant of $7x10^9$ $mol^{-s}.L.s^{-1}$, which is 3 times the rate of its reaction with SOD (Wolin, 2000). When NO level increases into the elevated nanomolar range, NO is able to compete with SOD for the scavenging of $O_2^{\bullet-}$ (Wink and Mitchell, 1998; Wolin *et al.*, 1998). These results in the production of peroxynitrite ($ONOO^-$, see equation 3) in amounts that can potentially interact with regulatory systems those are of biological significance.

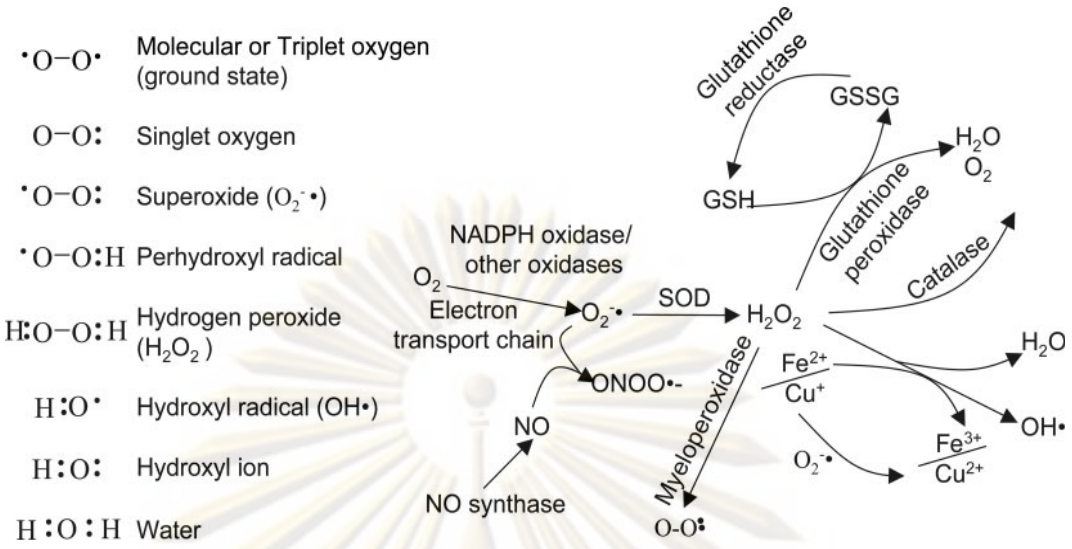


Figure 2.8. The charged states of oxygen and the formation and detoxification of oxygen radicals in cells. On the *left*, the various oxidative states of the molecule are illustrated to understand the terminology of free radicals. As molecular oxygen participates in biochemical reactions in the cell, electrons are shuttled between molecules, and highly reactive intermediates are produced and then removed through the activities of specific enzymes. These reactions are summarized in the schematic on the *right* (Vincent, 2004).

1.1 Sources of ROS in vascular cells

Although there are numerous enzymatic sources can potentially produce ROS in the vascular cell, four enzyme systems seem to predominate. These include the NADPH oxidase, xanthine oxidase, uncoupled NO synthase (NOS) and mitochondrial sources and the following section reviews each enzyme.

1.1.1 NADPH Oxidase

The NADPH oxidase enzymes, also known as the Nox enzymes, represent a major source of ROS in vascular cells. Nox1 protein levels are quite low in vascular cells. (Lassegue and Clempus, 2003). Nox2, previously known as $gp91^{phox}$, is the large catalytic subunit of the phagocyte cytochrome b558. It is expressed in endothelial and in the vascular smooth muscle cells of smaller vessels (Gorlach *et al*., 2000; Touyz *et al*., 2002). Nox4 is constitutively expressed and active in vascular smooth muscle and endothelial (Ago *et al*., 2004; Hilenski *et al*., 2004). In fact, all the Nox enzymes require $p22^{phox}$, which serves as a docking protein for other subunits and stabilizes the Nox proteins (Ambasta *et al*., 2004). Interestingly, pathophysiological stimuli can increase expression

of several of NADPH oxidase subunits, including $p22^{phox}$, Nox1 and Nox4, further promoting an increase in ROS production (Lassegue and Clempus, 2003).

1.1.2 Xanthine oxidase

The xanthine oxidoreductase is a molybdoenzyme capable of catalyzing of the oxidantion of hypoxanthine and xanthine in the process of purine metabolism. Xanthine oxidoreductase exists in 2 forms, as xanthine dehydrogenase (XDH) and as xanthine oxidase (XO) (Harrison, 2002). XDH uses NAD^+ to receive electrons from hypoxanthine and xanthine, yielding NADH and uric acid. In contrast, XO uses oxygen as an electron acceptor from these same substrates to form $O_2^{\bullet-}$ and H_2O_2. The ratio of XO to XDH in the cell is therefore critical to determine the amount of ROS produced by these enzymes. Conversion of XDH to XO is stimulated by inflammatory cytokines and also by oxidation of cysteine residues by oxidants, such as peroxynitrite (Sakuma *et al.*, 1997). There is interest in the studies that the XO present in endothelial cells originates from other organs and that the enzyme is probably taken up via heparin binding sites (Fukushima *et al*., 1995). Previous studies have shown that early stage of experimental atherosclerosis caused by diet-induced hypercholesterolemia are associated with increased $O_2^{\bullet-}$, presumably from xanthine oxidase, because $O_2^{\bullet-}$ production in this setting can be normalized by oxypurinol (Ohara *et al*., 1993).

1.1.3 Uncoupled endothelial nitric oxide synthase

eNOS is a cytochrome p450 reductase-like enzyme that catalyzes flavin-mediated electron transport from the electron donor NADPH to a prosthetic heme group. This enzyme requires tetrahydrobiopterin (BH_4), bound near this heme group, to transfer electrons to a guanidine nitrogen of L-arginine to form nitric oxide. However, in the absence of either L-arginine or BH_4, the eNOS are incapable of transferring electron to L-arginine and use oxygen as a substrate for $O_2^{\bullet-}$ formation (Vasquez-Vivar *et al*., 1998). Uncoupling of eNOS has been demonstrated in various pathophysiological conditions including diabetes. In line with these findings, the production of $O_2^{\bullet-}$ by eNOS has been demonstrated in STZ-diabetic rats (Hink *et al*., 2001). There is evidence that reduced NO production by eNOS in diabetes might be due to its substrate L-arginine (Pieper, 1998), a post translational modification of the eNOS on the Akt phosphorylation site (Pieper, 1997; Pieper, 1998). The latter results from decreased expression and activity of GTP-

cyclohydrolase I, the first and rate limiting enzyme in the *de novo* biosynthesis of BH_4, in diabetic cells (Meininger *et al*., 2000). In studies on the purified enzyme the lack of either L-arginine or BH_4 or both results in eNOS uncoupling (Vasquez-Vivar *et al*., 1998). It has been suggested that peroxynitrite oxidase BH_4, which can uncouple eNOS *in vivo* (Laursen *et al*., 2001) and thereby contributes in the endothelium to oxidative stress and endothelial dysfunction through at least 3 mechanisms. First, the enzymatic production of NO is diminished, thus the system lacks NO as an essential mediator molecule. Second, the enzyme produces $O_2^{\bullet-}$ and contributes to oxidative stress. Finally, it is likely that eNOS becomes partially uncoupled, so that both $O_2^{\bullet-}$ and NO are produced simultaneously. Under these circumstances, eNOS could become a peroxynitrite generator, leading to a dramatic increase in oxidative stress (Spitaler and Graier, 2002).

1.1.4 Mitochondrial electron transport

It is important to note that mitochondrial electron transport chain represents the predominant source of $O_2^{\bullet-}$ and consequently H_2O_2. It has been estimate that between 1-4% of oxygen reacting with the respiratory chain is incompletely reduced to $O_2^{\bullet-}$ Under hyperglycemia conditions, mitochondrial $O_2^{\bullet-}$ has been shown to activate sorbital accumulation by the aldos-reductase pathway (Williamson *et al.*, 1993) resulting in the activation of PKC (Ishii *et al*., 1998; Nishikawa *et al*., 2000) which, in turn, is responsible for endothelial dysfunction. In addition, mitochondrial $O_2^{\bullet-}$ overproduction inhibits glyceraldehydes-3-phosphate dehydrogenase (GAPDH) activity and activates the hexosamine pathway, presumably by diverting the upstream metabolite fructose-6-phosphate from glycolysis to glucosamine formation (Du *et al*., 2000). In addition to the role of mitochondria as a source of ROS, the mitochondria themselves can be damaged by oxidants. ROS mediate mitochondrial DNA damage, alterations in gene expression and mithochondrial dysfunction in cultured vascular endothelial cells and smooth muscle cells (Ballinger *et al*., 2000).

2. Link between ROS, PKC, COX-2 and NF-κB in diabetes-induced endothelial dysfunction

Among various possible mechanisms of hyperglycemia-induced vascular dysfunction, both PKC activation and oxidative stress have increasingly received attention in recent year (Hink *et al*., 2001; Kowluru, 2001; Gutterman, 2002; Cosentino *et al*., 2003; Pricci *et al*., 2003; Yakubu *et al*., 2004). There are several studies showing that the relationship between PKC and ROS in hyperglycemia-induced vascular dysfunction. Hink, *et al.* (2001) found that *in vivo* model; diabetes was induced with STZ in rats. Diabetes led to endothelial dysfunction and increased vascular $O_2^{\bullet-}$ production, as assessed by lucigenin- and coelenterazine-derived chemiluminescence. The bioavailability of vascular NO was reduced in diabetic aortas, although expression of endothelial NOS (mRNA and protein) was markedly increased. When they treated NOS inhibition with N^G-nitro-L-arginine reduced superoxide levels in diabetic vessels. These results identified NOS as a superoxide source. Similarly, they also found an activation of NADPH-oxidase and an increase in $gp91^{phox}$ mRNA in diabetic vessels. Interestingly, *in vivo* PKC inhibition with *N*-benzoyl-staurosporine prevented eNOS gene upregulation and NOS-mediated $O_2^{\bullet-}$ production. These findings point to a role of PKC in mediating the restoring vascular NO bioavailability and endothelial function. In agreement with Yakubu, *et al.* (2004), they proposed that glucose-induced attenuation of vascular relaxation involves PKC linked free radicals generation. Pretreatment with calphostin C, a PKC inhibitor, reduced high glucose- or xanthine-induced attenuation of ACh relaxation. This is consistent with Inoguchi, *et al*. (2000), hypothesized that high glucose level may stimulate ROS production through PKC-dependent activation of NADPH-oxidase in cultured both vascular endothelial cells and smooth muscle cells (Inoguchi *et al.*, 2000). These results have been shown treatment of the cells with phorbol myristic acid (PMA), a PKC activator, also increased free radical production. This increase was restored by diphenylene iodonium, a NADPH- oxidase inhibitor, suggesting ROS production through PKC-dependent activation of NADPH oxidase. Although the ability of PKC to induce ROS is well established, as discussed above, or *vice versa*, $O_2^{\bullet-}$ also activates PKC (Nishikawa *et al*., 2000). Hyperglycemia increases the production of $O_2^{\bullet-}$ production via the mitochondrial electron transport chain. $O_2^{\bullet-}$ then induces diacylglycerol and activates PKC inside cultured bovine aortic endothelial cells (Nishikawa *et al*., 2000; Nishikawa *et al*., 2000).

Although, numerous studies have shown that high glucose increases eNOS gene and protein expression (Cosentino *et al*., 1997). Because eNOS up-regulation is associated with increased $O_2^{\bullet-}$ production, suggesting that NO is inactivated by $O_2^{\bullet-}$. The reaction of NO with $O_2^{\bullet-}$ produced a potent oxidant peroxynitrie, which is 3 times the rate of its reaction with SOD. NO is able to compete with SOD for the scavenging of $O_2^{\bullet-}$. Peroxynitrite, resulting from the interaction of NO and $O_2^{\bullet-}$, oxidizes the NOS co-factor BH_4 (Laursen *et al*., 2001). This uncoupled NOS, which then preferentially increases $O_2^{\bullet-}$ production over NO production (Milstien and Katusic, 1999).

In diabetes, endothelial dysfunction is characterized not only by decreased NO but also by increased synthesis of vasoconstrictor prostanoids and endothelin (Tesfamariam, 1994; De Vriese *et al*., 2000). However, the precise molecular mechanisms by which high glucose increase $O_2^{\bullet-}$ production and vasoconstrictor prostanoids remain unknown. One possibility may involve activation of PKC, because hyperglycemia increases diacylglycerol, a strong activator of PKC (Inoguchi *et al*., 1992). In normal blood vessels, activation of PKC by phorbol ester impairs endothelium-dependent relaxation via release of $O_2^{\bullet-}$ and vasoconstrictor prostanoids (Tesfamariam *et al*., 1991). Prostanoids are generated by cyclooxygenase enzymes (COX) from arachidonic acid. Two isoforms of the enzymes which are encoded by distinct genes (Williams and DuBois, 1996). COX-1 is constitutively expressed in most tissues and is involved in maintenance of cellular homeostasis, including regulatation of vascular tone. In contrast, under normal conditions, COX-2 is expressed at low or undetectable levels but is readily up-regulated by inflammatory, mitogenic and physical stimuli (Williams and DuBois, 1996). However, the mechanisms of COX expressions regulation in endothelial cells exposed to high glucose are not completely understood. Early studies have shown *in vitro* incubation of rabbit arteries with high glucose concentration increases vasoconstrictor prostanoids. These effects were prevented by both cyclo-oxygenase inhibitors and prostaglandin H_2/thromoxane A_2 receptor antagonist, thereby restoring endothelium-dependent relaxation (Tesfamariam *et al*., 1990). Recent biochemical studies have proposed a possible role for enhanced COX-2 expression in high glucose-induced alterations in vasoconstrictor prostanoid production in cultured endothelial cells (Cosentino *et al*., 2003). Also, it has been demonstrated that upregulation of COX isoforms is associated with a significant elevation of vascular prostaglandin synthesis (Quilley and Chen, 2003). However, there

are only a limit number of studies investigating consequences of alteration microvascular prostanoid synthesis (Davidge, 2001).

Recently, the potential importance of the nuclear factor κB (NF- κB) system as a key role in the regulation of transcription of genes encoding mediators of inflammatory responses, including diabetes have received attention in the development of diabetic vascular complications (Barnes and Karin, 1997). Many cytokines, lysophosphatidic acid, and other agents activate NF-κB by stimulating the phosphorylation and degradation of I-κB, allowing subsequent translocation of the released NF dimer to the nucleus. One of the most potent stimuli for NF-κB activation is oxidative stress (Schreck *et al*., 1992). The oxidative stress seen in diabetes and hyperglycemia appears to cause increased expression of various genes which are NF-κB dependent (Morigi *et al*., 1998). Also, it has been demonstrated that oxidative stress due to hyperglycemia is responsible for the activation of NF-κB (Sharpe *et al.*, 1998).

The aforementioned prompted us to investigate the effects of oxidative stress along with hyperglycemia may interact with NF-κB activation, PKC activation and COX-2 expression. In addition, the specific role vasoconstrictor prostaglandin synthesis of alter endothelial function in STZ-induced diabetic rat.

2.1 Protein kinase C

Adding and removing phosphate groups is an important physiological mechanism for regulating intracellular proteins, including enzymes, receptors and second messengers. Thus, a variety of receptor-mediated response and metabolic pathways are activated and deactivated by intracellular kinases (enzymes that add phosphate groups) and phosphatases (enzymes that remove phosphate groups). Cellular kinases are broadly divided into two types: those that phosphorylate proteins at tyrosine residues (tyrosine kinase) and those that phosphorylate serine and threonine sites (serine/threonine kinases). There are three major serine/threonine kinases widely distributed in all tissues:

1. Cyclic AMP-dependent protein kinase (protein kinase A, PKA)
2. Akt also known as protein kinase B (PKB)
3. The calcium-phospholipid activated kinase (protein kinase C, PKC)

PKC is a multifunctional, cyclic nucleotide-dependent protein kinase that phosphorylates serine and threonine residues in many target proteins. This enzyme was first identified by Nishizuka and co-workers (Inoue *et al*., 1977; Takai *et al*., 1977). Further studies have indicated that members of the PKC family differ in their structure, cofactor requirement and substrate specificity, which may account for their functional differences. Furthermore, PKC isoforms display distinct cell and tissue- type expression patterns and supposedly differential activation according to their intracellular localization (Nishizuka, 1988; Newton, 1997).

2.1.1 PKC structure

The primary structure of a PKC molecule consists of a single polypeptide chain and can be divided into four conserved domains (C1-C4) separated by five variable regions (V1-V5) (Figure 2.9). The N-terminal of polypeptide (C1, C2, V1, V2 and part of V3) constitutes the regulatory domain, while the C-terminus (C3, C4, V4 and V5) forms the catalytic domain (Hug and Sarre, 1993). The V3 region act as a link between the regulatory and catalytic domains and also provides a site for proteolytic cleavage by calpains and trypsin, which results in constitutive and activator-dependent kinase activity (Mochly-Rosen *et al*., 1990). The C domains are conserved sequences throughout the PKC family. C1 features the diacylglycerol (DAG) and phosphatidylserine biding sites. At the N-terminus of the C1 domain is located a pseudosubstrate site that regulates PKC activity (Soderling, 1990). C2 is the Ca^{2+}-binding domain present only in cPKC's. The C-terminus of all PKCs features the highly conserved catalytic domain (the C3 and C4 regions), separated by a short variable (V4) insert present only PKCγ. The C3 region, which is essential for enzyme activity, represents the conserved ATP-binding site sequence found in all isoforms in which an invariant lysine residue (lys-380) is essential for kinase activity (Freisewinkel *et al*., 1991). The C4 domain represents the catalytic core of PKC's. In addition to C1 region contains a pseudosubstrate sequence, C1 is an autoinhibitory domain (Soderling, 1990) that binds to the substrate binding site on the C4 region in the catalytic domain to keep the enzyme in an inactive state in the absence of cofactors and activator (Orr *et al*., 1992). Moreover, all PKC family members possess a phosphatidylserine binding domain for membrane interaction.

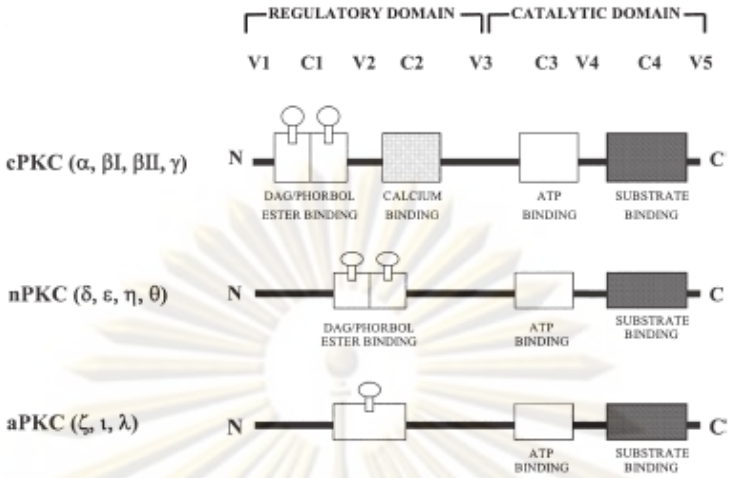


Figure 2.9. Structure of PKC isoforms according to subgroups. The regulatory and catalytic domains are displayed. Four conserved (C1 to C4) and five variable (V1 to V5) regions are indicated (Meier and King, 2000).

The PKC family can be divided into three groups that differ in their cofactor requirements.

1. **The conventional or classical PKC isoforms** (cPKC-α, β1, β2 and γ) are calcium-dependent and contain two cysteine-rich, zinc finger-like motifs (C1 region), which are binding sites of DAG and phorbol ester. The C2 region is rich in acidic residues and binds Ca^{2+}. The C3 and C4 regions form the ATP- and substrate-binding sites. Each PKC isoform is a separate gene product with the exception of PKC-βI and -βII, which are alternatively spliced variants of the same gene product.
2. **The novel PKC isoforms** (nPKC-δ, ε, η, and θ) are DAG/phorbol ester-sensitive isoforms. They lack the C2 region and therefore do not require Ca^{2+} for activation. However, the nPKCs include a C2-like domain close to the N-terminal end which is unable to bind Ca^{2+} (Sossin and Schwartz, 1993).
3. **The atypical PKC isoforms** (aPKC ζ and ι (its mouse homolog has been named PKC- λ)) have only one cysteine-rich motif in their C1 region, but can be activated by phosphatidylserine. aPKC are not affected by DAG, phorbol ester or Ca^{2+} (Nakanishi *et al*., 1993).

In addition, μPKC (also called PKD), displays multiple unique feature that makes it a distant relative in the PKC family (Johannes *et al*., 1994).

2.1.2 Mechanisms of PKC translocation

PKC bind to diacylglycerol or Ca^{2+} cause conformational change in the PKC molecule that result in remove the pseudosubstrate sequence from the C4 site on the catalytic domain. These effects increase the hydrophobicity of PKC, and facilitate their binding to membrane lipid (Newton, 1995). Classically, the activation of calcium-dependent PKC isoforms involves receptor-mediated activation of phospholipase C (PLC), resulting in generation of DAG and inositol (1,4,5) triphosphate (IP_3) from membrane-associated phosphotidyl inositol (4,5)-bisphosphate (PIP_2). Subsequently, IP_3 stimulates the release of intracellular calcium, which then binds to the C2 region of the PKC enzyme and promotes its translocation from the cytosol to the plasma membrane (Nishizuka, 1992). However, cofactor regulation within each PKC isozymes is similar, suggestin that additional mechanisms must be active in the differential modulation of the multiplicity of isoform function (Meier and King, 2000).

Hyperglycemia induces de novo synthesis of DAG-PKC pathway

Numerous *in vivo* and *in vitro* studies have suggested that increased DAG levels in vascular tissues are related to PKC activation in diabetes mellitus (Inoguchi *et al.*, 1992; Shiba *et al*., 1993; Ishii *et al.*, 1996). Hyperglycemia leads to elevate DAG levels was established using cultured vascular cells where elevating glucose levels from 5.5 mM to 22 mM increased DAG levels to a maximum in aortic endothelial and smooth muscle cells (Inoguchi *et al*., 1992).

Intracellular release of DAG is the primary step leading to activation and translocation of PKC. The increased levels of DAG can be generated from multiple pathways as described below (Figure 2.10).

1. The classical receptor-mediated, phospholiase C-catalysed hydrolysis of inositol phospholipids.
2. The release of DAG from phospholiase D-mediated hydrolysis of phosphatidylcholine (PC).
3. The release of non-esterified fatty acids from precursor lipids by the action of phospholipase A_2 (PLA_2).
4. *De novo* synthesis of DAG from phosphatidic acid (PA).

This latter pathway is mainly responsible for hyperglycemia-induced DAG formation (Idris *et al.*, 2001). In metabolic labeling studies (Craven *et al.*, 1990; Inoguchi *et al.*, 1992; Xia *et al.*, 1994), they have reported that the incorporation of glucose into the glycerol backbone of DAG was increased in the high glucose condition. These findings suggest that increased DAG contents were partly derived from a *de novo* synthesis of DAG from glycerol 3-phosphate (Figure 2.10).

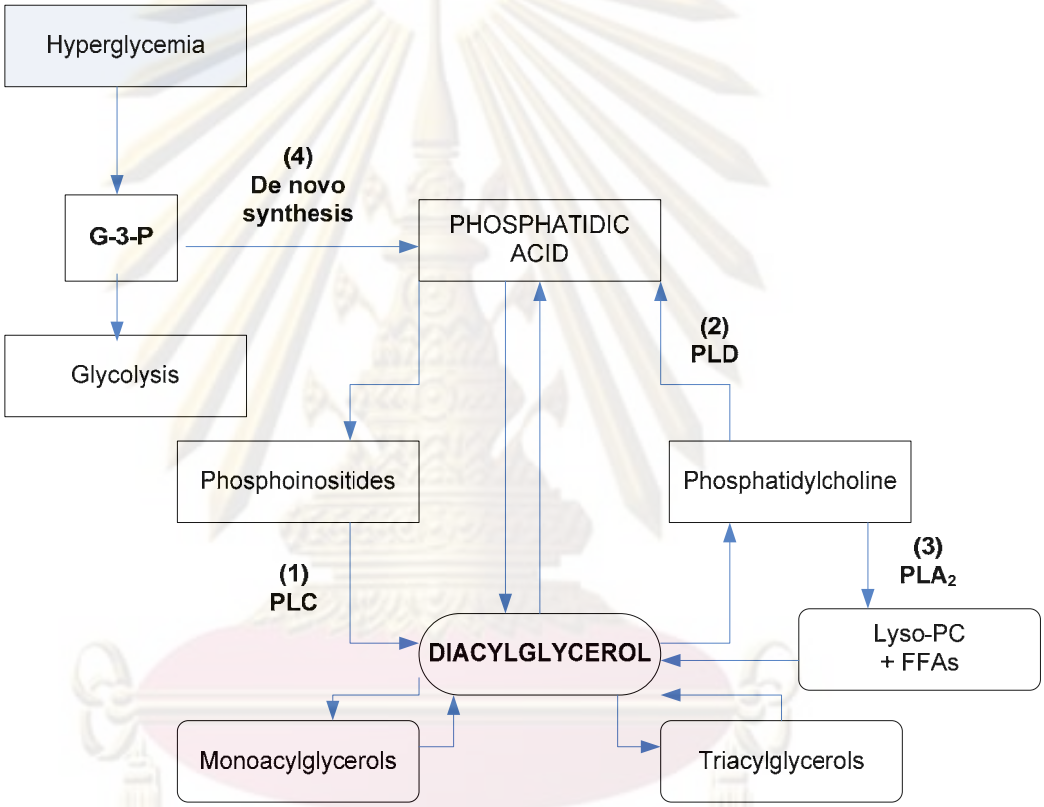


Figure 2.10. DAG are derived from multiple sources, including (1) phospholipase C (PLC) mediated hydrolysis of phosphoinositides, (2) phospholipase-D (PLD) mediated hydrolysis of phosphatidylcholine (PC) by phosphatidic acid, (3) release of free fatty acids from precursor lipids through phospholiapase A_2 (PLA_2) mediated, and (4) *de novo* synthesis of DAG from a glycolytic intermediate, glyceraldehydes-3-phosphate (G-3-P), under conditions of hyperglycemia (Adapted from Meier, 2000 and Idris, 2001).

Effect of diabetes mellitus on activating specific PKC isoforms

Among the various PKC isoforms in vascular cell, PKC-β and δ isoforms appear to be preferentially activated in the aorta and heart of diabetic rats (Inoguchi *et al*., 1992) and in cultured rat aortic smooth muscle cells (Kunisaki *et al*., 1994) exposed to high glucose level. This activation has been determined by immunoblot analysis of the membrane fraction which represents the active pool of PKC. In the retina of diabetic rats, PKC-α, β1, β2 and ε isoforms were reported to be activated (Shiba *et al*., 1993). However, in all the vascular tissues, PKC-β isoforms exhibit a greater increase in the membrane than the other isoforms (Meier and King, 2000). Possible mechanisms for the predominant isoform-specific activation of PKC-β in diabetes mellitus include cofactor activation (Nishizuka, 1995), regulation by phosphorylation (Keranen *et al.*, 1995; Konishi *et al*., 1997; Feng and Hannun, 1998), and target proteins (Ron and Kazanietz, 1999).

Hyperglycemia-induces PKC activation: development of diabetic vascular complications

It has been reported that PKC up-regulation on exposure to high glucose concentrations impairs NO-mediated vasodilatation in diabetes by inhibiting eNOS expression in cultured retinal microvascular endothelial cells (Chakravarthy *et al*., 1998). Furthermore, calcium flux and PKC activation induced by high glucose levels may inhibit the expression on iNOS in vascular smooth muscle cells (Muniyappa *et al.*, 1998). In large vessels of diabetic patients, the relaxation phase in peripheral arteries after acetylcholine stimulation appears to be delayed (McVeigh *et al*., 1992). The impaired vascular relaxation can be restored by PKC inhibitors and mimicked by phorbol ester (Tesfamariam *et al*., 1991), suggesting that PKC activation has an important role in causing abnormal peripheral hemodynamic in diabetes. In addition, PKC can also regulate vascular contractility in diabetes mellitus through altered expression of contractile proteins like caldesmon (Aiello *et al*., 1994), resulting in increased contraction and decrease vascular blood flow.

PKC inhibitors and their effects in vascular disease

According to their site of interaction with in the PKC protein structure, regulatory domain inhibitors may target the phospholipid or phorbol ester binding site, where as catalytic domain inhibitors are directed to either the substrate site or ATP-binding site. The majority of the compounds as selective PKC inhibitors are targeted to the ATP-binding site of the catalytic domain. They belong either to the indolocarbazole or bisindoylmaleinmide class of compounds. The bisindoylmaleinmide, Ruboxistaurin mesylate (LY333531), which specificially inhibit PKC-β, is the most selective and clinically promising agent developed to date (Ishii *et al.*, 1996; Jirousek *et al.*, 1996; Aiello *et al.*, 1997; Koya *et al.*, 1997). The specificity of PKC-β inhibitor LY333531 was evaluated by an *in vitro* study examining the PKC isoform-induced phosphorylation of myelin basic protein (Jirousek *et al.*, 1996). LY333531 inhibited PKC-β1 and –β2 with a half-maximal inhibitory constant (IC_{50}) of 4.5 nM and 5.9 nM, respectively, whereas the IC_{50} was 250 nM or greater for other isoforms (Jirousek *et al.*, 1996). The kinetic analysis showed that LY 333531 was a competitive inhibitor for ATP, with a K_i of 2 nM for PKC-β2 (Jirousek *et al.*, 1996). In addition, the abnormal retinal and renal hemodynamics and the increases in albuminuria in STZ-induced diabetic rats can be ameliorated using an orally administatered dosage (0.1-10 mg/kg) of LY333531 (Ishii *et al.*, 1996). Furthermore, its specificity was confirmed while demonstrating that the PKC-β-specific inhibitor LY333531 prevented the increase in phosphorylation of PKC-β1 but not the α-isoform in isolated glomeruli from control and STZ-induced diabetic rat (Kunisaki *et al.*, 1994). Recently, Koya *et al* provided the first *in vivo* evidence that the long-term inhibition of PKC activation by this PKC-β inhibitor can ameliorate glomerular pathologies in diabetic *db/db* mice (Koya *et al.*, 2000). LY333531 was also reported to inhibit PKC-β activity that shown to be beneficial in cardiac dysfunction. Administration of LY33531 improved depressed cardiomyocyte function by increasing myofilament responsiveness to calcium in hearts obtain from transgenic mice over-expressing PKC-β (Takeishi *et al.*, 1998). In addition, LY333531 significantly decreased elevated PKC activity in membrane fractions of heart (Bowling *et al.*, 1999). Since a few reports have shown that glucose-dependent PKC activation and translocation can be observed in patients with type2-diabetes mellitus (Ceolotto *et al.*, 1999). These findings provide strong evidence that the activation of PKC isoforms is involved in the development of diabetic vascular complications. Clinical trials are now ongoing to

evaluate the efficacy of LY333531 on pathological changes in cardiovascular and diabetic vascular disease.

2.2 Cyclooxygenase and prostaglandins in diabetes melliutus

The potential role of cyclooxygenase (COX) enzymes, particularly the COX-2 isoform, in several chronic inflammatory conditions seems to be well established. It has been demonstrated that a low-level vascular inflammation implicated in the development of diabetes vascular complications (Fernandez-Real and Ricart, 2003; Helmersson *et al*., 2004; Sjoholm and Nystrom, 2005; Wellen and Hotamisligil, 2005). In several early studies, it was found that diabetes mellitus alters the vascular synthesis of prostanoids (Harrison *et al*., 1978; Sterin-Borda *et al*., 1984; Myers *et al*., 1985). In mesenteric arteries of pancreatomized, diabetic dogs, exogenous arachidonic acid elicited thromboxane A_2 (TXA_2)-mediated constrictions, but evoked prostacyclin (PGI_2)-mediated dilatations in those of controls (Sterin-Borda *et al*., 1984). In the aorta of STZ-induced diabetic rats a reduction in PGI_2 synthesis has been regconized (Harrison *et al*., 1978). In contrast, significant elevation of the tissue levels of prostaglandin E_2 (PGE_2) and PGI_2 were reported in skeletal muscle microvessels of these rats (Myers *et al*., 1985). By now it is clear, that prostaglandins are primarily generated from arachidonic acid by COX-1 and COX-2 enzymes. COX-1 is constitutively expressed in most tissues, such as vascular endothelial cells, and is involved in maintenance of cellular homeostasis. Whereas the expression of COX-2 is very low in the endothelium and in smooth muscle cells under normal conditions (Davidge, 2001). Importantly, COX-2 can be readily up-regulated by inflammatory, mitogenic and physical stimuli (Parente and Perretti, 2003). However, there are only a few papers in the literature investigating the vascular expression of COX-2 and its functional consequences in diabetes mellitus, moreover these yielded controversial findings. In recent studies (Bagi *et al*., 2005; Guo *et al*., 2005) provided evidence that COX-2 protein expression is elevated in the aorta of *db/db* mice. In these diabetic mice, using large vessel ring preparations it has been found that up-regulation of COX-2 enhanced the synthesis of constrictor prostaglandins, TXA_2/PGH_2 during the agonist induced aortic contractions (Guo *et al*., 2005). In skeletal muscle resistance arteries increases in COX-2 activation resulted in an enhanced myogenic tone, primarily by COX-2 derived prostanoids, TXA_2/PGH_2 (Bagi *et al*., 2005). These findings concluded that diabetes was associated with the up-regulation of COX-2, both in large vessels and in

microvessels. In line with the findings on experimental animals, the recent studies have demonstrated in vascular expression of COX-2 is markedly elevated in coronary arterioles diabetic patients (Szerafin *et al*., 2006). Unexpectedly, in human coronary arterioles of diabetic patients COX-2 up-regulation was associated with the enhanced production of dilator prostaglandins, most likely PGI_2 or PGE_2 production, rather than constrictor prostanoid release (Szerafin *et al*., 2006). These findings underline the need for the investigations concerning the possible species and vascular bed specific differences in prostaglandin-mediation associated with COX-2 up-regulation. In this study, we would address the downstream enzymes, such as PGI_2 and TXA_2, responsible for the production of prostanoids in a vascular bed specific manner in diabetic animal.

Prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) play an essential role in the regulation of vascular tone. The production of these eicosanoids is regulated by the availability of arachidonic acid and the activity of prostaglandin H synthase (PGHS), also known as prostaglandin endoperoxide synthase or commonly called cyclooxygenase. PGHS is a bifunctional enzyme, which means it carries out two separate reactions. The first reaction incorporates two molecules of oxygen into arachidonate to a hydroperoxide called prostaglandin G_2. This activity is often called a cyclooxygenase (COX) activity. The second reaction is catalyzed 2-electron reduction of PGG_2 at a different site, the hydroperoxidase site, and the final product is prostaglandin H_2 (Figure 2.11).

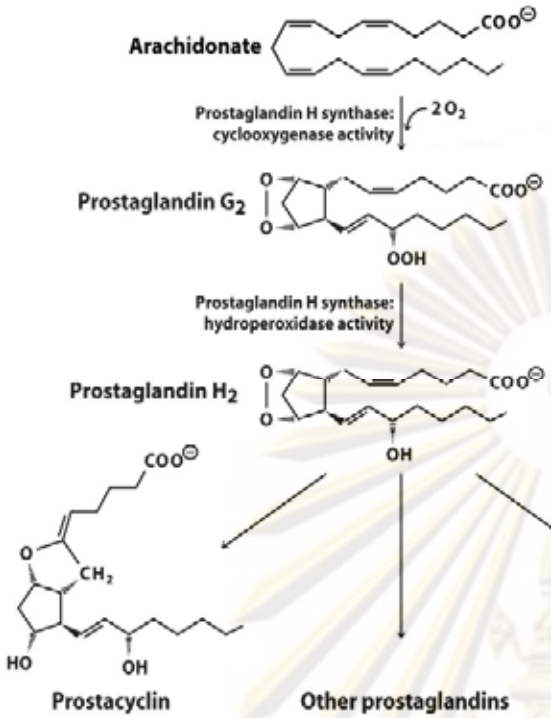


Figure 2.11. Prostaglandin H synthase (PGHS) pathway. Arachidonate is converted by PGH_2 by a cyclooxygenase activity and peroxidase activity. In the vasculature, PGI_2 is a poten vasorelaxant. In some conditions and vascular beds, PGHS-dependent vasoconstrictors predominate. It is important to note that TXA_2 is a vasoconstrictor (Pearson Prentice Hall, Inc., 2006).

PGI_2 is a vasodilator and an inhibitor of platelet aggregation, whereas TXA_2 is a vasoconstrictor and a promoter of platelet aggregation. As a consequence of their opposing roles, an imbalance in PGI_2 or TXA_2 production has been implicated in the pathophysiology of cardiovascular diseases. PGI_2 and TXA_2 are products of arachidonic acid metabolism by cyclooxygenase, followed by metabolism of the COX product, PGH_2, by the terminal synthase enzymes, prostacyclin or thromboxane synthase, respectively (Figure 2.12). Several different prostanoid terminal synthases can be present within the one cell. In monocytic cells, it has been observed that the ratio of PGE_2/ TXA_2 produced is not fixed, but varies according to which COX isoform is present (Matsumoto *et al*., 1997). It is considered that PGI_2 is the main prostanoid synthesized by vascular endothelium and TXA_2 is the main prostanoids produced by platelets. However, the endothelium has been reported to synthesize TXA_2 in addition to PGI_2 (Bustos *et al*., 1997). Endothelial COX-2 can be up-regulated in vitro by inflammatory stimuli (Bustos *et al*., 1997; Camacho *et al.*, 1998) and shear stress (Okahara *et al.*, 1998). The balance between PGI_2 and TXA_2 production is central in the maintenance of vascular tone and platelet aggregation. The determination of the roles of endothelial COX isozymes, particularly with regard to the contribution of COX-2 in the regulation of prostanoid biosynthesis by the endothelium, is important.

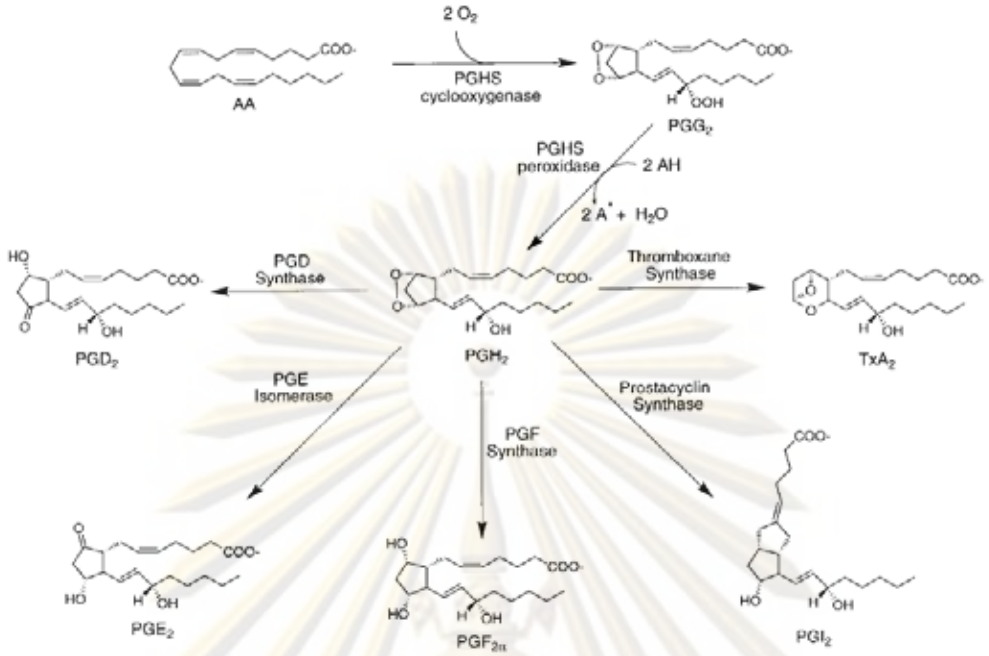


Figure 2.12. Synthesis of PGH_2 from arachidonate by PGHS and further processing of PGH_2 to prostanoids (Goodwin, 1999).

Both the endothelium and smooth muscle cell contain COXs, however, endothelial cells contain up to 20 times more COX than smooth muscle cells (DeWitt *et al.*, 1983). Interestingly, in bovine aortic endothelial cells, prostacyclin synthase and COX-1 were localized to the nuclear envelope and endoplasmic reticulum. However, there was a lack of colocalization of the COX-2 with prostacyclin synthase (Liou *et al.*, 2000). Overall in vascular pathologies, there may be an imbalance where COX-dependent vasoconstrictions become more predominant. Moreover, in a number of vascular complications there are common risk factors, such as oxidative stress, which are known modulators for COX-dependent function that lead to impairment of vascular function. For instance, an *in vivo* oxidative stress rat model demonstrated an increased COX-dependent vasoconstrictor that modified endothelium-dependent vascular response via PGH_2/TXA_2 receptor (Davidge *et al.*, 1993).

2.3 Nuclear factor -κB in diabetes melliutus

Another factor that contributes to COX-2 induction tin vascular cells is the nuclear factor-κB (NF-κB). NF-κB is necessary for transcription of COX-2 (Davidge, 2001). It is known that NF-κB is involved in the regulation of genes that control the immune and stress response, inflammation and appotosis (Barnes and Karin, 1997). The transcription factor NF-κB was first identified in mature B lymphocytes, where it interacted with the B site of the κ light chain gene enhancer (Baeuerle and Baltimore, 1996). It was soon found to be present in other cells as well, including vascular smooth muscle cells (Yerneni *et al*., 1999). NF-κB is a heterodimer composed of a 50-kDa and 65-kDa subunit. When inactive, NF-κB is sequestered in the cytoplasm with the inhibitor I-κB. I-κB compose of three I-κB protein; I-κBα, I-κBβ and I-κBε (Baeuerle and Baltimore, 1996). Many cytokines, lysophosphatidic acid and other agents activate NF-κB by stimulating the phosphorylation and degradation of I-κB (Baeuerle and Baltimore, 1996). The I-κBs are rapidly phosphorylated at two specific serine residues located at their N-terminal region (Ser-32 and Ser-36 for I-κBα, Ser-19 and Ser-23 for I-κBβ, Ser-157 and Ser-161 for -κBε) and then undergo ubiquitination and proteolysis by the 26S proteasome, resulting in release and translocation of NF-κB to the nucleus. In the nucleus, NF-κB activates transcription of specific target genes (Figure 2.13). Recently, the protein kinase of molecular mass 85,000 and 87,000 are identified and called I-κB kinase (IKKα and IKKβ, respectively) (Zandi *et al*., 1997). Both IKKα and IKKβ are rapidly activated by cytokines, with kinetics matching those of I-κBα phosphorylation and degradation. Interestingly, IKK activity depends on its phosphorylation, as it is inactivated by protein phosphatase 2A (DiDonato *et al*., 1997).

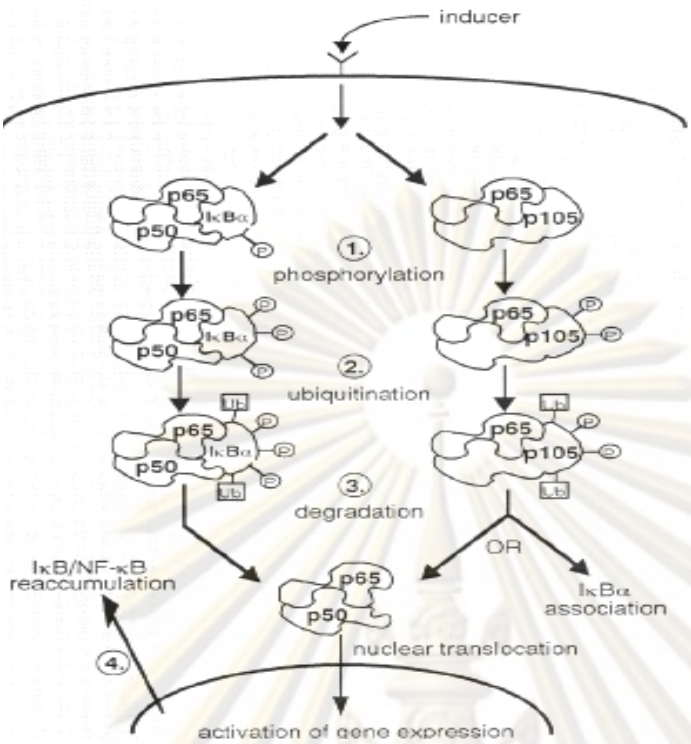


Figure 2.13. Generalized NF-κB activation. Following exposure of a cell to inducer, IκB (either IκBα or IκBβ) become phosphorylated (step1). The IκB form then becomes ubiquitinated (step2) and degraded by the proteasome (step3). NF-κB then translocates to the nucleus where it activates a variety of genes (step4) including IκBα (Baldwin, 1996).

Furthermore, NF-κB is known as one intracellular target of hyperglycemia and oxidative stress (Mercurio and Manning, 1999; Tak and Firestein, 2001). NF-κB can be activated by exogenous and endogenous stimuli including hyperglycemia (Barnes and Karin, 1997). The effect of hyperglycemia on NF-κB activity in porcine VSMC was examined and it was shown that treatment with high glucose (25mM) enhanced the DNA-binding activity of NF-κB by about 50%, compared to cells treated with normal glucose (5.5 mM) (Yerneni *et al*., 1999). In line with a potential role of PKC in hyperglycemia-induced responses, previous studies have demonstrated that treatment with calphostin C, a PKC inhibitor, completely attenuate NF-κB activation by high glucose (Yerneni *et al*., 1999). It is possible that NF-κB contributes to abnormal vascular function in diabetes (Srivastava, 2002). In addition, increasing evidence implicate NF-κB activation by hyperglycemia, oxidized LDL and oxidant stress in patients with diabetes could contribute to early development of atherosclerosis and its rapid progression (Collins, 1993; Bourcier *et al*., 1997).

Taken together, the effects of ROS, PKC, COX, and NF-κB from hyperglycemia are important contributors to endothelial dysfunction. An understanding of the intervention at the level of endothelial cells may improve the treatment of diabetic vascular complications.

Curcumin

In recent years, many herbs have made a cross-cultural penetration and in turn, have generated scientific curiosity about their biological effects and modes of action. One such wonder is the “turmeric” spice made from the plant *Curcuma longa* L. It is a tropical plant native to southern and southeastern tropical Asia. Turmeric measures up to 1 meter high with a short stem and tufted leaves (Figure 18A). Turmeric was described as *C. longa* by Linnaeus and its taxonomic position is as follows (Chattopadhyay *et al*., 2004):

Class	Liliopsida
Subclass	Commelinids
Orders	Zingiberales
Family	Zingiberaceae
Genus	*Curcuma*
Species	*Curcuma longa*

Chemical composition of turmeric

Turmeric contains protein (6.3%), fat (5.1%), minerals (3.5%), carbohydrates (69.4%) and moisture (13.1%). The essential oil (5.8%) obtained by steam distillation of rhizomes has α–phellandrene (1%), sabinene (0.6%), cineol (1%), borneol (0.5%), zingiberene (25%) sesquiterpines (53%) (Chattopadhyay *et al*., 2004). The parts used are the rhizome. Perhaps the most active component in turmeric is curcumin, it comprises 2–8% of most turmeric preparations (Sharma *et al.*, 2007).

Biological activity of turmeric and its compounds

Tumeric, *Curcuma longa* L rhizomes, has been widely used for centuries in indigenous medicine for treatment of a variety of inflammatory conditions and other diseases (Ammon and Wahl, 1991). Its medicinal properties have been attributed mainly to the curcuminoids which are responsible for the yellow color of turmeric. Curcuminoids includes 77% curcumin, 17% demethoxycurcumin and 3% bisdemethoxycurcumin. Curcumin was first isolated from turmeric in 1815 by Vogel and Pellatier and the structure was shown to be diferuloylmethane (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-hepadiene-3,5-dione) (Figure 2.14. B) in 1910 by Lampe (Chattopadhyay *et al*., 2004) .

Curcumin is hydrophobic in nature and it is soluble in ethanol, acetone, dimethylsulfoxide and oils. It has an absorption maximum around 420 nm and a melting point at 176-177°C. When it expose to acidic conditions, the color of curcumin turns from yellow to deep red (Aggarwal *et al.*, 2007).

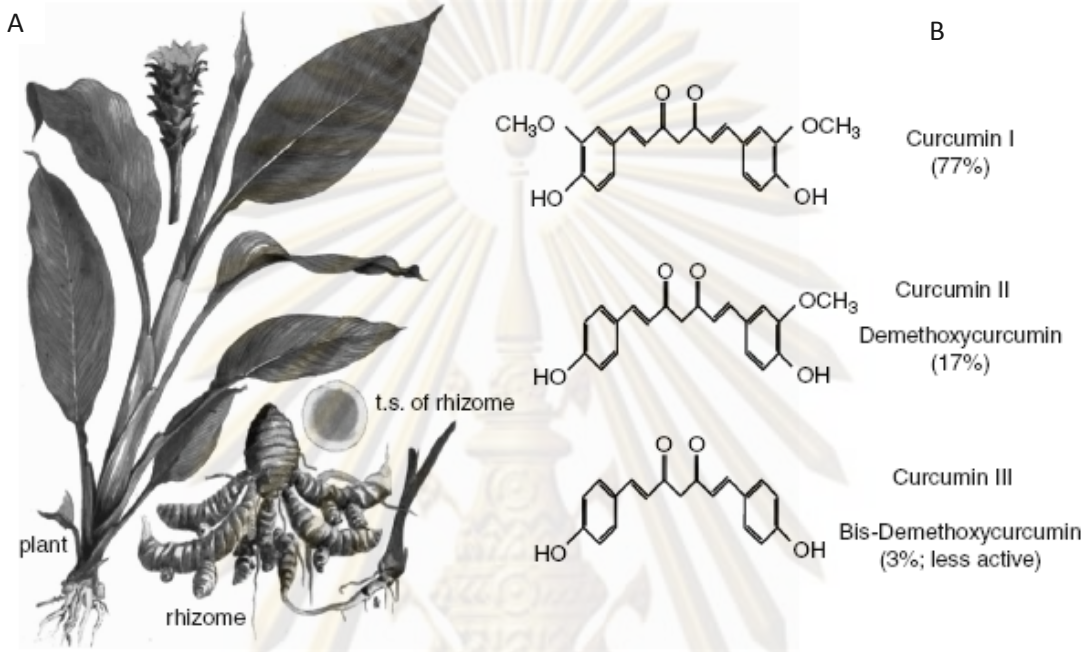


Figure 2.14. The plant Curcuma longa (panel A), from which curcumin is derived, and its structure (panel B) (Aggarwal, 2007).

Pharmacokienetics and metabolism of curcumin

The absorption, distribution, metabolism, and excretion of curcumin in rodents have been described in several studies over the past three decades. Collectively, these studies support that curcumin undergoes a rapid and efficient metabolism that decreased the availability of parent compound. In an early study in rats, a dietary dose (1 g/kg b.w) administered to rats resulted in detection in feces, whereas negligible amounts appeared in the urine (Wahlstrom and Blennow, 1978). In another early study, the pharmacokinetic properties of curcumin have been investigated in mice (Pan *et al.*, 1999). After intraperitoneal adminstration of curcumin (0.1 g/kg b.w) to mice, about 2.25 g/mL of curcumin appeared in the plasma in the first 15 min. One hour after administration, the levels of curcumin in the intestine, spleen, liver, and kidneys were 177, 26, 27, and 7.5 g/g tissue, respectively. Only traces (0.41 g/g tissue) were observed in the brain at 1 hour. To clarify the nature of the metabolites of curcumin, the plasma was analyzed by reversed-

phase high performance liquid chromatography (HPLC) and two conjugates were observed. Further treatment of the plasma with β-glucuronidase resulted in a decrease in the levels of these two conjugates and the concomitant appearance of the tetrahydrocurcumin and curcumin, respectively (Figure 2.15). To investigate the nature of these glucuronide conjugates *in vivo*, the plasma was analyzed by electrospray. The chemical structures of these metabolites were determined by mass spectrometry–mass spectrometry (MS/MS) analysis (Pan *et al*., 1999). The experimental results showed that curcumin was first bio-transformed to dihydrocurcumin and tetrahydrocurcumin and these compounds subsequently were converted to monoglucuronide conjugates.

These results suggest that curcumin–glucuronide, dihydrocurcumin–glucuronide, tetrahydrocurcumin-glucuronide, and tetrahydrocurcumin are major metabolites of curcumin in mice. The bioavailability of parent curcumin is low (Cheng *et al*., 2001), so its pharmacological activity can be mediated, in part, by curcumin metabolites. Interestingly, the gastrointestinal tract seems to be exposed more prominently to unmetabolized curcumin than any other tissue, the results support the clinical evaluation of curcumin as a colorectal cancer chemopreventive agent (Cheng *et al*., 2001).

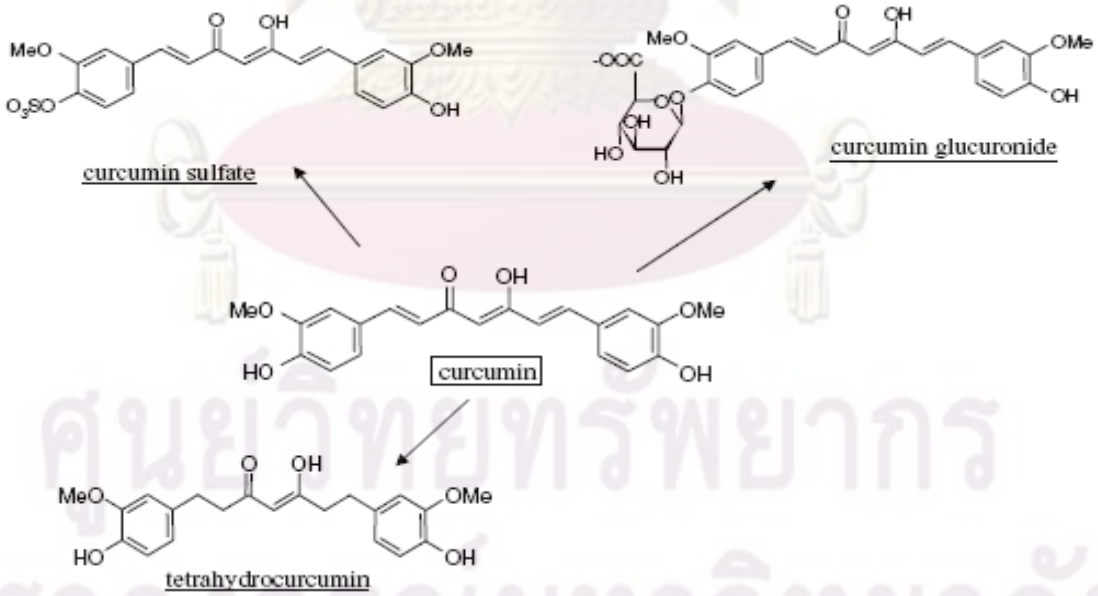


Figure 2.15. Major metabolites of curcumin detected inrodents and humans (Sharma, 2007).

Curcumin and diabetes

Base on oxidative stress as a consequence of hyperglycemia and impairs cellular function and alters vascular function. Curcumin has been shown to possess wide range of pharmacological activities including antioxidant and anti-inflammatory effects. Curcumin exhibits strong antioxidant activity, comparable to vitamin C and E (Toda *et al*., 1985; Ak and Gulcin, 2008). The antioxidant mechanism of curcumin is attributed to its unique conjugated structure, which includes two methoxylated phenols and an enol form of β-diketone; the structure shows typical radical-trapping ability as a chain-breaking antioxidant. Generally, the nonenzymatic antioxidant process of the phenolic material is thought to be mediated through the following two stages (Chattopadhyay *et al.*, 2004):

$$S\text{-}OO^{\bullet} + AH \longrightarrow SOOH + A^{\bullet}$$

$$A^{\bullet} + X^{\bullet} \longrightarrow \text{Non-radical materials,}$$

Where S is the substance oxidized, AH is the phenolic antioxidant, $A^{\bullet}$ is the antioxidant radical and $X^{\bullet}$ is another radical species or the same species as $A^{\bullet}$ and $X^{\bullet}$ dimerize to form the non-radical product.

In another study, it has shown the antioxidant activity of curcumin by radiation-induced lipid peroxidation in rat microsomes (Priyadarsini *et al.*, 2003). These results suggested that although the energetic to remove hydrogen from both phenolic OH and the CH group of the beta-diketo structure were very close, the phenolic OH was essential for both antioxidant activity and free radicals kinetics.

Numerous studies have shown that curcumin acts by enhancing the function of antioxidant. Antioxidant activities of curcumin might occur synergistically with glucose-lowering activity (Mahesh *et al*., 2004; Patumraj *et al*., 2006; Sharma *et al*., 2006). The antidiabetic action of curcumin seems to be mediated through 1) stimulation of the pancreas to produce and secrete insulin, 2) interference with dietary glucose absorption and 3) antioxidant and anti-inflammatory properties of curcumin (Menon and Sudheer, 2007). In addition, curcumin also decreases AGE-induced complications (Sajithlal et al., 1998) and prevent galactose-induced cataract formation in diabetic rats (Suryanarayana *et al*., 2003) .

However,curcumin was given to Wistar rats of both sexes at a dose of 300 mg/kg no pathological, behavioral abnormalities or lethality was observed (Shankar et al., 1980). Human clinical trials also indicate that curcumin has no toxicity when administered at dose of 1-8 g/day (Chainani-Wu, 2003) and 10 g/day (Aggarwal *et al*., 2003).

According to the aformation literature review, it might be summarized the idaea as that chronic hyperglycemia in diabetes leads to the overproduction of free radicals may play an important role in the etiology of diabetic vascular complications. These evidences prompted us to investigate the potential actions of cucumin on restoration endothelial function in diabetes-induced model through antioxidant, anti-inflammatory and PKC inhibitory properties.

The diagram shown in Figure 2.16 indicated the conceptual framework of the present study. This conceptual framework base on the target cells, diabetic arteriolar endothelial cells which were considered as an area for studying the molecular mechanisms of curcumin's action.

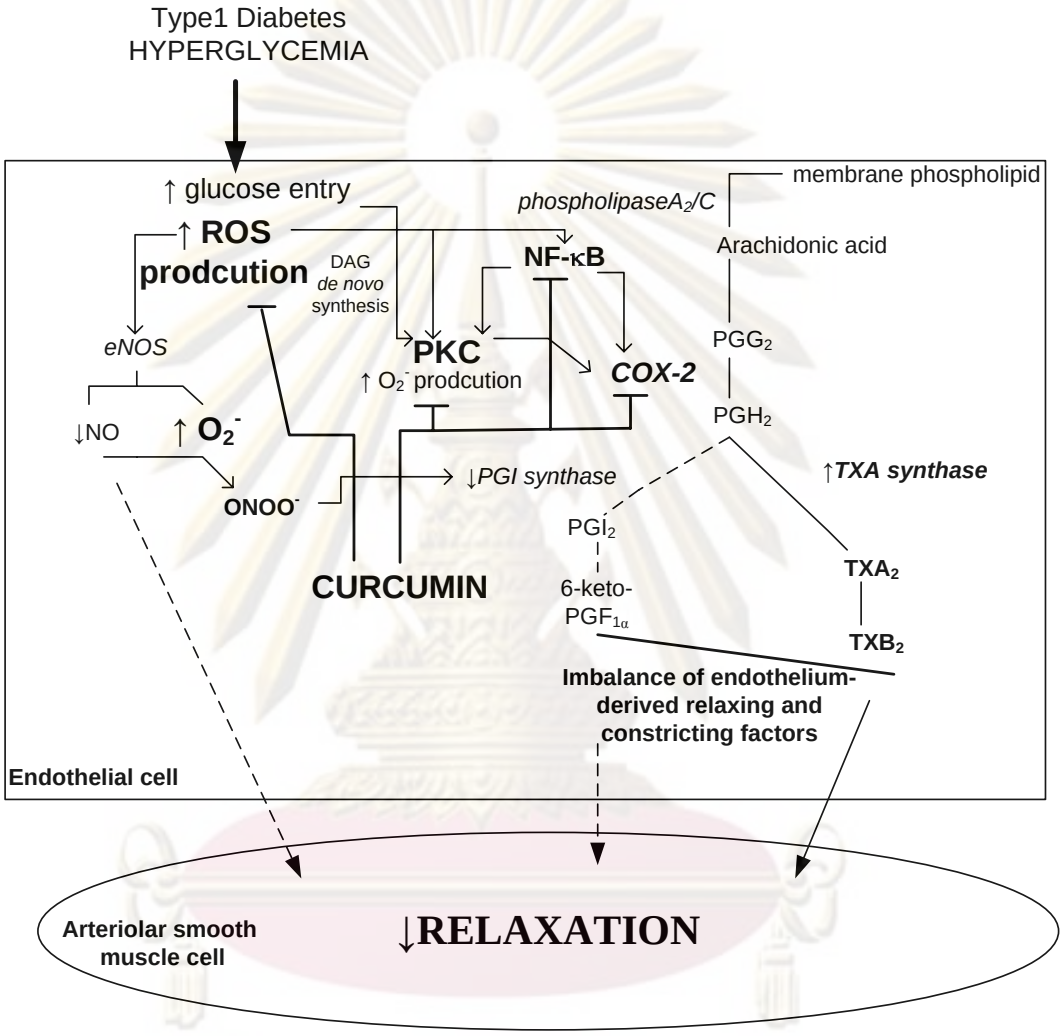


Figure 2.16. The conceptual framework of the present study is proposed under the hypothesis that curcumin could restore diabetes endothelial dysfunction through its antioxidant, anti-inflammatory and PKC inhibitory actions. Hyperglycemia will cause the increase in free radicals (ROS) which in turn they will enhance NF-κB, PKC and COX-2 activations. The increase in free radicals inside the arteriolar endothelial cells will consequently reduce vasodilatation through the reduction of NO bioavailability. ROS-induced decreased NO bioavailability may be attributed by; *a)* ROS causes the uncoupling of eNOS, therefore, $O_2^{\bullet-}$ was produced than NO, *b)* The increased $O_2^{\bullet-}$ from the PKC-induced $O_2^{\bullet-}$ production and from the other pathways can rapidly react with NO and $ONOO^-$. Curcumin which is a phenolic compound acts as a potent antioxidant, anti-inflammatory agent, and a PKC inhibitor. Therefore, it is proposed that the supplementation of curcumin will decrease the levels of free radicals, and NF-κB, PKC, COX-2 activities. Therefore, curcumin can restore arteriolar vasodilatation in diabetic rats. In association with the potential of curcumin in attenuating PKC and COX-2 activities, it is also able to retain PGI_2 mediation-dilatation in hyperglycemia.

CHAPTER III

MATERIALS AND METHODS

Animal Preparation

Male Wistar rats weighing 200-250 g were obtained from the National Laboratory Animal Center, Salaya Campus, Mahidol University, Thailand. Animals were allowed to rest for a week after arrival at the Animal Center, Faculty of Medicine, Chulalongkorn University before being used in the experimentation. They were housed under optimal conditions (constant room temperature 25°C, 12/12 hour light/dark cycle). Four animals were placed per cage (13x29x7.5 inch stainless steel cage). They were fed standard rat chow and drank tap water *ad libitum* throughout the experimental period.

The present study was conducted in accordance with the guidelines for animal experimentation established by the National Research Council of Thailand, 1999 (The National Research Council of Thailand (NRCT), 1999) and approved by the Institutional Animal Care and Use Committee of Chulalongkorn University.

Induction of experimental diabetes

The rats were randomly divided into non-diabetic and diabetic groups. The diabetic rats were induced by a single intravenous injection of streptozotocin (55 mg/kg, STZ, Sigma Chemical, USA). STZ was freshly prepared by dissolving in citrate buffer pH 4.5 (Sigma Chemical, USA) and immediately injected into the tail vein after 8 hours of fasting. Control rats were received citrate buffer of the same volume instead. Within 48 hours of injection, fasting blood glucose level was measured by a glucometer (ACCU-CHECK, Advantage, Roche Diagnostics, Germany). STZ-induced diabetic rats were included and retained for the experiments if their blood glucose was greater than 200 mg/dL (Panes *et al.*, 1996). Rats produced experimental diabetes characterized by polyuria, polydipsea and elevated blood glucose levels, whereas their body weights were reduced compared to non-diabetic group.

Experimental design

On the basis of the aforementioned studies prompted us to investigate the mechanisms underlying the effects of curcumin on restoring endothelial dysfunction in STZ-induced diabetic rat through antioxidant, anti-inflammatory and PKC inhibitory properties. Therefore, the experimental protocol of this study was divided into two parts.

Experimental protocol 1: To determine the effects of curcumin supplementation on endothelial dysfunction in diabetes-induced rat. Physiological characteristics and functional vascular responses in all animal groups were examined. These providing evidences support the beneficial effects of curcumin on restoration of endothelial dysfunction in diabetes.

Experimental protocol 2: To study the mechanisms underlying the beneficial effect of curcumin on diabetes-induced endothelial dysfunction. The methods for this protocol were based on the hypothesis and conceptual framework of the present study. Chemical analysis and immunohistochemistry analysis were performed.

Protocol 1: To determine the effects of curcumin on restoration of endothelial dysfunction in STZ-induced rat.

Six weeks after the STZ injection, animals were separated into five groups: (1) diabetes (DM; n=15), (2) DM-treated with curcumin (Cayman Chemical, USA) 30 mg/kg (DM+cur30; n=15), (3) DM-treated with curcumin 300 mg/kg (DM+cur300; n=15), (4) control (con; n=15) and (5) control-treated with curcumin 300 mg/kg (con+cur300; n=15), as shown in Figure 3.1. The daily oral feeding of curcumin was started at six weeks after the STZ injection. Since it has been shown that endothelial dysfunction in STZ induced-rat was occurred at six-week after STZ injection (Sridulyakul *et al*., 2006).

Twelve weeks after the STZ injection (or vehicle), the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). After tracheostomy, polyethylene tubes were inserted into the external jugular vein and the common carotid artery for injection of fluorescence tracers and monitoring of mean arterial blood pressure (mABP), respectively. Moreover the diabetes-induced endothelial dysfunction was evaluated under epi-illumination fluorescence video-microscopy and image-analysis software for determining real-time arteriolar diameter changes after acetylcholine (ACh) and sodium nitorpusside (SNP) applications.

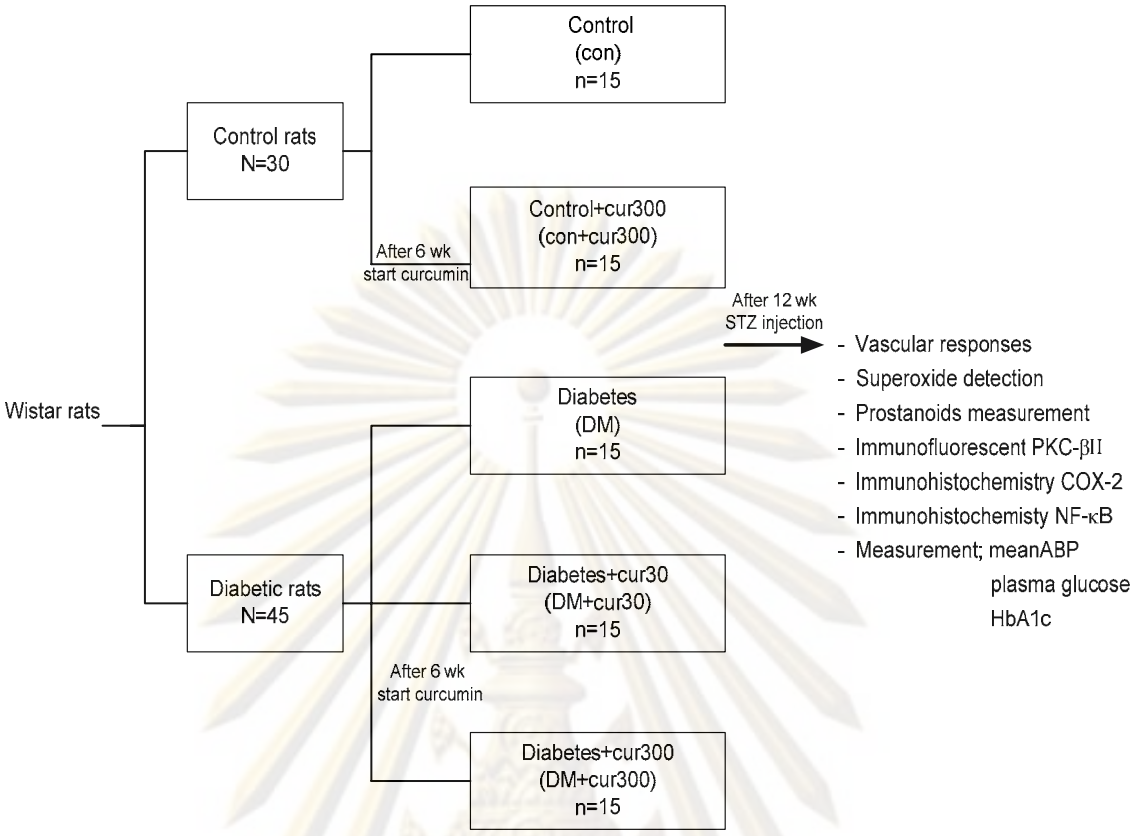


Figure 3.1. The experimental design was conducted in order to test the hypothesis based on the present conceptual framework.

1.1 Determination of physiological characteristics and biochemical parameters

In the present study, the physiological characteristics were determined, including body weight (BW) and mABP. In unconscious rats, systolic and diastolic blood pressures were measured via a tube inserted into the carotid artery using a Statham pressure transducer connected to the Polygraph system (Nihon Koden, Japan). mABP was also calculated from diastolic pressure + 1/3 (systolic pressure – diastolic pressure).

At the end of each experiment, the blood sample of each rat was collected for further plasma glucose and glycosylated hemoglobin (HbA1c) determination, using enzymatic method and turbidimetric immunoinhibition method, respectively (Bangkok RIA Laboratory Co., Bangkok, Thailand).

1.2 Arteriolar response to acetylcholine and sodium nitropusside

Experiments were conducted on mesenteric arterioles of rat, as described previously by Chakraphan, 2002 (Chakraphan, 2002). In this study, the mesenteric microcirculation was used to observed the endothelial dysfunction in diabetic rats due to this tissue model is available and easy to observe the function of microvessel *in situ* in particular using the intravital fluorescence microspcope. Moreover, the functional changes in this mesentery could reflect the generalized vascular responses as the other tissues under diabetic condition.

After overnight fasting, rat was anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). The rat was allowed to stabilize for 20 to 30 minutes following surgery. The abdominal cavity was opened via midline incision. The rat was placed on its right side on a microscope stage. A small loop of intestine was exteriorized (Figure 3.2). The ileocecal portion of the mesentery was carefully spread on a Plexiglass chamber with continuously perfused by 1 mL/min Kreb-Ringer buffer solution (37°C, pH 7.4, composition in mmol/L: 135.7 NaCl, 4.7 KCl, 2.52 $CaCl_2$, 1.18 KH_2PO_4, 1.64 $MgSO_4.7H_2O$, and 7.14 $NaHCO_3$) throughout the experiment.

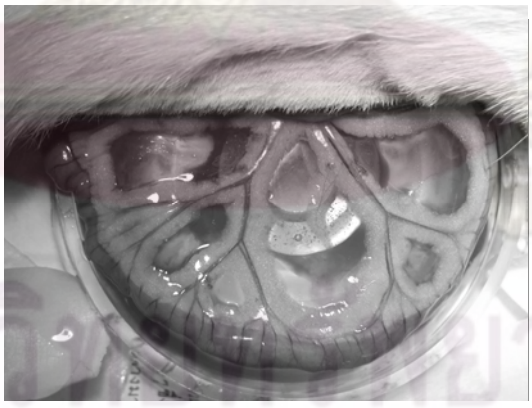


Figure 3.2. The mesenteric vascular bed for studying vascular responses and perfusates collection

To directly visualize the arteriolar response to vasodilators, the second-order mesenteric arterioles (20 to 30 μm in diameter) were selected for study. Arterioles could be identified by its divergence flow direction. During the experiment, the real time image of mesenteric microcirculation was recorded by an epi-illumination fluorescence video-microscopy 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 video timer (VTG-33, For-A, Japan) and a 20x objective lens (CF Plan Fluor, Nikon, Japan). Un-branched segments of mesenteric arterioles were visualized by fluorescein isothiocyanate-labeled dextran (FITC-Dextran 250, 5 μg/mL, Sigma Chemical, USA).

The mesenteric arterioles were allowed to stabilize for 30 minutes and then topical application of norepinephrine (10^{-5} mol/L, NE) was used for inducing 1-min pre-constriction. The topical applications of two vasodilators, acetylcholine (10^{-5} mol/L, ACh) which is endothelium dependent vasodilator and NO donor sodium nitroprusside (10^{-5} mol/L, SNP), endothelium independent vasodilator, were used to test the functions of the endothelium and smooth muscle of mesenteric arterioles, respectively. Video images of the studied area were taken at one minute after NE and three minutes after ACh application. At the end of this experiment, the mesenteric arteriole was perfused with Krebs-Ringer solution until its diameter was return to basal diameter (Figure 3.3). To evaluate diabetes altered the vascular smooth muscle function in the regulation of microvascular tone. SNP, an endothelium-independent vasodilatation, was applied topically on the mesenteric arteriole after the pre-constriction with NE.

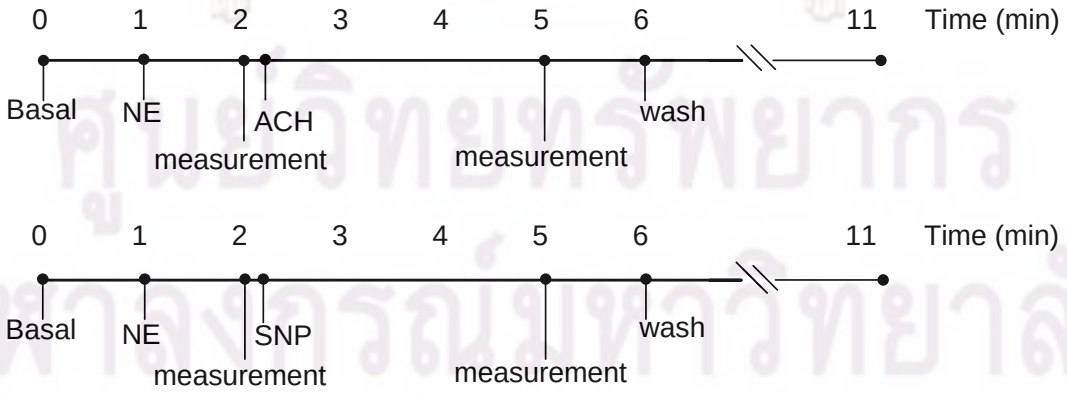


Figure 3.3. The experimental design for the examinations of ACh- and SNP- vascular responses.

After challenging by each vasodilator, the changes of mesenteric arteriolar diameter were off-line analyzed using the digital image software (Image-Pro Plus; Media Cybernetics, Inc, USA). For each rat, at least three different video frames by using the same reference point as a marker for measuring at the same position in each frame were used for mean diameter estimation, as shown in Figure 3.4. Changes of arteriolar diameters were expressed as the percentage of relaxation after pre-constricted with NE by using the equation: Changes of arteriolar dimeters = $\mathbf{[(D_{ACh/SNP} - D_{NE})/D_{NE}\ X100]}$

Where $D_{ACh/SNP}$ is the diameter at three minutes after the application with ACh or SNP

D_{NE} is the diameter at one minute after the application with NE.

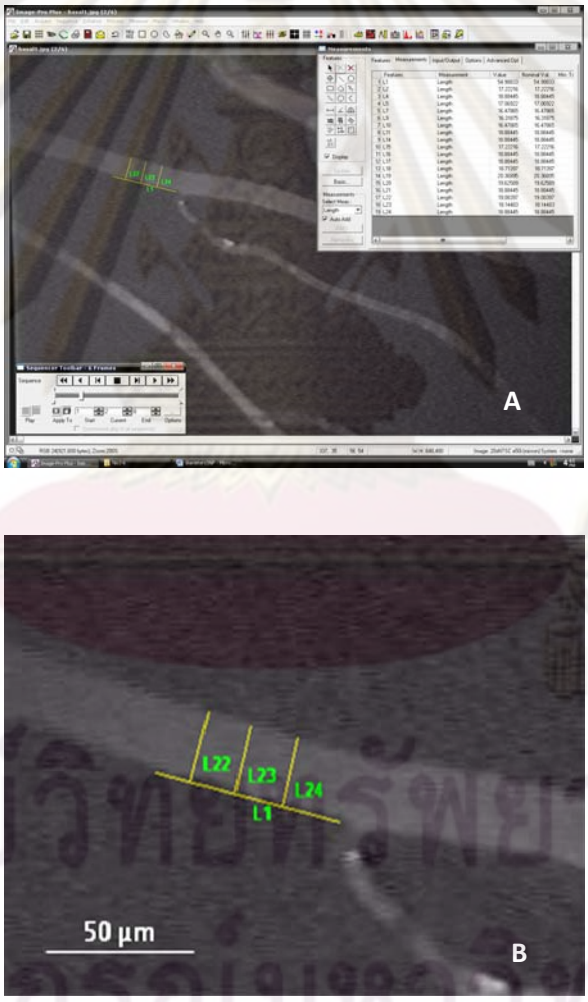


Figure 3.4. The intravital video-microscopic image showing change of arteriolar diameter (A) and the averaged arteriolar diameter was obtained from three diameters measured at three different consecutive positions on each arteriole in each rat (B).

Protocol 2: To study the mechanisms underlying the beneficial effects of curcumin on diabetes-induced endothelial dysfunction.

Based on the above we hypothesized that due to hyperglycemia increased free radicals, reduced vasodilatation, being mediated in part by NF-κB, PKC and COX-2 activation, was impaired in vascular prostanoids synthesis in the regulation of mesenteric arteriolar tone. Thus, to test this hypothesis, intracellular superoxide production was detected in mesenteric arteriole in all groups. To determine whether NF-κB, PKC and COX-2 were involved in diabetes induced-endothelial dysfunctions and curcumin could restore these alterations through antioxidant, anti-inflammatory and inhibitory PKC actions.

2.1 In situ detection of superoxide by using hydroethidine

Mesenteric arteriole was subjected to intracellular $O_2^{\bullet-}$ measurement based on the fluorescence detection of the DNA-binding fluorophore ethidium bromide, which is formed by oxidation of hydroethidine (dihydroethidium). Hydroethidine, the sodium borohydride-reduced derivative of ethidium bromide (Thomas and Roques, 1972), which permeates the cell membrane easily. It is an oxygen radical-sensitive fluorescent probe. The intracellular hydroethidine can be directly oxidized to form red fluorescent ehtidium bromide, which in turn is trapped in the nucleus by intercalation into DNA (Rothe and Valet, 1990). Hydroethidine is more specific to $O_2^{\bullet-}$ than hydrogen peroxide or hydroxyl radical (Carter *et al*., 1994; Benov *et al*., 1998).

Under pentobarbital sodium anesthesia, the mesentery was exposed and prepared for *in situ* intravital fluorescent microscopic observation. Single unbranched mesenteric arteriole with diameter between 20 to 30 μm was selected for study. The mesenteric arteriole was visualized with a 20x objective lens using a CCD camera which set sensitivity at constant values.

After an initial stabilization period, the mesenteric preparation was perfused with a 37°C Krebs-Ringer buffered solution saturated with a 95% N_2 and 5% CO_2 gas mixture to minimize the production of oxygen free radicals by exposure of the tissues. A background fluorescence image in the selected arteriole area was recorded. The prepation was then perfused with a buffer solution containing hydroethidine (5 µmol/L, Polysciences, USA) for 60 minutes according to the modified method described by Suzuki *et al.* (Suzuki *et al*., 1995; Suzuki *et al*., 1998). During the experimental periods, the shutter for the excitation light was kept closed. The fluorescent ethidium bromide was monitored at 490 nm excitation and 590 nm emission wavelength (Thomas and Roques, 1972).

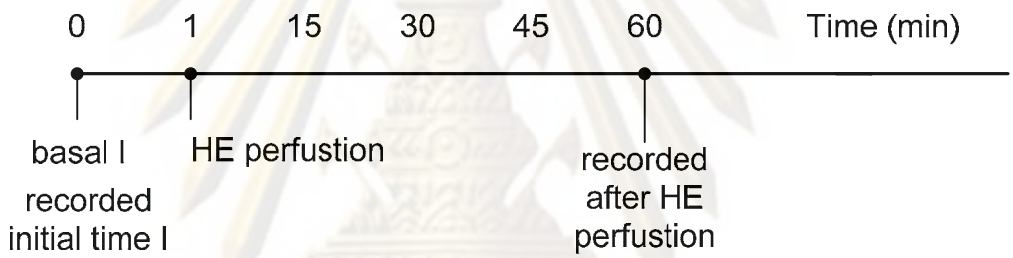


Figure 3.5. Experimental design to detect intracellular superoxide anion.

The numbers of nuclei labeled with ethidium bromide along arteriole were off-line analyzed. The EB-stained nuclei in arteriole were considered to have a longitudinally oriented shape and were positioned on the inner lining of the vascular wall, suggesting that they were endothelial cells (Suzuki *et al.*, 1995). These EB-positive nuclei were counted by using the digital image software (Image-Pro Plus; Media Cybernetics, Inc, USA).

The numbers of EB-stained nuclei were counted per 100 µm vessel length as shown in Figure 3.6. The selection of microvessel was limited to arterioles with diameter range between 20 to 35 µm. The results were confirmed by the other examinator who was blinded to grouping in counting.

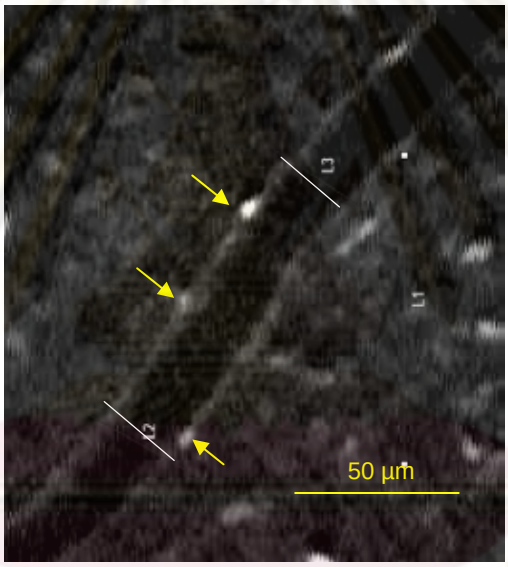


Figure 3.6 Numbers of EB-positive nuclei were counted along vascular length. The white lines depict the length of the vessels that were analyzed in each vessel. (Bar represents 50 µm).

2.2 *Determination of prostanoid level*

To elucidate the role of curcumin in COX-derived prostaglandins which contribute to impaired vasodilatation in diabetes. The COX activity was probed by the measurement of the level of PGI_2 and TXA_2 from the mesenteric perfusate.

PGI_2 is formed from arachidonic acid primarily by the vascular endothelium and renal cortex (Moncada *et al*., 1976; Whorton *et al*., 1978). It is a vasodilator and inhibitor of platelet aggregation. PGI_2 is hydrolyzed non-enzymatically to form 6-keto-$PGF_{1\alpha}$. It was chosen as an index of vasodilatory prostaglandin, whereas TXB_2 is the hydrolysis product of TXA_2 was determined as an index of vasoconstrictor prostaglandin. TXA_2 is also produced from arachidonic acid and causes platelet aggregation and vascular contraction (Hamberg *et al*., 1975; Ellis *et al*., 1976). It is important to note that TXA_2 is not circulating hormone. It is formed in response to local stimuli and exerts its effects within a short distance of its biosynthesis. Therefore, TXB_2 measurement is better suited for sample as perfusates, tissue/cell culture (Cayman Chemical).

Under pentobarbital sodium anesthesia, the mesentery was exposed and prepared for the measurement of existing 6-keto-$PGF_{1\alpha}$ and TXB_2 levels in mesenteric perfusates. . The mesenteric perfusates were collected from mesenteric vascular bed as shown in Figure 3.2. These perfusate were collected for three minutes before and after the stimulation with 10^{-5} mol/L ACh (14) according to the experimental procedure shown in Figure 3.7. These prostanoids were determined by using enzyme immunoassay kits, according to the protocols provided by the manufactures (Cayman Chemical, USA).

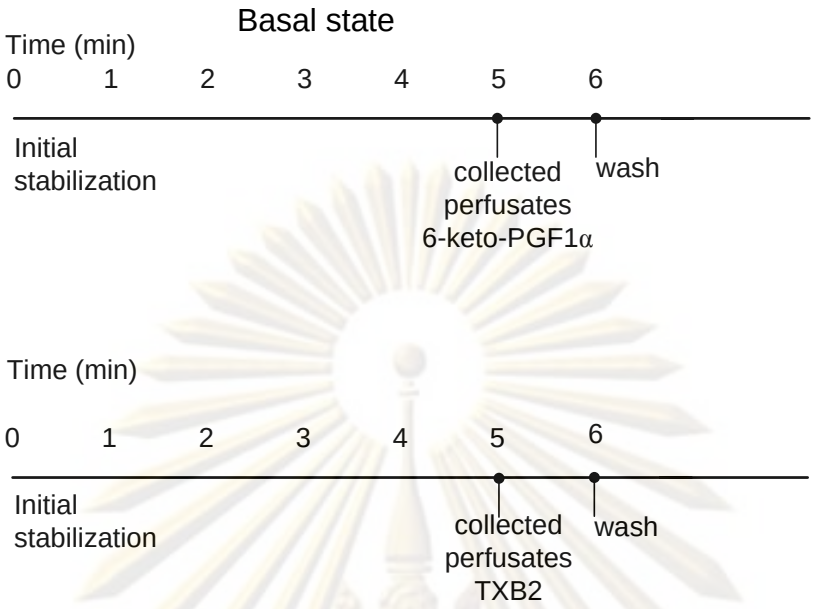


Figure 3.7. The experimental designs for mesenteric perfusates collection.

2.3 Immunohistochemistry analysis

The present protocol was conducted to verify the roles of PKC, COX-2 and NF-κB in diabetes-induced endothelial dysfunction. In addition we also investigated whether the potential of curcumin on restoration of diabetes-induced endothelial dysfunction was in association with those roles of PKC, COX-2 and NF-κB or not. Immunohistochemistry was used to identify PKC, COX-2 and NF-κB presented in mesenteric microvasculature.

2.3.1 Immunofluorescent staining for PKC-βII

Under pentobarbital sodium anesthesia, the rat mesentery was exposed. Single unbranched small mesenteric artery with diameter between 100-120 μm was selected for study. Immunohistochemical staining of small mesenteric artery was performed according to the protocols provided by the Department of Pathology, Faculty of Medicine, Chulalongkorn University. The selected microvessel was then dissected and cleared of connective tissue and briefly rinsed in ice-cold PBS. After the collection, mesenteric arteries were then immediately fixed in 4% paraformaldehyde for 24 hour and were embedded in paraffin. These specimens were then deparaffinized in xylene, rehydrated in graded ethanol and distilled water, and antigen unmasked with sodium citrate (10 mmol/L,

pH 6.0, Dako, Denmark), followed by microwave heat source for high power 3 minutes and following low power 10 minutes. After PBS wash, non specific background was blocked with 3% normal horse serum at room temperature for 20 minutes. Incubation with anti-PKC-βII (1:100 dilutions; SC-210, Santa Cruz Biotechnology, CA) was performed at room temperature for 60 minutes. Anti-α-smooth muscle actin (1:200 dilutions; Dako, Denmark) was also used to colocalize microvascular smooth muscle. Sections were then washed in PBS and incubated with the secondary antibody swine anti-rabbit IgG-TRITC (1:50 dilution; R0156, Dako, Denmark) for PKC-βII and rabbit anti mouse IgG-FITC (1:50 dilution; R0261, Dako, Denmark) for smooth muscle actin at room temperature for 30 minutes. Arteries received two 3-min washes in PBS and covered with mounting medium containing DAPI (Vector Laboratories) to counterstain nuclei and antifade and then cover slipped. Labeling of the arteries with secondary alone was used as negative controls. Images were obtained using a laser scanning confocal microscopy (E800, Nikon, Japan) to establish the localization of PKC-βII and α-smooth muscle actin in small mesenteric arteries

2.3.2 Immunohistochemistry for COX-2 and NF-κB p65

After collection, mesenteric arteries were fixed by the same protocol. Paraffin-embedded sections of small mesenteric arteries were sequentially exposed to the following solutions as previously described. COX-2 immunohistochemically detected by incubation with rabbit anti-COX-2 (1:500 dilutions; RP111, Diagnostic Biosystems) followed by the appropriate secondary antibody; anti-rabbit IgG horseradish peroxidase.

Activated NF-κB was detected by incubation with anti- NF-κB p65 rabbit polyclonal antibody (1:150 dilution, SC-109, Santa Cruz Biotechnology, CA), which was recognized as an epitope accessible only when NF-κB is bound to DNA. This was followed by incubation with a horseradish peroxidase-conjugated secondary antibody. The data were expressed as positive signal in vasculature. The stained sections were examined under a microscopy system (Optiphot 2, Nikon, Japan) equipped with Nikon Digital Sight DS-Fi1 and DS-L2 and 20x objective lens (CF Plan Fluor, Nikon, Japan).

Image analysis for COX-2 and NF-κB p65

The intensity of each image of COX-2 and NF-κB p65 was analyzed quantitatively by image analysis using Global Lab Image/2 (GLI/2) software. GLI/2 is an object-oriented scientific imaging software product that powerful, expandable, able to definite of contrasting areas, and improved precision in measurements, using the histogram tool to quantitate positive immunoreactions brown precipitate of COX-2 or NF-κB p65. Results can be exported to Excel spreadsheets and expressed in number of pixels for COX-2 or NF-κB p65.

An example showed how to quantify COX-2 and NF-κB p65 by GLI/2.

1. Opened a File Manager Tool. Selected image file. The example of one image file of COX-2 expression was selected and digitized by GLI/2. (Figure 3.8)

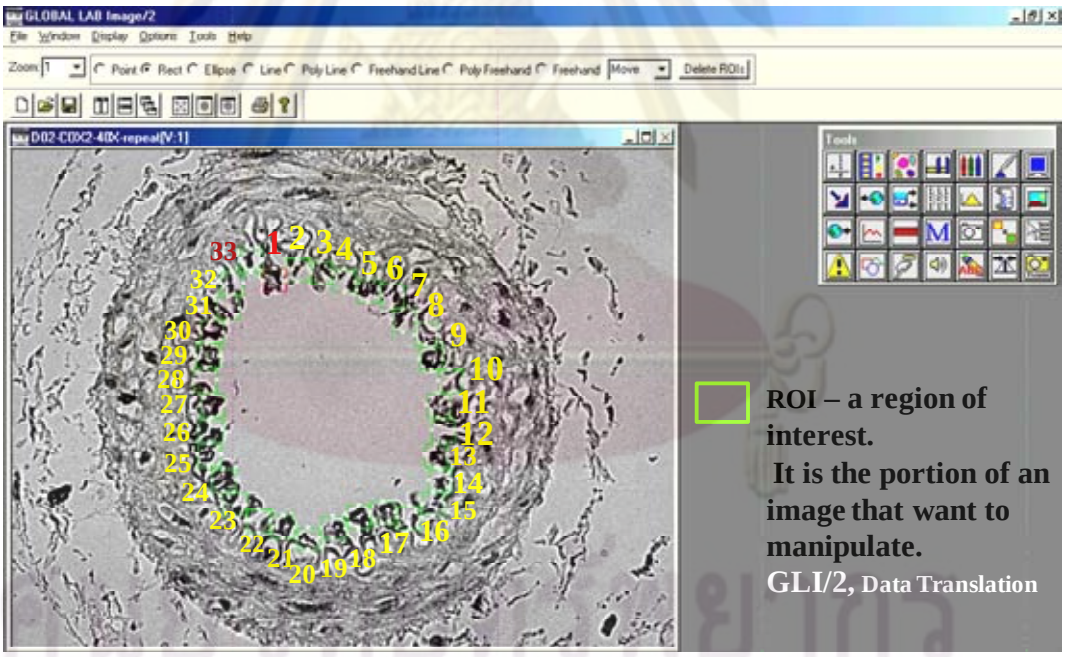


Figure 3.8. The image file of COX-2 expression activated ROI squares around the immunoreactions brown precipitate of COX-2.

2. Select ROI (region of interest) Menu bar and click Draw in the ROI actions drop-down list (See Figure 3.8).

3. Create squares that cover around all positive immunoreactions brown precipitate of COX-2 or NF-κB p65 in the endothelium layer (inner layer) of small mesenteric arteries. There were 33 ROI cover around all positive immunoreactions brown precipitate of COX-2 endothelium layer as shown in Figure 3.8.

4. Open the Histogram Tool and click Add Histogram button to add a histogram to the graph.

5. Select the Function Menu/Show statistics. The statistic values are calculated with regard to the range of pixel values. Then record mean value (the average pixels value in the selected range) for set specifying of the maximum threshold limits that appropriate for this image.

6. From the total 33 ROIs, the brown precipitate of COX-2 or NF-κB p65 were added up and defined as numbers of pixels within that brown area. Then the average number of pixels for COX-2 and NF-κB p65 expression were represented as the average number by equation (Figure 3.9).

   Average of the number of pixels for COX-2 expression = $\frac{x_1+x_2+x_3+\ldots.+x_{33}}{33}$

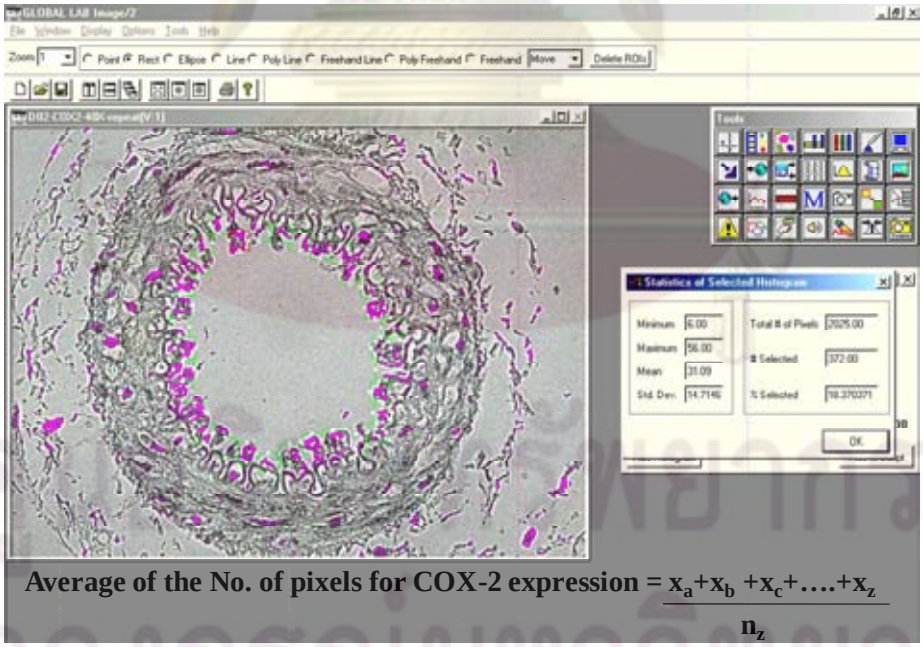


Figure 3.9. Average of the number of pixels for COX-2 expression was determined by GLI/2 software.

Statistical analysis

Data were expressed as mean and standard errors of mean (mean±SEM). For comparison among groups of animals, one-way analysis of variance (one-way ANOVA) were used and followed by Tukey post hoc test. $P<0.01$ and 0.05 were considered statistically significant. The data were analyzed using the SPSS program (version 16.0) for Windows.

CHAPTER IV

RESULTS

In this section, two major results were described according to our experimental design as follow:

Part 1. The results of functional responses of mesenteric arterioles to vasodilators, ACh and SNP, in diabetic and curcumin treated diabetic rats.

Part 2. The results of; *a) in situ* detection of intracellular $O_2^{\bullet-}$ in mesenteric arterioles of each animal group, *b)* The possible molecular mechanisms underlying the beneficial effects of curcumin in associate with COX-2-derived prostanoids, PKC-βII, and NF-κB.

Part I

Determination of physiological characteristics

Previously, the single intravenous injection of STZ in to male Wistar rats (200-250 g) caused beta cell of pancreatic islet cells to damage, resulting in hyperglycemia within 48 hours. The criterion to decide on diabetic rats was the blood glucose concentration greater than 200 mg/dL. Moreover, rats produced experimental diabetes characterized by polyuria, polydipsea, and polyphagia throughout the twelve-week experimental period.

In the present study, we had found that body weights of twelve-week diabetic rats (285.3±8.8 g) were significantly decreased when compared to age-matched control rats (398.7±12.3 g, $P<0.01$). Both doses of curcumin supplementation 30 mg/kg (292.7±17.8 g, $P<0.01$) and 300 mg/kg (325.7±13 g, $P<0.05$) did not have any effects on the body weight restoration in diabetic rats. The supplementation of curcumin (300 mg/kg) in controls (con+cur300) did not alter their body weight (403.8±17 g, P=NS) as compared to control+vehicle rats (Table 4.1).

Mean arterial blood pressure (mABP) obtained from mean systolic and diastolic pressure data were calculated for each group as shown in Table 4.1. In comparison with control group, mABP was significantly increased in DM group ($mABP_{con}$ 103.1±3.5 mmHg and $mABP_{DM}$ 151.6±9.6 mmHg, $P<0.01$, respectively). The increased mABP in diabetes was significantly attenuated by a high dose of curcumin supplementation (122.2±8.6 mmHg, $P<0.05$). There were no significant differences between mABPs in the two groups of control rats treated with curcumin supplementation (con+cur300) and control+vehicle rats (106.1±3.9 and 103.1±3.5 mmHg, respectively).

Table 4.1. Body weight (BW) and mean arterial blood pressure (mABP).

	BW (g)	mABP (mmHg)
con	398.7±12.3	103.1±3.5
con+cur300	403.8±17.0^{NS}	106.1±3.9^{NS}
DM	285.3±8.8**	151.6±9.6**
DM+cur30	292.7±17.8**	128.3±3.6 ^{NS}
DM+cur300	325.7±13.0*	122.2±8.6†

Values are mean±SEM (n=10 for each group).

NS, not significant difference compared to control rats

***P*<0.01 and **P*<0.05 significantly difference compared to control and control treated with curcumin rats

† significant difference from diabetic rats (*P*<0.05)

Determination of biochemical parameters

Twelve weeks after the injection of STZ, plasma glucose and HbA1c values were significantly elevated in DM rats compared with control rats (Table 4.2). Supplementation of curcumin for six weeks in con+cur300 rats did not alter the plasma glucose level and HbA1c compared to control rats. Interestingly, the high dose supplementation of curcumin in DM+cur300 significantly lower both values of plasma glucose and HbA1c as compared to DM group (P<0.05). The low dose of curcumin supplementation (30 mg/kg) was slightly lower plasma glucose than the DM group, but did not reach the statistical significance. However, this low dose of curcumin could significantly lower HbA1c as compare to DM group (P<0.05).

Table 4.2. Plasma glucose and mean glycosylated hemoglobin (HbA1c) at 12 week after STZ injection.

	Plasma glucose (mg/dL)	**HbA1c (%)**
control	101.8±4.89	3.68±0.17
con+cur300	106.8±0.92 ^{NS}	4.08±0.41 ^{NS}
DM	459.0±24.40**	10.73±0.32**
DM+cur30	360.8±35.82**	8.20±0.88**,†
DM+cur300	310.00±32.73**,†	7.90±0.97**,†

Values are mean±SEM (n=10 for each group).

NS, not significant difference as compared to control rats

**P<0.01 and *P<0.05 significantly difference compared to control and control treated with curcumin rats

† significant difference from diabetic rats (P<0.05)

Effect of curcumin supplementation on arteriolar responses

The dilatory response of the mesenteric arterioles to ACh (10^{-5} mol/L) was significantly decreased in diabetic group (8.11±0.44%) as compared to control (12.82±0.2%, $P<0.01$), (Table 4.3 and Figure 4.1). Both low and high doses of curcumin supplementation (DM+cur30; 10.56±0.2% and DM+cur300; 11.88±0.52%) significantly restore the arteriolar dilatation to ACh (10^{-5} mol/L) as compared to DM rats (8.11±0.44%, $P<0.01$). However, the supplementation of curcumin in con+cur300 did not show any effects on ACh-induced arteriolar dilatation as compared to control (12.58±1.07% and 12.82±0.2%, respectively).

Table 4.3 and Figure 4.2 demonstrated that the impaired vasodilatation in mesenteric arterioles of DM rats appeared to involve only upon the endothelial cells function not smooth muscle function, since the vasodilatation response to SNP-activation was not altered in DM (12.55±0.95%), DM+cur30 (14.71±0.38%), and DM+cur300 groups (13.54±1.40%).

Table 4.3. Percentage changes of mesenteric arteriolar diameter in responses to ACh and SNP.

	% change of arteriolar diameter	
	ACh-induced responses	**SNP-induced responses**
control	12.82±0.2	15.97±0.6
con+cur300	12.58±1.0 *NS*	16.45±1.3 *NS*
DM	8.11±0.4**	12.55±0.9 *NS*
DM+cur30	10.56±0.2 *NS, ††*	14.71±0.3 *NS*
DM+cur300	11.88±0.52 *NS, ††*	13.54±1.4 *NS*

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

NS, not significant difference compared to control arterioles

** $P<0.01$, significant difference compared to control arterioles

†† $P<0.01$, significant difference compared to diabetic arterioles

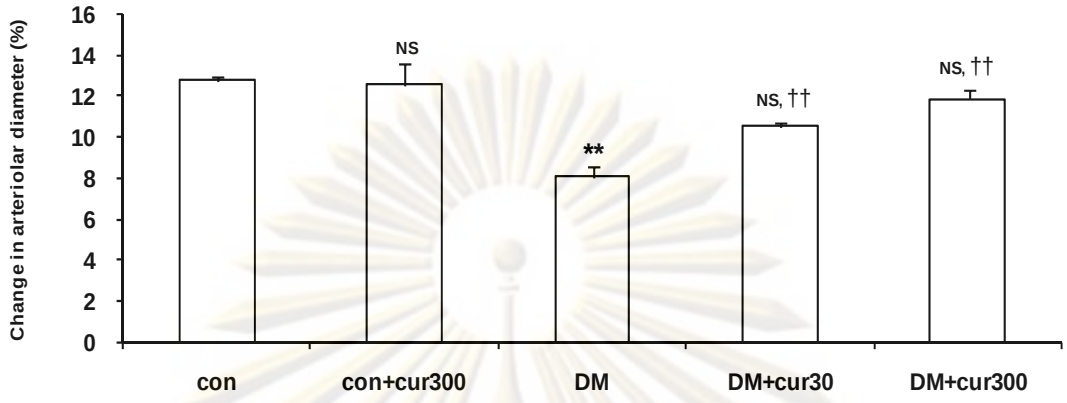


Figure 4.1. Acetylcholine-induced changes in mesenteric arteriolar diameters taken from control (con), diabetes (DM) and treatment with curcumin in diabetic and control rats (DM+cur30, DM+cur300 and con+cur300).

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

NS, not significant difference compared to control arterioles

** $P<0.01$, significant difference compared to control arterioles

†† $P<0.01$, significant difference compared to diabetic arterioles

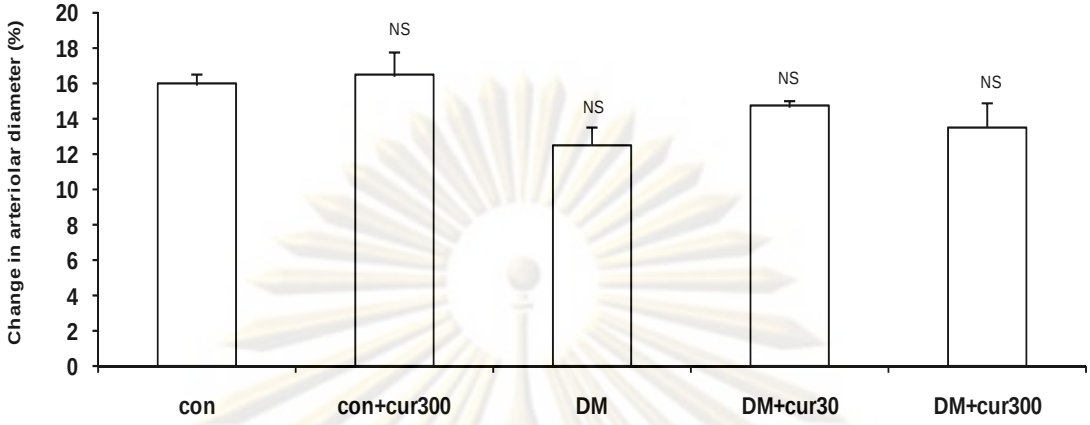


Figure 4.2. Sodium nitroprusside-induced change in mesenteric arteriolar diameters taken from control (con), diabetes (DM) and curcumin treated groups (con+cur300, DM+cur30, DM+cur300).

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

NS, not significant difference compared to control arterioles.

Conclusion remark from Part I

The findings are that diabetic rats have shown the common characters of increased plasma glucose, glycosylated HbA1c, and elevated mABP. In addition to these typical diabetic abnormalities, the alteration of vascular reactivity was also demonstrated by using the endothelium-dependent vasodilator, acetylcholine (10^{-5} mol/L). As the result, it showed that ACh-induced mesenteric arteriolar response in DM rat was significantly decreased when compared to its age-matched control. Interestingly, both supplement doses of curcumin (30 mg/kg and 300 mg/kg) were able to restore these diabetic impairments.

Part II

Base on our hypothesis which believed that the antioxidant property of curcumin should be the underlying mechanism of that beneficial effect of curcumin on vascular activity as shown in Part I. Therefore, we further examined whether the diabetes-induced intracellular superoxide ($O_2^{\bullet-}$) evaluation was reduced by curcumin treatments or not.

Effect of curcumin supplementation on vascular superoxide anion production

By using hydroethidine-sensitive vascular superoxide, the results showed that the number of EB-positive nuclei were significantly increased along the arteriolar wall of DM rats (19.6±0.5%) as compared to control rats (4.6±0.5%, $P<0.01$) (Table 4.4 and Figure 4.3). The number of EB-positive nuclei observed in DM+cur30 and DM+cur300 groups (10.8±1.2% and 11.2±1.8%, respectively) were significantly reduced as compared to DM (19.6±0.5%, $P<0.01$), but remained higher than controls (4.6±0.5%, $P<0.05$) (Table 4.4 and Figure 4.3). There was no significant difference between the EB-positive nuclei in control and con+cur300 arterioles (4.6±0.5% and 5.2±0.5%, respectively).

Table 4.4. The ethidium bromide-positive nuclei along the mesenteric arterioles.

	EB-positive nuclei/ 100 µm vessel length
control	4.6±0.5
con+cur300	5.2±0.5 ^{NS}
DM	19.6±0.8 **
DM+cur30	10.8±1.2*,††
DM+cur300	11.2±1.8*,††

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

NS, not significant difference compared to control arterioles

** P<0.01 an * P<0.05 significant difference compared to control arterioles

†† P<0.01, significant difference compared to diabetic arterioles

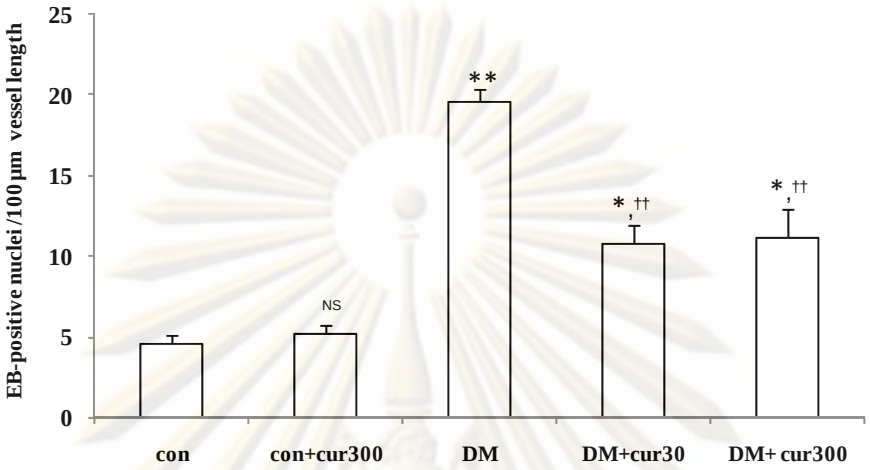


Figure 4.3. Histogram showing the ethidium bromide-positive nuclei along the mesenteric arterioles of control (con), control treated with curcumin (con+cur300) rats. Diabetes treated with low (DM+cur30) and high (DM+cur300) curcumin and untreated diabetic rats (DM).

Data are expressed as mean±SEM (n=5 for each group)

NS, not significant difference compared to control arterioles

**P<0.01 and *P<0.05 significant difference compared to control arterioles

†† P<0.01, significant difference compared to diabetic arterioles

Correlation between intracellular superoxide production and arteriolar vasodilatation

Next we have made further study, in order to examine the correlation between the intracellular superoxide production and endothelial vascular response. Figure 4.4 showed the plot between the data set of superoxide production and ACh-induced arteriolar vasodilatation determined from each group. The results indicated that the EB-positive nuclei along the mesenteric arterioles and % changes of arteriolar diameters stimulated by ACh have a significantly inversely correlation with correlation coefficient equal to 0.78 (P<0.01). And they could be fitted by the linear equation:

$$y = -0.29x + 14.5 \ (R^2 = 0.78, P<0.01)$$

Where x is percentage of EB-positive nuclei and y is percentage of arteriolar-diameter change.

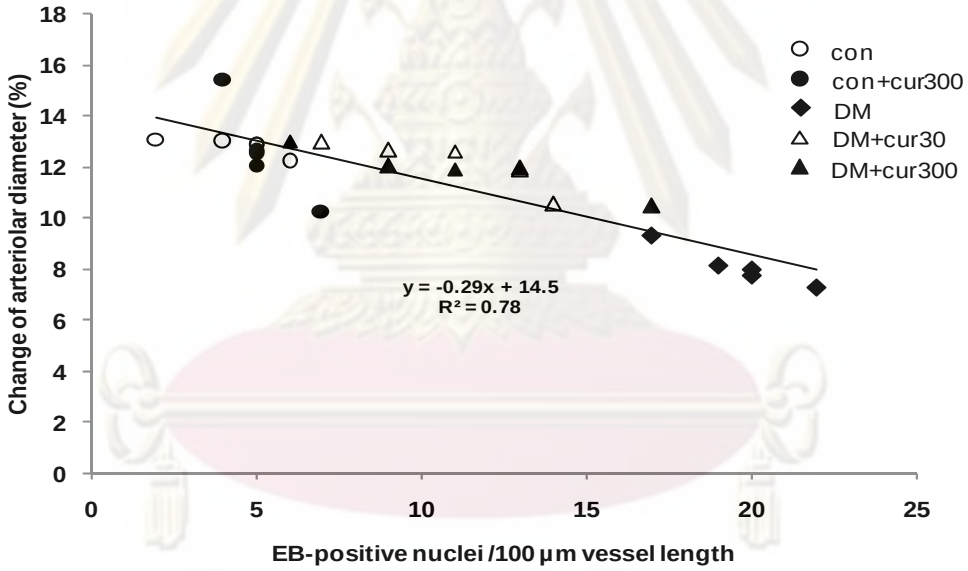


Figure 4.4. Inversely relationship between EB-positive nuclei per 100 µm vessel length and % ACh-induced change in arteriolar diameter for control (con), control treated with 300 mg curcumin (con+cur300), diabetes (DM), diabetes treated with 30 and 300 mg curcumin (DM+cur30 and DM+cur300, respectively).

Effect of curcumin supplementation on prostanoids biosynthesis in mesenteric arterioles

Base on our hypothesis which believed that the anti-inflammatory property of curcumin should be one of the underlying mechanism of that beneficial effect of curcumin on vascular activity. Therefore, we further elucidate the role of curcumin in ACh-induced prostanoid biosynthesis in control, diabetic and diabetes treated with curcumin rats were examined. It is to be noted that this experiment used only the high dose supplementation of curcumin (DM+cur300). Due to this high dose could be the more effective dose of curcumin supplementation to restore the impairment of endothelium dependent vasodilatory response than the low dose of curcumin supplementation (curcumin 30 mg/kg).

Table 4.5. The basal levels of 6-keto-$PGF_{1\alpha}$ and TXB_2.

	6-keto-$PGF_{1\alpha}$ (pg/mL)	**TXB_2** (pg/mL)
Con	536.9±53.3	29.41±4.3
DM	407.6±37.1*	58.44±5.8*
DM+cur300	502.32±27.6 ^{NS}	47.43±7.2^{NS}

Data are expressed as mean±SEM (n=5 for each group)

NS, not significant difference compared to control rats

*P<0.05 significant difference compared to control rats

Table 4.5 and Figure 4.5A demonstrated that the un-stimulated (basal) level of 6-keto-$PGF_{1\alpha}$ (stable metabolite of PGI_2) was significantly reduced in diabetic (DM, 407.6±37.1 pg/mL) compared to control (con) rats (536.9±53.3 pg/mL, **P*<0.05) whereas there was not different between control and diabetes treated with curcumin 300 mg/kg (DM+cur) rats (502.32±27.6 pg/mL, *P*=NS). On the other hand, the basal level of TXB_2 (stable metabolite of TXA_2) in the perfusate from DM rats (58.44±5.8 pg/mL) was significantly elevated as compared with control rats (29.41 pg/mL, **P*<0.05) whereas those of DM+cur (47.43±7.2 pg/mL) was not different (Table 4.5 and Figure 4.5B). Interestingly, DM rats showed a simultaneous lower of 6-keto-$PGF_{1\alpha}$ and higher of TXB_2 prostanoids release at basal state.

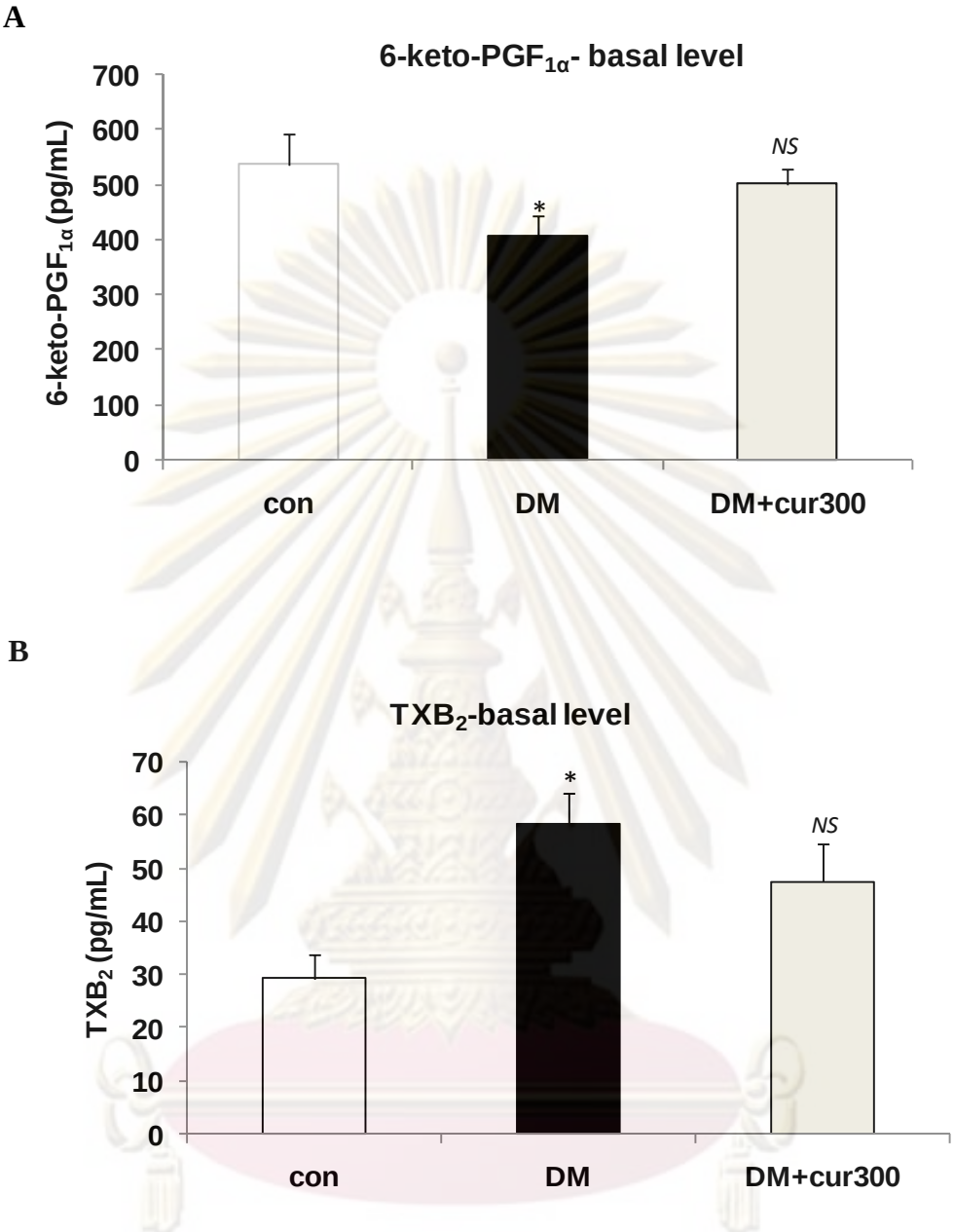


Figure 4.5. The basal levels of 6-keto-$PGF_{1\alpha}$ (A) and TXB_2 (B) measured in the perfusate from mesenteric arteriolar bed of control (con), diabetic (DM) and diabetes treated with curcumin 300 mg/kg (DM+cur) rats.

Data are expressed as mean±SEM (n=5 for each group).

NS, not significant difference compared to control rats

*P<0.05 significant difference compared to control rats

Table 4.6. Basal levels of 6-keto-$PGF_{1\alpha}$ and TXB_2 ratio in mesenteric arteriolar bed.

	6-keto-$PGF_{1\alpha}$ level (pg/mL)	**TXB_2 level (pg/mL)**	**6-keto-$PGF_{1\alpha}$ /TXB_2 ratio**
con	536.9±53.3	29.4±4.3	18.4
DM	407.6±37.1*	58.4±5.8*	7.0*
DM+cur300	502.3 ± 27.6^{NS}	47.4 ± 7.2^{NS}	10.7^{NS}

NS, not significant difference compared to control arterioles

*$P<0.05$ significant difference compared to control arterioles

Table 4.6 showed the ratio of 6-keto-$PGF_{1\alpha}$ to TXB_2 release in mesenteric arteriolar bed. These ratios seem to be decreased in DM rats compare to control rats. (7.0 and 18.4, respectively). These results suggested that DM rats release more TXB_2 level than 6-keto-$PGF_{1\alpha}$ level. On the other hand, DM+cur group increased this ratio to 10.7. Therefore, supplementation with curcumin could increase 6-keto-$PGF_{1\alpha}$ whereas decreased TXB_2 level.

Expression of PKC-βII in mesenteric artery

Immunofluorescent staining of small mesenteric arteries displayed a strong signal for PKC-βII in DM rat (Figure 4.6C). In contrast, the TRITC signals of anti-PKC-βII antibodies were weak in control and DM+cur rat vessels (Figure 4.6B and 4.6D, respectively). Negative control displayed a minimal detectable fluorescence when the secondary antibodies were used alone (Figure 4.6A). Double-immunofluorescent staining with specific antibodies for PKC-βII and α–smooth muscle actin showed that the diabetic mesenteric arteries also contained PKC-βII in the vascular smooth muscle cells (Figure 4.7). These results suggested that mesenteric vessels in DM had higher levels of PKC-βII both in endothelium and smooth muscle cells. Interestingly, supplementation with curcumin (300 mg/kg) could reduce PKC-II expression in DM+cur comparable to control rats.

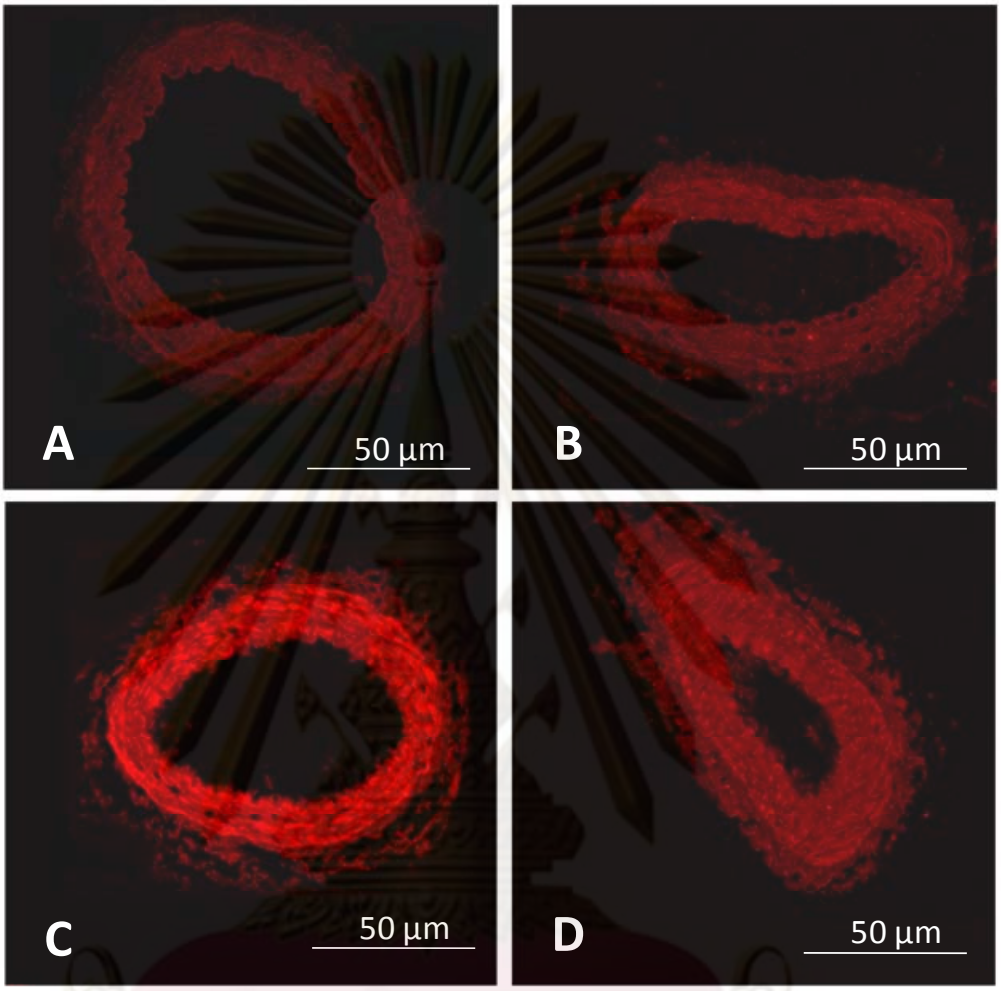


Figure 4.6 Immunofluorescent staining of PKC-βII in the mesenteric arteries of control (B), DM (C) and DM+cur (D) rats. Respective negative controls is displayed on the left upper panel (A). Magnification: x400.

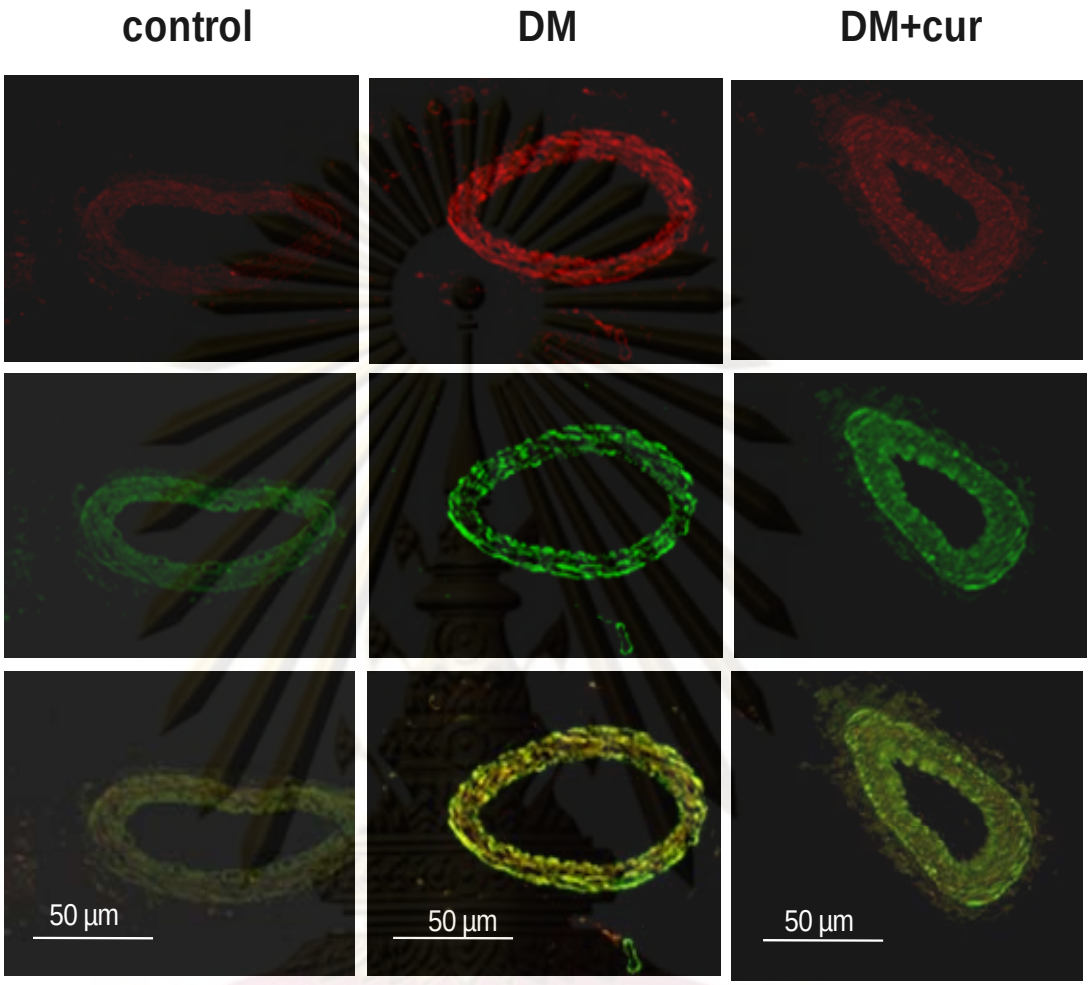


Figure 4.7. Coimmunofluorescent staining for PKC-βII and vascular smooth muscle in DM rats. Double-immunolabeled was performed to demonstrate PKC-βII in the mesenteric arteries (red fluorescence; A), α-smooth muscle actin (green fluorescence; B) and merged image (yellow; C). Magnification: x400.

Histological study

A histological study was performed by hematoxylin and eosin staining in paraffin sections by the standard method of Department of Pathology, Faculty of Medicine, Chulalongkorn University. Figure 4.8 shows the characteristic features in mesenteric artery.

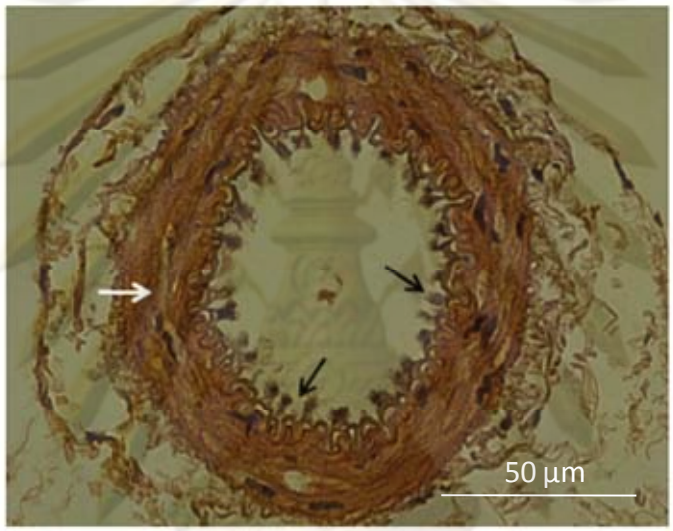


Figure 4.8. Histological features in small mesenteric artery. Section of arterial segment showed the endothelial cell layer inwards (black arrows) and smooth muscle cell layer (white arrow). Magnification: x400.

Expression of COX-2 in mesenteric artery

In control mesenteric arteries, a weak COX-2 immunopositive label was detected (Figure 4.9- left upper panel). In contrast, a marked enhancement in COX-2 immunonstaining was observed in endothelial cells in vessels from DM rats (Figure 4.9- right upper panel). As expected, supplementation with curcumin could reduce COX-2 expression in mesenteric microvessels (DM+cur30 and DM+cur300), as shown in Figure 4.9- lower panels), although these reduced COX-2 expression seem to be not completely abolishing the COX-2 expression in DM arteries. However, these results suggest that curcumin could inhibit COX-2 expression in mesenteric endothelial cells in treated-DM rats.

A quantitative comparison using image analysis (Global Lab II software) was used to allow for measurement of the COX-2 expression. Figure 4.10 demonstrated the mean COX-2 expression of low and high doses of curcumin supplementation (DM+cur30; 5.59±2% and DM+cur300; 3.55±1%, respectively) significantly decreased as compared to DM rats (12.41±.2%).

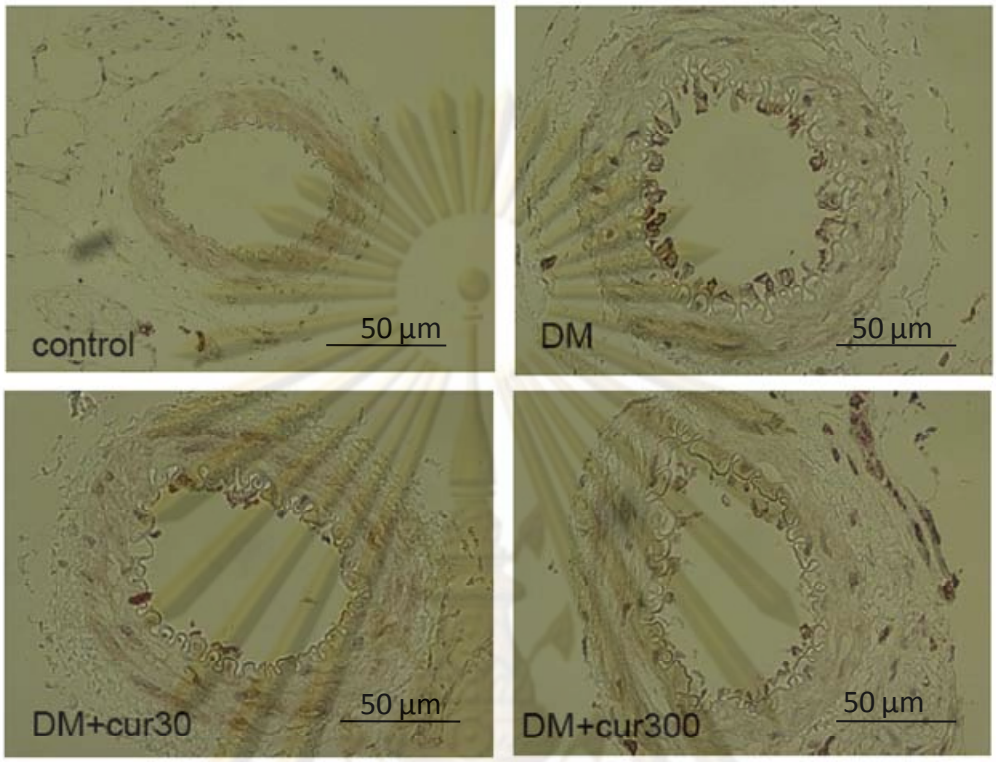


Figure 4.9. Immunostaining for COX-2 in small mesenteric arteries. Sections of arterial segments show the endothelial cell layer inwards. Positive immunoreactions are observed as brown precipitate. Magnification: x400.

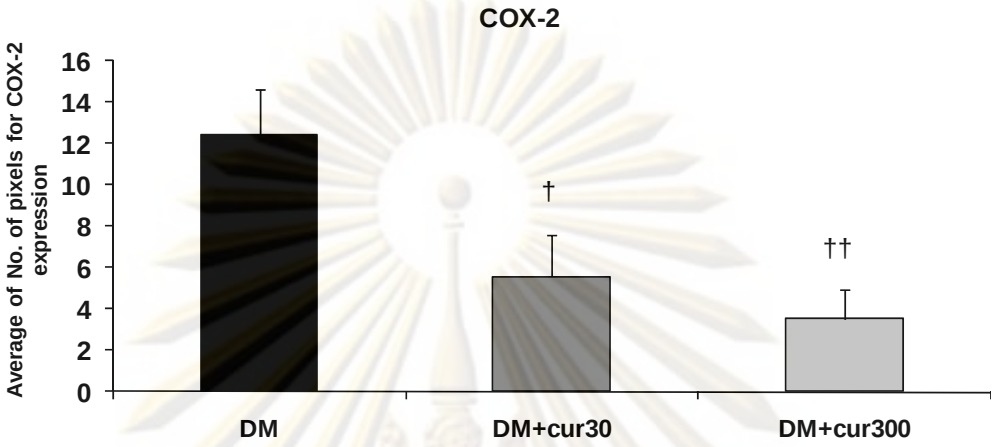


Figure 4.10. Expression of COX-2 in endothelium layer in small mesenteric arteries using image analysis (GLI/2) measurement in diabetes (DM) and diabetes treated with curcumin 30 mg/kg (DM+cur30) and 300 mg/kg (DM+cur300) group.

Data are expressed as mean±SEM (n=4 for each group).

† $P<0.05$ significant difference compared to diabetic arteries

†† $P<0.01$ significant difference compared to diabetic arteries

Expression of NF-κB in mesenteric artery

Figure 4.11 showed the expression of inflammation-related factor (NF-κB) in small mesenteric arteries in con, DM, DM+cur30 and DM+cur300 rats. NF-κB p65 was more detectable in the DM arteries than in the control arteries. Both doses of curcumin supplementation (DM+cur30 and DM+cur300) were little immunoreactivity of these NF-κB p65 compared with untreated DM rats. These results suggested that curcumin could inhibit NF-κB activity in mesenteric endothelial cells in treated-DM rats.

A quantitative comparison using image analysis (Global Lab II software) was used to allow for measurement of the NF-κB p65 expression. Figure 4.12 demonstrated the mean NF-κB p65 expression of high doses of curcumin supplementation (DM+cur300; 7.13±0.4%, $P<0.05$) significantly decreased as compared to DM group (19.07±.4%). However, the supplementation of curcumin in DM+cur30 (11.78±2%) did not difference in NF-κB p65 expression as compared to diabetes group.

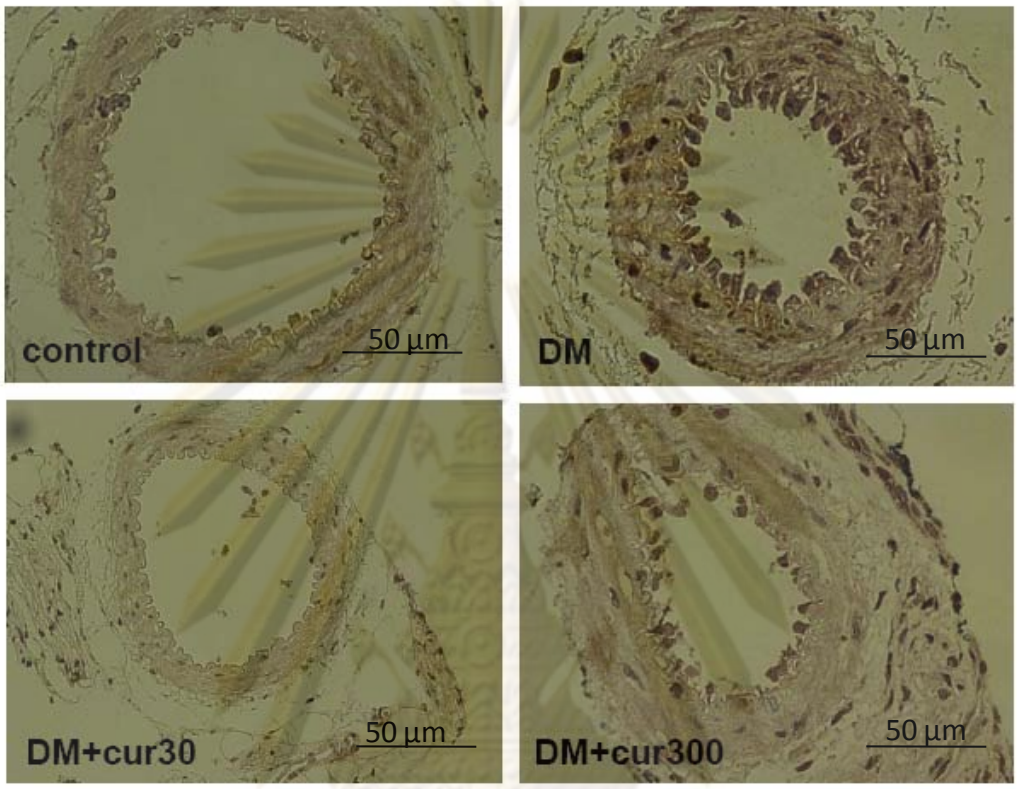


Figure 4.11. The expression of nuclear factor-κB p65 (NF-κB p65) in small mesenteric arteries in control (left upper panel), DM (right upper panel), DM+cur30 (left lower panel) and DM+cur300 (right lower panel) rats. Positive immunoreactions are observed as a brown precipitate. Magnification: x400.

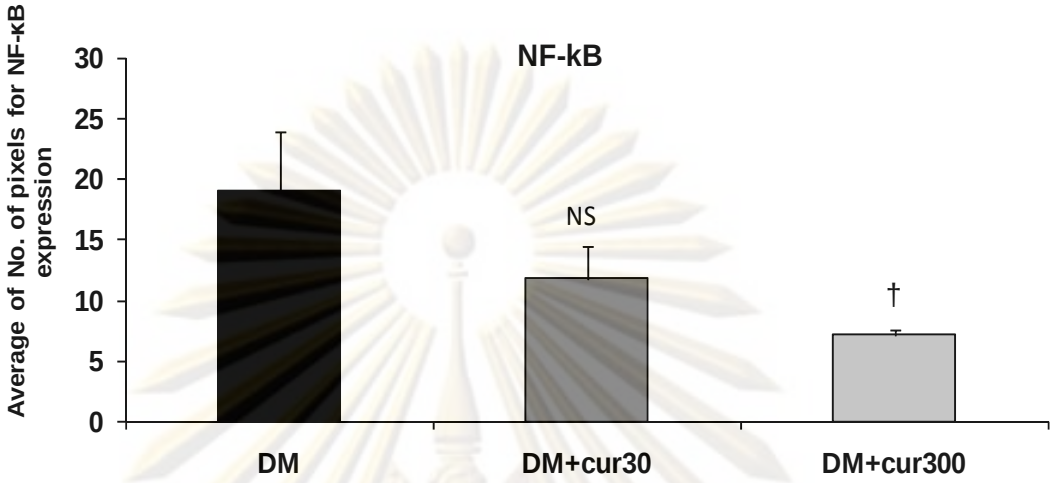


Figure 4.12. Expression of NF-κB in endothelium layer in small mesenteric arteries using image analysis (GLI/2) measurement in diabetes (DM) and diabetes treated with curcumin 30 mg/kg (DM+cur30) and 300 mg/kg (DM+cur300) group.

Data are expressed as mean±SEM (n=4 for each group).

NS, not significant difference compared to diabetic arteries

† $P<0.05$ significant difference compared to diabetic arteries

CHAPTER V

DISCUSSION

The new findings from the present study are that the effects of curcumin supplementation on improving diabetes-induced endothelial dysfunction in STZ-induced diabetic rats. The beneficial effects of curcumin are associated with its potential on; 1) antioxidant property, especially superoxide scavenger, 2) anti-inflammatory property via suppression of NF-κB and COX-2 and 3) PKC inhibitory property.

In the present study, we have shown that STZ-injection rats demonstrated typical characteristic of diabetes mellitus; such as hyperglycemia, polyuria, polydipsia and polyphagia and weight loss. In diabetes, there is hyperosmolarity in urine which is developed by a large amount of secreted glucoses and then increased urinary volume (polyuria). Following dehydration and the hyperosmolarity of the body fluids stimulates the osmoreceptors at the thirst center in brain causing polydipsia (Smith and McFall, 2005). In type1 diabetes, insulin deficiency promotes protein catabolism which lead to a negative energy balance, and in turn induces the excessive intake (polyphagia) (Cotran, 1999). Also weight loss was found in DM rats significantly decreased compared with control (285.3±8.8 and 398.7±12.3 g, respectively, $P<0.01$).

Role of curcumin in anti-diabetic action in diabetes

Twelve weeks after STZ injection, diabetic rats exhibited increased plasma glucose compared non-diabetic rats. Our findings were consistent with others that treatment with curcumin in diabetic rats leading to lower plasma glucose level (Patumraj *et al*., 2006; Sharma *et al*., 2006). Supplementation of curcumin 30 and 300 mg/kg per day could lower blood glucose in diabetic group down to 18.73% and 30.26%, respectively. Thus, curcumin exerted the anti-diabetic effect could be detected at low dose of curcumin (30 mg/kg) for six-week oral daily administration (Table 4.2).

Moreover, oxidative stress as a consequence of hyperglycemia and changes in energy metabolism and inflammatory mediators play an important role in the pathophysiology of diabetes in association with depleted cellular antioxidant defense systems and enhanced production of ROS (Tesfamariam, 1994). Our findings have indicated that an increased superoxide production in diabetic vasculature and these effects could be attenuated by daily oral curcumin supplemention (discuss below). In addition, the antioxidant effect of curcumin in this study may be partially attributed by the hypoglycemic effect of curcumin as well. It has been suggested that curcumin might act by sparing or enhancing the function of the endogenous antioxidants (Mahesh *et al*., 2004). In line with these findings, Sharma, *et al*. (2006) showed that chronic administration of curcumin in diabetic rats significantly reversed the decrease in the antioxidant enzymes superoxide dismutase (SOD) and catalase and reduced glutathione (GSH) in the kidney. Antioxidant activities of curcumin might occur synergistically with glucose-lowering activity. Curcumin was also found to increase insulin level (Halim and Hussain, 2002). These findings are consistent with the suggestion that the anti-diabetic action of curcumin seems to be mediated through the stimulation of the pancreas to produce and secrete insulin (Menon and Sudheer, 2007).

Recently, the mechanisms of curcumin mediate anti-diabetic effects have been examined. The anti-diabetic effects of curcumin have been shown to be partially attributed by the reduction hepatic glucose production in isolated hepatocytes (Fujiwara *et al*., 2008). These results have demonstrated that curcumin inhibited both hepatic gluconeogenesis and glycogenolysis by suppressing both hepatic glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK). The AMP kinase was involved in the activate cascade by curcumin. The action of AMP protein kinase is known to suppress gene expression of G6Pase and PEPCK and to inhibit hepatic glucose output (Lochhead *et al*., 2000; Yamauchi *et al*., 2002).

In consistent with increased plasma glucose level, diabetic rats also showed higher HbA1c in twelve-week diabetic development. These HbA1c was increased up to three-fold higher than normal HbA1c. Interestingly, the supplementation of curcumin in DM+cur30 and DM+cur300 groups significantly lower HbA1c values as compared to DM group.

It has long been suspected that high glucose is harmful in all complications, including microvascular and macrovascular. In recent times, glycosylated hemoglobin has been established as the marker of assessing glycemic control in diabetic subjects. Glycated hemoglobin is formed by a post-traslational, non-enzymatic, substrate-concentration dependent irreversible process of combination of aldehyde group of glucose with the amino-terminal valine of the chain of hemoglobin (Chandalia and Krishnaswamy, 2002). The major component of hemoglobin (Hb) is HbA_0 ($\alpha_2\beta_2$) comprises over 90% the total protein, with the other two minor components being HbA_2 ($\alpha_2\delta_2$) and HbF ($\alpha_2\gamma_2$) with non-α units being products of different globin genes. Hemoglobin A1c (HbA1c) is the most abundant minor component, arising from post-translational modification of HbA_0 (Bunn et al., 1978). While high concentration of glucose in the environment, under diabetes condition, brings about the reaction on account of the carbonyl groups on sugar forming Schiff base adduct with amino groups of protein. Thus HbA1c represents the formation of a non-enzymatical glycosylated form of human hemoglobin (Dixon, 1972). Traditionally, HbA1c has been thought to represent average glycemia over the past 12 to 16 weeks (Shaklai *et al*., 1984). In fact, glycation of hemoglobin occurs over the entire 120-day life span of the red blood cell (Bunn *et al*., 1978). Indeed, theoretical models and clinical studies has been suggested that a patient in stable control will have 50% of their HbA1c formed in the month before sampling, 25% in the month before that, and the remaining 25% in months two to four prior to sampling (Tahara and Shima, 1995). Thus, one patient with wildly fluctuating glucose concentrations could have the same HbA1c value as one whose glucose varies little throughout the day. Our findings provided the evidence that curcumin was a good anti-diabetic action by six-week daily curcumin supplementation (DM 10.73%, DM+cur30 8.20% and DM+cur300 7.9%, $P<0.05$, Table 4.2).

The antioxidant effect of curcumin on diabetes-induced endothelial dysfunction

In the present study, we have shown that the effect of curcumin supplementation on restoring diabetes-induced endothelial dysfunction is closely associated with its antioxidant property. The supplementation of either low or high doses of curcumin appears to improve the diabetic endothelial dysfunction as shown by the increase in ACh-activated vasodilatation (Figure 4.1 and Table 4.3). However, there was no significant difference between restoring effect of both low and high doses. In contrast with this increased ACh-vasorelaxation, the endothelium-independent relaxation to the NO donor, SNP, was not affected by neither diabetes nor curcumin supplementation (Figure 4.2 and Table 4.3). Therefore, it implied that during this twelve-week STZ-induced diabetes, the NO-stimulated downstream cGMP/PKG signaling in arteriolar smooth muscle was not a primary affected target in diabetic condition.

In the present study, hemodynamic parameter was observed in unconscious rats. Using direct measurement via carotid arterial cannulation, we found a significant rise in mean arterial blood pressures in DM rats compared with non-diabetic rats (Table 4.1). These results were in accordance with previous clinical observations of the prevalence of high blood pressure in patients with diabetes mellitus (Szerafin *et al*., 2006). Mean arterial blood pressure was significantly decreased in curcumin-treated DM rats compared with DM rats. Our present data suggest that a lower systemic blood pressure is associated with reducing high glucose and increasing ACh-activated vasodilatation in DM+cur300 rats.

It is well established that hyperglycemia can produce ROS production by a series of cellular events and further leads to diabetic complications through oxidative stress (Brownlee, 2001; Guzik *et al.*, 2002). The increased dibetes-induced ROS was indirectly demonstrated by using lipid peroxidation end-products, malondialdehyde (MDA) level, as an indicator (Jariyapongskul *et al*., 2002; Patumraj *et al*., 2006; Sharma *et al*., 2006). In particular, one type of ROS, superoxide anion ($O_2^{\bullet -}$), can be generated by several enzymatic or chemical systems, mainly in vascular wall through the activation of NADPH oxidase (Inoguchi *et al.*, 2000; Pacher *et al.*, 2007). Moreover, it is believed that the sustained production of $O_2^{\bullet -}$ at high levels will lead to oxidative damage to diabetic blood vessels (Inoguchi *et al.*, 2000; Wolin *et al.*, 2002). The increased $O_2^{\bullet -}$ in diabetic vasculature can inactivate nitric oxide (NO) and lead to the impairment of vasorelaxation

to endothelium-dependent agonists, since $O_2^{\bullet-}$ is the precursor of hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and other strong oxidizing species (Mugge *et al*., 1991).

In order to examine the dynamical process of such diabetes-stimulated ROS production and its corresponding with endothelial dysfunction, our present study was conducted by hydroethidine-sensitive vascular superoxide detection. One of the most frequently used assays for the detection of intracellular $O_2^{\bullet-}$ production is the detection method with hydroethidine as an intracellular probe (Carter *et al*., 1994; Bizyukin and Soodaeva, 1995). In the presence of $O_2^{\bullet-}$, hydroethidine is rapidly converted to ethidium bromide, which binds to DNA and is detected by its red fluorescence light with minimal oxidation induced by H_2O_2, $ONOO^-$, or $HOCl^-$ (Benov *et al*., 1998). The *in situ* nuclei label with ethidium bromide along arteriolar wall could be observed and the amount of EB-positive nuclei could be quantitatively estimated for each arteriole as shown in Table 4.4 and Figure 4.3.

The results showed that superoxide production along the arteriolar wall of DM rats was about 4.4 times higher than control. This excessive increased vascular superoxide could destroy vascular endothelial lining, causing the decreased in ACh response at about 0.67 times less than control.

The increase in hyperglycemia induced oxygen-derived free radicals was believed as a major contributor in reducing NO bioavailability in diabetes. The interaction between NO and $O_2^{\bullet-}$ occur at an extremely rapid rate, three times faster that the reaction rate for $O_2^{\bullet-}$ with SOD (Benz *et al.*, 2002). Therefore, this hyperglycemia induced $O_2^{\bullet-}$ may quench NO and cause the inefficacy of endothelium-dependent vasodilatation (Meininger *et al*., 2000; Jariyapongskul *et al.*, 2003; Johnston and DeMaster, 2003).

Interestingly, our findings have indicated that this increased $O_2^{\bullet-}$ production in diabetic vasculature could be attenuated by daily oral curcumin supplementation. In addition, the results also showed that both low and high doses of curcumin could decrease diabetic vascular superoxide production down to 44.9% and 42.9%, respectively. However, there was no difference between low and high doses of curcumin on reducing superoxide production at diabetic vascular wall. Simultaneously, they could increase ACh-activated vasodilatation up to 30.22% and 46.47%, respectively. In order to confirm the effect of curcumin action on $O_2^{\bullet-}$ productoon, the correlation between the HE-sensitive

superoxide production and ACh-induced arteriolar vasodilatation for all five groups was examined. As the result shown in Figure 4.4 confirmed the strong correlation between both parameters with the correlation coefficient, 0.78, and $P<0.01$.

Even though, our study did not monitor the NO production directly, however, it can be demonstrated by endothelial-dependent vasodilatation and implied that curcumin supplementation could enhance endothelial-dependent relaxation in diabetic rat. By using *in vitro* chemical reaction testing, the mechanism of curcumin on protecting NO against oxidation by sequestering the reaction intermediates was suggested by Johnson, *et al.* (2003). The IC_{50} for curcumin with $1.0x10^{-6}$ mol/L DEA/NO was calculated to be $13x10^{-6}$ mol/L (Johnston and DeMaster, 2003). Moreover, they also suggested that the ability of curcumin was actually attributed by its ability to sequester NO_2, but not NO. Therefore, curcumin supplementation may be useful for enhancing NO bioavailability in various biological systems.

In another study was investigated the treatment of diabetic animals with curcumin prevents diabetes-induced NOS up-regulation and oxidative stress by Farhangkhoee, *et al* (2006). Treatment of human microvascular endothelial cells (HMECs) exposed to $25x10^{-6}$ mol/L glucose to varying curcumin concentrations (0.1, 10 and 100 $x10^{-6}$mol/L) prevented both eNOS and iNOS expression and transcription factor activity (Farhangkhoee *et al*., 2006). Also, oxidative protein and DNA damage were assessed by immunohistochemical analysis of nitrotyrosine and 8-hydroxy-2'-deoxyguanosine (8-OHdG). These findings demonstrated that curcumin prevented NOS alteration and oxidative stress in a dose-dependent manner which was mediated by NF-κB and activating protein-1 (AP-1). In line with these findings, our previous study also suggested that topical application of curcumin (10^{-6} mol/L) significantly increased the dilatory response to ACh in mesenteric arteriole in STZ-induced diabetes rats (Rungseesantivanon *et al.*, 2007).

Even though, the molecular mechanisms of how curcumin could decrease $O_2^{\bullet-}$ and promote NO bioavailability still need to be explored in detail. Nevertheless, our results have warranted that anti-diabetic and antioxidant properties of curcumin are significantly related to its potential on improving endothelial-dependent vasodilatation.

Curcumin has been reported as a potent scavenger of a variety of ROS (Reddy and Lokesh, 1994). In several investigations, antioxidant activities of curcumin have been studied both *in vitro* and *in vivo*. Curcumin has a unique conjugated structure including two methoxylated phenols and an enol form of β–diketone, and the structure shows a typical radical trapping ability as a chain-breaking antioxidant.

Generally, the nonenzymatic antioxidant process of the phenolic material is thought to be divided into the following two stages (Frankel, 1998):

(1) radical trapping stage $S\text{-}OO^{\bullet} + AH \rightleftharpoons SOOH + A^{\bullet}$

(2) radical termination stage $A^{\bullet} + X^{\bullet} \longrightarrow$ non radical materials

where S is the substance oxidized, AH is the phenolic antioxidant, $A^{\bullet}$ is the antioxidant radical, and $X^{\bullet}$ is another radical species or the same species as $A^{\bullet}$. Non radical materials such as A-A (dimer of A), AOOS (substrate antioxidant peroxide), and Aox (oxidized A).

Although the first stage is a reversible process, the second stage is irreversible and must produce stable radical termination compounds. The chemical structure of such a termination compound would give important information in order to elucidate the antioxidant mechanism of the chain-breaking antioxidant. The antioxidant mechanism based on the chemical structures of the radical termination products, Masuda, *et al* (2001) have demonstrated that curcumin reacted with the peroxyl radicals of linoleate to produce stable cyclic compounds by a unique pathway through the AOOS termination step (Masuda *et al*., 2001). They also found that radical reaction of curcumin under non-lipidic conditions gave a dimer as an A-A termination compound. From the quantitative data for the production of these termination products, the formation rate of the AOOS termination products was almost constant regardless of the curcumin concentration, while the rate of the dimer production depended on the concentration (Masuda *et al*., 2002).

Recently, curcumin has attracted much attention due to its significant beneficial potential. Ak and Gulcin (2008) determined the antioxidant activity of curcumin by employing various *in vitro* antioxidant assays including, superoxide anion radical scavenging when compared to standard antioxidant compounds such as BHA, BHT, α-tocopherol, a natural antioxidant and trolox (Ak and Gulcin, 2008). Curcumin was found to be a marked antioxidant effect in linoleic acid emulsion. In addition, curcumin had an effective ferrous ions (Fe^{2+}) chelating, H_2O_2 scavenging and $O_2^{\bullet-}$ scavenging capacities. Reactive radicals scavenging and antioxidant activity of curcumin was interpreted as originating by H-atom abstraction from the free hydroxyl group. These results suggested that H-atom donation from phenolic group which was responsible for the superb antioxidant properties of curcumin.

In recent study, the antioxidant activities of curcumin, the demethoxy derivatives (Dmc and Bdmc) and metabolite hydrogenated derivatives (THC, HHC and OHC) have been compared using three *in vitro* models; DDPH, AAPH induced linoleic oxidation and AAPH induced red blood cell hemolysis assays (Somparn *et al.*, 2007). The DPPH scavenging assay is a widely used method to primarily evaluate free radical scavenging activity. The effects of antioxidants on DPPH stable radical are thought to be due to their hydrogenating ability (Chen *et al*., 2006). Another assays, AAPH induced linoleic acid peroxidation and RBC hemolysis were used as lipid peroxidation and biomembrane damage models, respectively. These results in all models demonstrated the lower antioxidant activity of the demethoxy derivatives, suggesting the ortho-methoxyphenolic groups of curcumin are involved in antioxidant activities. On the other hand, hydrogenated derivatives of curcumin showed a remarkably higher activity than curcumin, suggesting that the hydrogenation at conjugated double bonds of the central seven carbon chain and β–diketone of curcumin improved antioxidant activities (Somparn *et al*., 2007).

The anti-inflammatory effect of curcumin on diabetes-induced endothelial dysfunction

In the next experiments, we aimed to elucidate the possible underlying mechanisms responsible for the impair vasodilatation in diabetes. Recently, a key role for chronic, low-level vascular inflammation has received great attention in the development of diabetic vascular complications (Libby *et al*., 2002; Helmersson *et al.*, 2004). Among other factors, prostaglandin are important mediators of several inflammatory mechanisms (Higgs *et al*., 1984), however, it is also known that many prostaglandin derivatives have specific vasoactive properties, particularly contributing to the local regulation of arteriolar diameter (Koller and Kaley, 1990; Koller and Kaley, 1991). In recent study, a role for prostanoid-mediated vascular inflammation has been shown to be associated with the development of vascular complications in type2 diabetes (Helmersson *et al*., 2004; Bagi *et al.*, 2005; Szerafin *et al.*, 2006). Prostanoids generated by prostaglandin H synthase (PGHS), also knonw as prostaglandin endoperoxide synthase or cyclooxygenase. PGHS is a rate-limiting enzyme that exhibits a cyclooxgenase activity that incorporates two molecules of oxygen into arachidonic acid to form PGG_2 and a peroxidase activity catalyzing a 2-electron reduction of PGG_2 to PGH_2. Cell-specific isomerization of PGH_2 produces biologically active endproducts, such as prostacyclin (PGI_2) and thromboxane (TXA_2) (Davidge, 2001). Two isoforms of the cyclooxygenase enzyme (COX), were encoded by distinct genes. COX-1 is constitutively expressed in most tissues, such as vascular endothelial cells, and is involved in the maintenance of cellular homeostasis (Davidge, 2001). In contrast, under normal conditions, COX-2 is expressed only at low or undetectable levels but it readily upregulated by inflammatory, mitogenic and physical stimuli (Parente and Perretti, 2003). Early investigations reported enhanced release of a constrictor prostanoid from diabetic vessels (Sterin-Borda *et al.*, 1984). Also, PGH_2/TXA_2 receptor antagonist increased the diameter of arterioles of T2 DM mice back to control levels, whereas it did not affect the diameter of vessels from control animals. In agreement with our previous study also suggested that increased vascular tone in mesenteric arterioles from diabetic rats could inhibited by SQ29548 (PGH_2/TXA_2 receptor antagonist) (Rungseesantivanon *et al*., 2006). These findings indicated that endogenous release of constrictor prostanoid, PGH_2/TXA_2, might be responsible for the reduced diameter of arterioles from diabetes.

In our study, the un-stimulated (basal) level of 6-keto-$PGF_{1\alpha}$ (stable metabolite of PGI_2) in diabetic rats was markedly reduced compared with those of control rats. While curcumin enhance 6-keto-$PGF_{1\alpha}$ production up to almost basal level of control rats (Table 4.4 and Figure 4.6A). On the other hand, the basal levels of TXB_2 (stable metabolite of TXA_2) in DM rats were significantly elevated as compared with those of control rats. Compared with untreated DM rats, we have found a slightly increased TXB_2 level in DM+cur rats. These findings suggested that in mesenteric arterioles of diabetes prostaglandin release predominantly via COX-2 which produce more TXA_2 than PGI_2 level. In addition, we also found a marked COX-2 immunostaining in mesenteric arteries of DM animals, which localized in endothelial layer of arterial wall (Figure 4.9 and 4.10). These results are in accordance with other study obtained in both diabetic animal model (Bagi *et al.*, 2005) and human diabetes (Szerafin *et al*., 2006).

Furthermore, we found a decreased PGI_2/TXA_2 ratio in DM animals. These data might indicate that a higher activation of COX-2 has occurred in diabetic rats. Interestingly, supplementation with curcumin could improve the ratio of prostanoids change to higher PGI_2 than TXA_2 level (Table 4.6). According to prostanoid levels, slightly appear immunostaining for COX-2 was detected in arteries of DM supplement with curcumin (Figure 4.9). Thus, curcumin has been found to be effective in inhibiting TXA_2 synthesis in inflammatory by modulating the COX-2-pathway.

Collectively, our results showed that curcumin supplementation significantly decreased diabetic vascular superoxide production, simultaneously the suppression of COX-2 expression and augmented PGI_2 levels in mesenteric vessels. In line with our findings, curcumin increased the level of PGI_2 (Srivastava, 1986)

Recent studies have demonstrated that the eukaryotic transcription factor nuclear factor-κB (NF-κB) was involved in the regulation of COX-2. Surh, *et al.* (2001) studied the molecular mechanism underlying the anti-inflammatory activity of curcumin. They suggested the downregulation of COX-2 through suppression of NF-κB. Repression of the degradation of the inhibitory unit IκBα, which hampers subsequent nuclear translocation of the functionally active subunit of NF-κB, might be responsible for the inhibition of NF-κB by curcumin (Surh *et al.*, 2001; Nanji *et al.*, 2003). Our findings showed that immunohistochemical analysis of DM rats reveal an enhanced expression of COX-2 and NF-κB p65 (Figure 4.9 and 4.11) localized in the endothelial layer, which was markedly suppressed by a curcumin supplementation. Several the effects of curcumin are consistent with its ability to inhibit the activity of NF-κB. The modulatory potential of curcumin on NF-κB signaling pathways prevent phosphorylation of IκB by inhibiting the IκB –kinases (Iκκs) (Jobin *et al.*, 1999; Plummer *et al.*, 1999).

Another important molecular mechanism by which curcumin regulates the COX-2 is through the modulating of NO/iNOS. Some studies suggested that COX-mediated arachidonic acid oxygenation (by free radical mechanisms) requires $NO/ONOO^-$ for catalysis (Marnett *et al.*, 1999; Marnett *et al.*, 2000). It is possible that curcumin might directly act on the arachidonic acid oxygenation or reaction nitrogen species due to its antioxidant property (Rao, 2007).

The PKC inhibitory effect of curcumin on diabetes-induced endothelial dysfunction

However, the molecular mechanisms responsible for the up-regulation of COX-2 linking oxidative stress and endothelial dysfunction in diabetes remain to be clearified. A key role for high glucose concentrations on COX-2 expression has been previously proposed in *in vitro* experiment. It has been found that glucose augmented membrane-bound PKC activity. Furthermore, phorbol-12-myrostate-13-acetate (PMA), PKC activator, increased COX-2 expression to a similar as high glucose. These effects of glucose and PMA were totally reversed by the PKC inhibitor, calphostin C (Cosentino *et al.*, 2003). Phorbal esters mimic the action of second messenger DAG with respect to binding to specific motifs within the PKC regulatory domain and consequently, stimulating catalytic activity (Nishizuka, 1992). Indeed, DAG production in response to physiological factors is transient due to rapid metabolic conversion of DAG. In contrast, phorbal esters are metabolically stable in the cells. As a consequence, PKC activation by phorbal esters is much more prolonged than the transient activation that occurs with physiological regulators. Interestingly, the promoter region of the human eNOS gene contains a phorbal ester responsive element (Marsden *et al.*, 1993). In normal blood vessels, PKC activation by phorbal ester impairs endothelium-dependent relaxation via release of and vasoconstrictor prostanoids (Tesfamariam *et al.*, 1991). The adverse effects of high glucose level on ACh-induced relaxation of rabbit aorta and rat pial arterioles can be restored by PKC inhibitors (Tesfamariam *et al.*, 1991; Mayhan and Patel, 1995). Also, *in vivo* treatment with PKC inhibitor ameliorates vascular complications in diabetic rats (Ishii *et al.*, 1996).

In the present study, we demonstrate that high glucose cause PKC-dependent up-regulation of COX-2 (Figure 4.6C, 4.7 and 4.9) as well as selective increase of TXA_2 production (Table 4.5 and Figure 4.5B) and decreased of PGI_2 (Table 4.5 and Figure 4.5A) and reduced ACh-induced vasodilatation (Table 4.3 and Figure 4.1). Cosentino, *et al.* (2003) provided the evidence for an involvement of PKC in COX activation by glucose. This elevation glucose increased the expression of COX-2. This effect was also elicited by PMA as well as abolished by calphostin C, PKC inhibitor. In addition, glucose-induced COX-2 upregulation was associated with a shift in the balance of vasodilatory and vasoconstricting prostanoids produced by the endothelial cells in favor of the latter. This

imbalance of prostanoids leads to alter prostanoid-mediated vasodilatation. However, increased formation of the NO/ $O_2^{\bullet-}$ reaction produce $ONOO^-$ was associated with tyrosine nitration of prostacyclin synthase (PGIS). This nitration is a mechanism of selective inactivation of PGIS by $ONOO^-$ (Zou *et al.*, 1997; Zou and Bachschmid, 1999). Hink, *et al.* (2001) addressed a role of PKC in eNOS expression and vascular superoxide production. *In vitro* PKC inhibition with chelerythrine, PKC inhibitor, reduced vascular $O_2^{\bullet-}$ in diabetic vessels by chemiluminescence. *In vivo* PKC inhibition did not affect glucose levels in diabetic rats but prevent NOS-mediated $O_2^{\bullet-}$ production, thereby restoring vascular nitric oxide bioavailability and endothelial function. In agreement with Yakubu, *et al*. (2004), also demonstrated that glucose-induced attenuation of vascular relaxation involves PKC linked free radical generation. Pre-incubation of aortic ring from Sprague-Dawley rats with calphostin C, PKC inhibitor, in the high glucose level restored the ACh response relaxation. The reduced NO production was also restored by pretreatment with calphostin C by the Griess method (Yakubu *et al.*, 2004). In previous study, we provided evidence that application of chelerythrine, PKC inhibitor, improve ACh-induced vasodilatation in mesenteric arterioles from diabetic rats (Rungseesantivanon *et al.*, 2006). These findings indicate that PKC-dependent mechanism is the triggering step by which hyper glycemia also induces oxidative stress and in turn endothelial dysfunction.

Experimental evidence demonstrates that PKC is one of a group of cell-signaling molecules that are sensitive targets for redox modificaiton (Gopalakrishna *et al.*, 1995). The structure and function of PKC domains have been extensively studied. All PKC consist of *N*-terminal regulatory domains and *C*-terminal catalytic domains.

In resting cells, PKC assume an inactive conformation. This is maintained by an intramolecular interaction between an autoinhibitory sequence (the pseudosubstrate) in the regulatory domain and the substrate binding region of the catalytic domain. Activation is triggered by receptor-mediated stimulation of phospholipase C, which generates second messenger DAG. There is evidence that other lipids such as arachidonic acid can also stimulate PKC activity (Nishizuka, 1992). DAG binds to PKC regulatory domains, increases the affinity of PKC for membrane lipids and consequently stabilizes PKC membrane association. Several unique structural motifs of PKC make it a direct target for oxidants as well as antioxidants. Both the regulatory and catalytic domains of PKC contain cystein-rich regions that are targets for redox regulation (Gopalakrishna *et al.*, 1995).

Interestingly, the *C*-terminal catalytic domains are targets for chemopreventive antioxidants such as selenocompounds and polyphenolic agents such as curcumin (Boone *et al.*, 1990) to inhibit cellular PKC activity (Gopalakrishna and Jaken, 2000). Curcumin is a polyphenolic compound. It inhibited phorbal ester-induced expression of *c*-jun, *c*-fos, and NF-κB activation (Lu *et al.*, 1994). These results suggested that the activities of curcumin are mediated through interference with PKC signaling. Our findings demonstrated that markedly inhibition of PKC by curcumin supplementation (Figure 4.6 and 4.7). It is consistent with oxidized curcumin is a potent PKC inhibitor (IC_{50} = 3 μM) (Chen *et al.*, 1996). Collectively, these results suggest that as phenolic antioxidant, curcumin are converted to their oxidized state, this ability to react with the catalytic domain and thereby, inactivate PKC activities.

In conclusion, the findings indicated that endothelial dysfunction in diabetic rats was associated with increased intracellular superoxide production, PKC-βII and NF-κB activation and COX-2 up-regulation. Importantly, our findings provided the *in vivo* evidence that curcumin supplementations (30 mg/kg and 300 mg/kg) improved diabetes-induced endothelial dysfunction significantly related to its potential to $O_2^{\bullet-}$ scavenging, COX-2 and NF-κB suppression, and PKC inhibition. It is possible that curcumin supplementation might be beneficial for diabetic patients by improving microvascular functions and preventing the consequence of cardiovascular complications. Therefore, curcumin might be considered as a pharmaceutical/phytoceutical agent used for treatment diabetes vascular complication in diabetes patients in the future.

The proposed mechanisms for the effects of curcumin on diabetes-induced endothelial dysfunction: role of PKC, COX-2 and NF-κB

The proposed mechanisms of curcumin restore diabetes-induced endothelial dysfunction through its antioxidant, anti-inflammatory and PKC inhibitory actions (Figure 5.1) as follow:

1) The anti-diabetic action of curcumin reduced hyperglycemia.

2) The antioxidant of curcumin reduced intracellular ROS through its $O_2^{\bullet-}$ scavenger.

3) Decreased $O_2^{\bullet-}$ production, in turn, reduced PKC activation. Simultaneously, the PKC inhibitions of curcumin inactivated PKC activities at catalytic domain of PKC.

4) The curcumin inhibition of NF-κB activation was accompanied by the inhibition of p65 translocation to the nucleus and IκBα degradation.

5) The anti-inflammation of curcumin downregulated COX-2 through suppression of COX-2 activity and NF-κB expression and PKC activities.

6) Curcumin restored arteriolar vasodilatation via enhanced NO bioavailability and retained PGI_2 mediated-dilatation in diabtes.

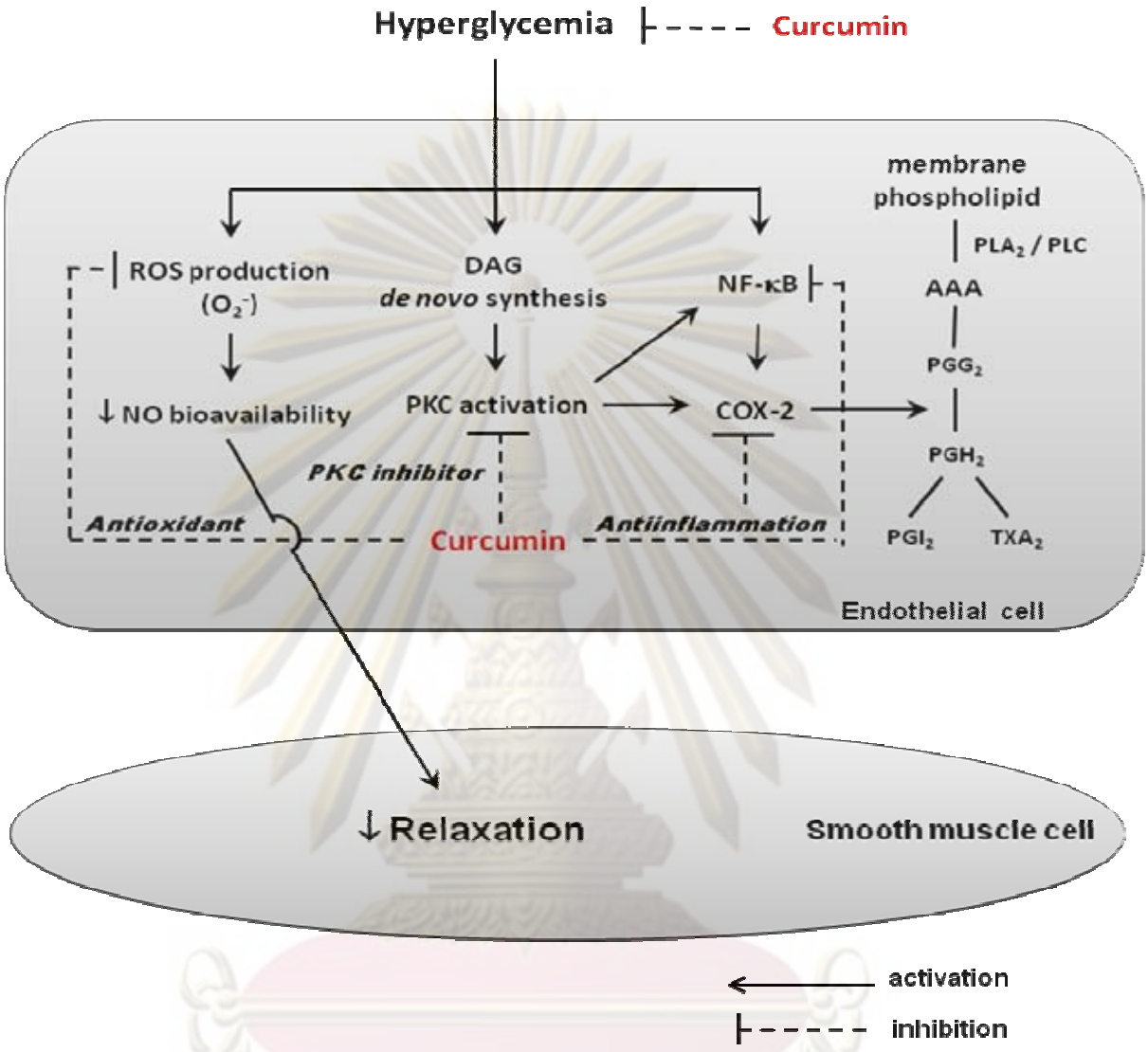


Figure 5.1. The proposed mechanism for curcumin restore diabetes endothelial dysfunction through it antioxidant, anti-inflammatory and PKC inhibitory actions.

CHAPTER VI

CONCUSSION

The new findings in this present study are demonstrated that the beneficial effects of curcumin supplementation on improving endothelial dysfunction in STZ-induced diabetic rats.

These significant findings could be summarized as follows;

1. Twelve weeks after STZ injection, DM rats exhibited higher both plasma glucose level and glycated hemoglobin HbA1c. Both DM+cur30 and DM+cur300 rats had lower plasma glucose and HbA1c values at the doses of 30 and 300 mg/kg on six-week curcumin supplementaion.

2. The endothelial dysfunction was determined by the functional responses of mesenteric arterioles to vasodilators, ACh and SNP using real time intravital fluorescence videomicroscopy. The dilatory responses to ACh significantly decreased in DM arterioles compared to control arterioles. No difference among groups when used SNP. ACh-responses were significantly improved by both low and high doses (30 and 300 mg/kg, respectively) of curcumin supplementation.

3. An oxygen radical-sensitive fluorescent probe, hydroethidine, was used to detect intracellular $O_2^{\bullet-}$ production in endothelial cells. $O_2^{\bullet-}$ production was markedly increased in DM arterioles, but it was significantly reduced by supplementation of either low or high doses of curcumin.

4. Basal level, DM rats demonstrated higher TXB_2 (stable metabolite of TXA_2) and lower 6-keto-$PGF_{1\alpha}$ (stable metabolite of PGI_2) levesl compared to control rats, whereas curcumin supplementation significantly increase 6-keto-$PGF_{1\alpha}$ level.

5. The ratio of PGI_2/ TXA_2 prostaglandins decreased in DM rats compared to control rats. Curcumin supplementation caused a shift in PGI_2/ TXA_2 ratio in diabetic rats back to control rats.

6. Immunohistochemical analysis comfirmed DM rats showed marked expression of PKC-βII, COX-2 and NF-κB. These suggested that PKC-dependent mechanism was the triggering step by which hyperglycemia also induces oxidative stress and in turn prostaglandins-mediated dilatation in diabetes.

7. Curcumin inhibited the activation of PKC- βII at the dose of 300 mg/kg on six-week curcumin supplementation in diabetic rats.

8. Curcumin had anti-inflammatory action in downregulate COX-2 activity and suppress NF-κB expression in diabetic rats at the doses of 30 and 300 mg/kg on six-week curcumin supplementation.

9. Curcumin supplementations improvd diabetes-induced endothelial dysfunction significantly related to its potential to $O_2^{\bullet-}$ scavenging, COX-2 and NF-κB suppression, and PKC inhibitory effects. It is possible that curcumin supplementation may be beneficial for diabetic patients by improving microvascular functions and preventing the consequence of cardiovascular complication.

REFERENCES

Abordo, E. A. and Thornalley, P. J. (1997). Synthesis and secretion of tumour necrosis factor-alpha by human monocytic THP-1 cells and chemotaxis induced by human serum albumin derivatives modified with methylglyoxal and glucose-derived advanced glycation endproducts. *Immunol Lett.*58(3): 139-47.

Aggarwal, B. B., Kumar, A. and Bharti, A. C. (2003). Anticancer potential of curcumin: preclinical and clinical studies. *Anticancer Res.*23(1A): 363-98.

Aggarwal, B. B., Sundaram, C., Malani, N. and Ichikawa, H. (2007). *Curcumin: the india solid gold,* Springer.

Ago, T., Kitazono, T., Ooboshi, H., Iyama, T., Han, Y. H., Takada, J., *et al.* (2004). Nox4 as the major catalytic component of an endothelial NAD(P)H oxidase. *Circulation.*109(2): 227-33.

Aiello, L. P., Bursell, S. E., Clermont, A., Duh, E., Ishii, H., Takagi, C., *et al.* (1997). Vascular endothelial growth factor-induced retinal permeability is mediated by protein kinase C in vivo and suppressed by an orally effective beta-isoform-selective inhibitor. *Diabetes.*46(9): 1473-80.

Aiello, L. P., Robinson, G. S., Lin, Y. W., Nishio, Y. and King, G. L. (1994). Identification of multiple genes in bovine retinal pericytes altered by exposure to elevated levels of glucose by using mRNA differential display. *Proc Natl Acad Sci U S A.*91(13): 6231-5.

Ak, T. and Gulcin, I. (2008). Antioxidant and radical scavenging properties of curcumin. *Chem Biol Interact.*174(1): 27-37.

Akamine, E. H., Urakawa, T. A., de Oliveira, M. A., Nigro, D., de Carvalho, M. H., de Cassia, A. T. R., *et al.* (2006). Decreased endothelium-dependent vasodilation in diabetic female rats: role of prostanoids. *J Vasc Res.*43(5): 401-10.

Ambasta, R. K., Kumar, P., Griendling, K. K., Schmidt, H. H., Busse, R. and Brandes, R. P. (2004). Direct interaction of the novel Nox proteins with p22phox is required for the formation of a functionally active NADPH oxidase. *J Biol Chem.*279(44): 45935-41.

Ammon, H. P. and Wahl, M. A. (1991). Pharmacology of Curcuma longa. *Planta Med.*57(1): 1-7.

Anderson, D. K. G. and Svaardsudd, K. (1995). Long-term glycemic control relates to mortality in type II diabetes. *Diabetes Care.*18: 1534-1543.

Athkinson, M. A., Maclaren, N. K., Riley, W. J., Winter, W. E., Fisk, D. D. and Spillar, R. P. (1986). Are insulin autoantibodies markers for insulin-dependent mellitus? *Diabetes.*35: 894-898.

Baeuerle, P. A. and Baltimore, D. (1996). NF-kappa B: ten years after. *Cell.*87(1): 13-20.

Bagi, Z., Erdei, N., Toth, A., Li, W., Hintze, T. H., Koller, A., *et al.* (2005). Type 2 diabetic mice have increased arteriolar tone and blood pressure: enhanced release of COX-2-derived constrictor prostaglandins. *Arterioscler Thromb Vasc Biol.*25(8): 1610-6.

Bakker, W., Eringa, E. C., Sipkema, P. and van Hinsbergh, V. W. (2009). Endothelial dysfunction and diabetes: roles of hyperglycemia, impaired insulin signaling and obesity. *Cell Tissue Res.*335(1): 165-89.

Balasubramanyam, M., Koteswari, A. A., Kumar, R. S., Monickaraj, S. F., Maheswari, J. U. and Mohan, V. (2003). Curcumin-induced inhibition of cellular reactive oxygen species generation: novel therapeutic implications. *J Biosci.*28(6): 715-21.

Ballinger, S. W., Patterson, C., Yan, C. N., Doan, R., Burow, D. L., Young, C. G., *et al.* (2000). Hydrogen peroxide- and peroxynitrite-induced mitochondrial DNA damage and dysfunction in vascular endothelial and smooth muscle cells. *Circ Res.*86(9): 960-6.

Barnes, P. J. and Karin, M. (1997). Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med.*336(15): 1066-71.

Beckman, J. A., Goldfine, A. B., Gordon, M. B., Garrett, L. A. and Creager, M. A. (2002). Inhibition of protein kinase Cbeta prevents impaired endothelium-dependent vasodilation caused by hyperglycemia in humans. *Circ Res.*90(1): 107-11.

Bedoya, F. J., Solano, F. and Lucas, M. (1996). N-Monomethyl-arginine and nicotinamide prevent streptozotocin-induced double strand DNA break formation in pancreatic rat islets. *Experientia.*52: 344-347.

Bengmark, S. (2006). Curcumin, an atoxic antioxidant and natural NF-κB, cyclooxygenase-2, lipooxygenase, and inducible nitric oxide synthase inhibitor: A shield against acute and chronic diseases. *Journal of Parenteral and Enteral Nutrition.*30(1): 45-51.

Benov, L., Sztejnberg, L. and Fridovich, I. (1998). Critical evaluation of the use of hydroethidine as a measure of superoxide anion radical. *Free Radic Biol Med.*25(7): 826-31.

Benz, D., Cadet, P., Mantione, K., Zhu, W. and Stefano, G. (2002). Tonal nitric oxide and health--a free radical and a scavenger of free radicals. *Med Sci Monit.*8(1): RA1-4.

Bizyukin, A. V. and Soodaeva, S. K. (1995). Study of oxidative phagocyte metabolism using the fluorescent indicator hydroethidine. *Pharmaceu Chem J* 29: 236-240.

Boone, C. W., Kelloff, G. J. and Malone, W. E. (1990). Identification of candidate cancer chemopreventive agents and their evaluation in animal models and human clinical trials: a review. *Cancer Res.*50(1): 2-9.

Bourcier, T., Sukhova, G. and Libby, P. (1997). The nuclear factor kappa-B signaling pathway participates in dysregulation of vascular smooth muscle cells in vitro and in human atherosclerosis. *J Biol Chem.*272(25): 15817-24.

Bowling, N., Walsh, R. A., Song, G., Estridge, T., Sandusky, G. E., Fouts, R. L., *et al.* (1999). Increased protein kinase C activity and expression of Ca2+-sensitive isoforms in the failing human heart. *Circulation.*99(3): 384-91.

Brownlee, M. (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature.*414(6865): 813-20.

Brownlee, M. (2005). The pathobiology of diabetic complications. A unifying mechanism. *Diabetes.*54: 1615-1625.

Bunn, H. F., Gabbay, K. H. and Gallop, P. M. (1978). The glycosylation of hemoglobin: relevance to diabetes mellitus. *Science.*200(4337): 21-7.

Bustos, M., Coffman, T. M., Saadi, S. and Platt, J. L. (1997). Modulation of eicosanoid metabolism in endothelial cells in a xenograft model. Role of cyclooxygenase-2. *J Clin Invest.*100(5): 1150-8.

Cai, H. and Harrison, D. G. (2000). Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res.*87(10): 840-4.

Camacho, M., Lopez-Belmonte, J. and Vila, L. (1998). Rate of vasoconstrictor prostanoids released by endothelial cells depends on cyclooxygenase-2 expression and prostaglandin I synthase activity. *Circ Res.*83(4): 353-65.

Carter, W. O., Narayanan, P. K. and Robinson, J. P. (1994). Intracellular hydrogen peroxide and superoxide anion detection in endothelial cells. *J Leukoc Biol.*55(2): 253-8.

Ceolotto, G., Gallo, A., Miola, M., Sartori, M., Trevisan, R., Del Prato, S., *et al.* (1999). Protein kinase C activity is acutely regulated by plasma glucose concentration in human monocytes in vivo. *Diabetes.*48(6): 1316-22.

Chainani-Wu, N. (2003). Safety and anti-inflammatory activity of curcumin: a component of tumeric (Curcuma longa). *J Altern Complement Med.*9(1): 161-8.

Chakraphan, D. (2002). Roles of vitamin C and exercise training on endothelial dysfunction in diabetic rats. Graduate School. Bangkok, Chulalongkorn University. Doctor of Philosophy in Physiology**:** 186.

Chakravarthy, U., Hayes, R. G., Stitt, A. W., McAuley, E. and Archer, D. B. (1998). Constitutive nitric oxide synthase expression in retinal vascular endothelial cells is suppressed by high glucose and advanced glycation end products. *Diabetes.*47(6): 945-52.

Chandalia, H. B. and Krishnaswamy, P. R. (2002). Glycated hemoglobin. *Current Science.*83(12): 1522-1532.

Charonis, A. S., Reger, L. A., Dege, J. E., Kouzi-Koliakos, K., Furcht, L. T., Wohlhueter, R. M., *et al.* (1990). Laminin alterations after in vitro nonenzymatic glycosylation. *Diabetes.*39(7): 807-14.

Chattopadhyay, I., Biswas, K., Bandyopadhyay, U. and Banerjee, R. K. (2004). Turmeric and curcumin: Biological actions and medicinal applications. *Curr Sci.*87: 44-53.

Chen, W. F., Deng, S. L., Zhou, B., Yang, L. and Liu, Z. L. (2006). Curcumin and its analogues as potent inhibitors of low density lipoprotein oxidation: H-atom abstraction from the phenolic groups and possible involvement of the 4-hydroxy-3-methoxyphenyl groups. *Free Radic Biol Med.*40(3): 526-35.

Chen, Z., Gundimeda, U. and Gopalakrishna, R. (1996). Curcumin irreversibly inactivates protein kinase C activity and phorbal ester binding: its possible role in cancer c hemoprevention. *Proc Am Assoc Cancer Res.*37: A1922.

Cheng, A. L., Hsu, C. H., Lin, J. K., Hsu, M. M., Ho, Y. F., Shen, T. S., *et al.* (2001). Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res.*21(4B): 2895-900.

Collins, T. (1993). Endothelial nuclear factor-kappa B and the initiation of the atherosclerotic lesion. *Lab Invest.*68(5): 499-508.

Cosentino, F., Eto, M., De Paolis, P., van der Loo, B., Bachschmid, M., Ullrich, V., *et al.* (2003). High glucose causes upregulation of cyclooxygenase-2 and alters prostanoid profile in human endothelial cells: role of protein kinase C and reactive oxygen species. *Circulation.*107(7): 1017-23.

Cosentino, F., Hishikawa, K., Katusic, Z. S. and Luscher, T. F. (1997). High glucose increases nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. *Circulation.*96(1): 25-8.

Cotran, R. S. (1999). *Robbins Pathologic basis of disease*. Philadelphia, W.B. Saunders Company.

Craven, P. A., Davidson, C. M. and DeRubertis, F. R. (1990). Increase in diacylglycerol mass in isolated glomeruli by glucose from de novo synthesis of glycerolipids. *Diabetes.*39(6): 667-74.

Davidge, S. T. (2001). Prostaglandin H synthase and vascular function. *Circ Res.*89(8): 650-60.

Davidge, S. T., Hubel, C. A. and McLaughlin, M. K. (1993). Cyclooxygenase-dependent vasoconstrictor alters vascular function in the vitamin E-deprived rat. *Circ Res.*73(1): 79-88.

De Vriese, A. S., Verbeuren, T. J., Van de Voorde, J., Lameire, N. H. and Vanhoutte, P. M. (2000). Endothelial dysfunction in diabetes. *Br J Pharmacol.*130(5): 963-74.

Delaney, C. A., Dunger, A., Di Matteo, M., Cunningham, J. M., Green, M. H. and Green, I. C. (1995). Comparison of inhibition of glucose-stimulated insulin secretion in rat islets of Langerhans by streptozotocin and methyl and ethyl nitrosoureas and methanesulphonates. Lack of correlation with nitric oxide-releasing or O^6-alkylating ability. *Biochem Pharmacol.*50: 2015-2020.

DeWitt, D. L., Day, J. S., Sonnenburg, W. K. and Smith, W. L. (1983). Concentrations of prostaglandin endoperoxide synthase and prostaglandin I2 synthase in the endothelium and smooth muscle of bovine aorta. *J Clin Invest.*72(6): 1882-8.

DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E. and Karin, M. (1997). A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. *Nature.*388(6642): 548-54.

Dixon, H. B. (1972). A reaction of glucose with peptides. *Biochem J.*129(1): 203-8.

Droge, W. (2002). Free radicals in the physiological control of cell function. *Physiol Rev.*82(1): 47-95.

Du, X. L., Edelstein, D., Rossetti, L., Fantus, I. G., Goldberg, H., Ziyadeh, F., *et al.* (2000). Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. *Proc Natl Acad Sci U S A.*97(22): 12222-6.

Eizirik, D. L., Sandler, S., Sener, A. and Malaisse, W. J. (1988). Defective catabolism of D-glucose and L-glutamine in mouse pancreatic islets maintained in culture after streptozotocin exposure. *Endocrinology.*123: 1001-1007.

Ellis, E. F., Oelz, O., Roberts, L. J., 2nd, Payne, N. A., Sweetman, B. J., Nies, A. S., *et al.* (1976). Coronary arterial smooth muscle contraction by a substance released from platelets: evidence that it is thromboxane A2. *Science.*193(4258): 1135-7.

Endemann, D. H. and Schiffrin, E. L. (2004). Endothelial dysfunction. *J Am Soc Nephrol.*15(8): 1983-92.

Farhangkhoee, H., Khan, Z. A., Chen, S. and Chakrabarti, S. (2006). Differential effects of curcumin on vasoactive factors in the diabetic rat heart. *Nutr Metab (Lond).*3: 27.

Feletou, M. and Vanhoutte, P. M. (1996). Endothelium-derived hyperpolarizing factor. *Clin Exp Pharmacol Physiol.*23(12): 1082-90.

Feng, X. and Hannun, Y. A. (1998). An essential role for autophosphorylation in the dissociation of activated protein kinase C from the plasma membrane. *J Biol Chem.*273(41): 26870-4.

Fernandez-Real, J. M. and Ricart, W. (2003). Insulin resistance and chronic cardiovascular inflammatory syndrome. *Endocr Rev.*24(3): 278-301.

Frankel, E. N. (1998). *Lipid oxidation.* Dundee, Scotland, The Oily Press.

Freisewinkel, I., Riethmacher, D. and Stabel, S. (1991). Downregulation of protein kinase C-gamma is independent of a functional kinase domain. *FEBS Lett.*280(2): 262-6.

Fujiwara, H., Hosokawa, M., Zhou, X., Fujimoto, S., Fukuda, K., Toyoda, K., *et al.* (2008). Curcumin inhibits glucose production in isolated mice hepatocytes. *Diabetes Research and Clinical Practice.*80(2): 185-191.

Fukushima, T., Adachi, T. and Hirano, K. (1995). The heparin-binding site of human xanthine oxidase. *Biol Pharm Bull.*18(1): 156-8.

Furchgott, R. F. and Vanhoutte, P. M. (1989). Endothelium-derived relaxing and contracting factors. *FASEB J.*3(9): 2007-18.

Furchgott, R. F. and Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature.*288(5789): 373-6.

Gabbay, K. H., Merola, L. O. and Field, R. A. (1966). Sorbital pathway: presence in nerve and cord with substrate accumaulation in diabetes. *Science.*151: 209-210.

Gelati, K. N., Peiffer, R. L. J. and Williams, L. W. (1979). Diabetic relinopathy. . Spontaneous Animal Models of Human Diseases. E. J. Andrews, B. C. Ward and W. H. Airman. New York, *Academic Press*. 1.

Gopalakrishna, R., Chen, Z. H. and Gundimeda, U. (1995). Modification of cysteine-rich regions in protein kinase C induced by oxidant tumor promoters and the enzyme specific inhibitors. *Meth Enzymol.*252: 134-148.

Gopalakrishna, R. and Jaken, S. (2000). Protein kinase C signaling and oxidative stress. *Free Radic Biol Med.*28(9): 1349-61.

Gorlach, A., Brandes, R. P., Nguyen, K., Amidi, M., Dehghani, F. and Busse, R. (2000). A gp91phox containing NADPH oxidase selectively expressed in endothelial cells is a major source of oxygen radical generation in the arterial wall. *Circ Res.*87(1): 26-32.

Griendling, K. K. and FitzGerald, G. A. (2003). Oxidative stress and cardiovascular injury: Part I: basic mechanisms and in vivo monitoring of ROS. *Circulation.*108(16): 1912-6.

Griendling, K. K. and FitzGerald, G. A. (2003). Oxidative stress and cardiovascular injury: Part II: animal and human studies. *Circulation.*108(17): 2034-40.

Guo, Z., Su, W., Allen, S., Pang, H., Daugherty, A., Smart, E., *et al.* (2005). COX-2 up-regulation and vascular smooth muscle contractile hyperreactivity in spontaneous diabetic db/db mice. *Cardiovasc Res.*67(4): 723-35.

Gutterman, D. D. (2002). Vascular dysfunction in hyperglycemia: is protein kinase C the culprit? *Circ Res.*90(1): 5-7.

Guzik, T. J., Mussa, S., Gastaldi, D., Sadowski, J., Ratnatunga, C., Pillai, R., *et al.* (2002). Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase. *Circulation.*105(14): 1656-62.

Halim, E. and Hussain, M. A. (2002). Hypoglycemic, hypolipidemic and antioxidant properties of combination of Curcumin from CURCUMA LONGA, Linn, and artially purified product from ABROBA AUGUSTA, Linn, in streptozotocin induced diabetes. *Indian J Clinical Biochem.*17: 33-43.

Halliwell, B. and Gutteridge, J. M. C. (1999). *Free radicals in biology and medicine.* Oxford, Oxford University Press.

Hamberg, M., Svensson, J. and Samuelsson, B. (1975). Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc Natl Acad Sci U S A.*72(8): 2994-8.

Harris, M. I. (1989). Impaired glucose tolerance in the U.S. populaiton. *Diabetes Care.*12: 464-474.

Harrison, H. E., Reece, A. H. and Johnson, M. (1978). Decreased vascular prostacyclin in experimental diabetes. *Life Sci.*23(4): 351-5.

Harrison, R. (2002). Structure and function of xanthine oxidoreductase: where are we now? *Free Radic Biol Med.*33(6): 774-97.

Helmersson, J., Vessby, B., Larsson, A. and Basu, S. (2004). Association of type 2 diabetes with cyclooxygenase-mediated inflammation and oxidative stress in an elderly population. *Circulation.*109(14): 1729-34.

Higgs, G. A., Moncada, S. and Vane, J. R. (1984). Eicosanoids in inflammation. *Ann Clin Res.*16(5-6): 287-99.

Hilenski, L. L., Clempus, R. E., Quinn, M. T., Lambeth, J. D. and Griendling, K. K. (2004). Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol.*24(4): 677-83.

Hink, U., Li, H., Mollnau, H., Oelze, M., Matheis, E., Hartmann, M., *et al.* (2001). Mechanisms underlying endothelial dysfunction in diabetes mellitus. *Circ Res.*88(2): E14-22.

Huang, W., Connor, E., DelaRosa, T., Muir, A., Schatz, D., Silverstein, J., *et al.* (1996). Although DR3-DQB1 may be associated with multiple component disease of the autoimmune polyglandular syndromes, the human leukocyte antigen DR4-DQB110302 haploytype is implicated only in beta cell autoimmunity. *J Clin Endocrinol Metab.*81: 1-5.

Hug, H. and Sarre, T. F. (1993). Protein kinase C isoenzymes: divergence in signal transduction? *Biochem J.*291 (Pt 2): 329-43.

Huijberts, M. S., Wolffenbuttel, B. H., Boudier, H. A., Crijns, F. R., Kruseman, A. C., Poitevin, P., *et al.* (1993). Aminoguanidine treatment increases elasticity and decreases fluid filtration of large arteries from diabetic rats. *J Clin Invest.*92(3): 1407-11.

Idris, I., Gray, S. and Donnelly, R. (2001). Protein kinase C activation: isozyme-specific effects on metabolism and cardiovascular complications in diabetes. *Diabetologia.*44(6): 659-73.

Inoguchi, T., Battan, R., Handler, E., Sportsman, J. R., Heath, W. and King, G. L. (1992). Preferential elevation of protein kinase C isoform beta II and diacylglycerol levels in the aorta and heart of diabetic rats: differential reversibility to glycemic control by islet cell transplantation. *Proc Natl Acad Sci U S A.*89(22): 11059-63.

Inoguchi, T., Li, P., Umeda, F., Yu, H. Y., Kakimoto, M., Imamura, M., *et al.* (2000). High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C--dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes.*49(11): 1939-45.

Inoue, M., Kishimoto, A., Takai, Y. and Nishizuka, Y. (1977). Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium-dependent protease from rat brain. *J Biol Chem.*252(21): 7610-6.

Ishii, H., Jirousek, M. R., Koya, D., Takagi, C., Xia, P., Clermont, A., *et al.* (1996). Amelioration of vascular dysfunctions in diabetic rats by an oral PKC beta inhibitor. *Science.*272(5262): 728-31.

Ishii, H., Koya, D. and King, G. L. (1998). Protein kinase C activation and its role in th development of vascular complications in diabetes mellitus. *J Mol Med.*76(1): 21-31.

Jariyapongskul, A., Patumraj, S. and Niimi, H. (2003). Cerebral endothelial dysfunction in diabetes: intravital microscopic analysis using streptozotocin-induced diabetic rats. *Clin Hemorheol Microcirc.*29(3-4): 331-5.

Jariyapongskul, A., Patumraj, S., Yamaguchi, S. and Niimi, H. (2002). The effect of long-term supplementation of vitamin C on leukocyte adhesion to the cerebral endothelium in STZ-induced diabetic rats. *Clin Hemorheol Microcirc.*27(1): 67-76.

Jirousek, M. R., Gillig, J. R., Gonzalez, C. M., Heath, W. F., McDonald, J. H., 3rd, Neel, D. A., *et al.* (1996). (S)-13-[(dimethylamino)methyl]-10,11,14,15-tetrahydro-4,9:16,21-dimetheno1H,13Hdibenzo[e,k]pyrrolo[3,4h][1,4,13]oxadiazacyclo hexadecene -1,3(2H)-d ione (LY333531) and related analogues: isozyme selective inhibitors of protein kinase C beta. *J Med Chem.*39(14): 2664-71.

Jobin, C., Bradham, C. A., Russo, M. P., Juma, B., Narula, A. S., Brenner, D. A., *et al.* (1999). Curcumin blocks cytokine-mediated NF-kappa B activation and proinflammatory gene expression by inhibiting inhibitory factor I-kappa B kinase activity. *J Immunol.*163(6): 3474-83.

Johannes, F. J., Prestle, J., Eis, S., Oberhagemann, P. and Pfizenmaier, K. (1994). PKCu is a novel, atypical member of the protein kinase C family. *J Biol Chem.*269(8): 6140-8.

Johnston, B. D. and DeMaster, E. G. (2003). Suppression of nitric oxide oxidation to nitrite by curcumin is due to the sequestration of the reaction intermediate nitrogen dioxide, not nitric oxide. *Nitric Oxide.*8(4): 231-4.

Kaiser, N., Sasson, S., Freener, E. P., Boukobza-Vardi, N., Higashi, S., Moller, D. E., *et al.* (1993). Differential regulation of glucose transport and transporters by glucose in vascular endothelial and smooth muscle cells. *Diabetes.*42: 80-89.

Karunanayake, E. H., Baker, J. R., Christian, R. A., Hearse, D. J. and Mellows, G. (1976). Autoradiographic study of the distribution and cellular uptake of (^{14}C)-streptozotocin in the rat.. *Diabetologia.*12: 123-128.

Katusic, Z. S. and Vanhoutte, P. M. (1989). Superoxide anion is an endothelium-derived contracting factor. *Am J Physiol.*257(1 Pt 2): H33-7.

Kaufman, D., Erlander, M., Clare-Salzler, M., Atkinson, M., Maclaren, N. and Tobin, A. (1992). Autoimmunity to two forms of glutamate decarboxylase in insulin-dependent mellitus. *J Clin Invest.*89: 283-292.

Keranen, L. M., Dutil, E. M. and Newton, A. C. (1995). Protein kinase C is regulated in vivo by three functionally distinct phosphorylations. *Curr Biol.*5(12): 1394-1403.

Kim, Y. K., Lee, M. S., Son, S. M., Kim, I. J., Lee, W. S., Rhim, B. Y., *et al.* (2002). Vascular NADH oxidase is involved in impaired endothelium-dependent vasodilation in OLETF rats, a model of type 2 diabetes. *Diabetes.*51(2): 522-7.

Kirstein, M., Aston, C., Hintz, R. and Vlassara, H. (1992). Receptor-specific induction of insulin-like growth factor I in human monocytes by advanced glycosylation end product-modified proteins. *J Clin Invest.*90(2): 439-46.

Kitamoto, S. and Egashira, K. (2004). Endothelial dysfunction and coronary atherosclerosis. *Curr Drug Targets Cardiovasc Haematol Disord.*4(1): 13-22.

Koller, A. and Kaley, G. (1990). Prostaglandins mediate arteriolar dilation to increased blood flow velocity in skeletal muscle microcirculation. *Circ Res.*67(2): 529-34.

Koller, A. and Kaley, G. (1991). A new role for prostaglandins in the regulation of peripheral resistance. *Adv Prostaglandin Thromboxane Leukot Res.*21B: 595-8.

Konishi, H., Tanaka, M., Takemura, Y., Matsuzaki, H., Ono, Y., Kikkawa, U., *et al.* (1997). Activation of protein kinase C by tyrosine phosphorylation in response to H2O2. *Proc Natl Acad Sci U S A.*94(21): 11233-7.

Kowluru, R. A. (2001). Diabetes-induced elevations in retinal oxidative stress, protein kinase C and nitric oxide are interrelated. *Acta Diabetol.*38(4): 179-85.

Koya, D., Haneda, M., Nakagawa, H., Isshiki, K., Sato, H., Maeda, S., *et al.* (2000). Amelioration of accelerated diabetic mesangial expansion by treatment with a PKC beta inhibitor in diabetic db/db mice, a rodent model for type 2 diabetes. *FASEB J.*14(3): 439-47.

Koya, D., Jirousek, M. R., Lin, Y. W., Ishii, H., Kuboki, K. and King, G. L. (1997). Characterization of protein kinase C beta isoform activation on the gene expression of transforming growth factor-beta, extracellular matrix components, and prostanoids in the glomeruli of diabetic rats. *J Clin Invest.*100(1): 115-26.

Koya, D. and King, G. L. (1998). Protein kinase C activation and the development of diabetic complications. *Diabetes.*47(6): 859-66.

Kunisaki, M., Bursell, S. E., Umeda, F., Nawata, H. and King, G. L. (1994). Normalization of diacylglycerol-protein kinase C activation by vitamin E in aorta of diabetic rats and cultured rat smooth muscle cells exposed to elevated glucose levels. *Diabetes.*43(11): 1372-7.

Lan, M. S., Wasserfall, C., Maclaren, N. and Notkins, A. L. (1996). IA-2, a transmembrane protein of the protein tyrosine phosphatase family, is a major autoantigen in insulindependent diabetes mellitus. *Proc Natl Acad Sci USA.*93: 6367-6370.

Lassegue, B. and Clempus, R. E. (2003). Vascular NAD(P)H oxidases: specific features, expression, and regulation. *Am J Physiol Regul Integr Comp Physiol.*285(2): R277-97.

Laursen, J. B., Somers, M., Kurz, S., McCann, L., Warnholtz, A., Freeman, B. A., *et al.* (2001). Endothelial regulation of vasomotion in apoE-deficient mice: implications for interactions between peroxynitrite and tetrahydrobiopterin. *Circulation.*103(9): 1282-8.

Lee, A. Y. and Chung, S. S. (1999). Contributions of polyol pathway to oxidative stress in diabetic cataract. *FASEB J.*13: 23-30.

Lenzen, S. (2008). The mechanisms of alloxan-and streptozotocin-induced diabetes. *Diabetologia.*51: 216-226.

Libby, P., Ridker, P. M. and Maseri, A. (2002). Inflammation and atherosclerosis. *Circulation.*105(9): 1135-43.

Liou, J. Y., Shyue, S. K., Tsai, M. J., Chung, C. L., Chu, K. Y. and Wu, K. K. (2000). Colocalization of prostacyclin synthase with prostaglandin H synthase-1 (PGHS-1) but not phorbol ester-induced PGHS-2 in cultured endothelial cells. *J Biol Chem.*275(20): 15314-20.

Lochhead, P. A., Salt, I. P., Walker, K. S., Hardie, D. G. and Sutherland, C. (2000). 5-aminoimidazole-4-carboxamide riboside mimics the effects of insulin on the expression of the 2 key gluconeogenic genes PEPCK and glucose-6-phosphatase. *Diabetes.*49(6): 896-903.

Lu, J., Li, Q., Xie, H., Chen, Z., Borovitskayam, A. E., Maclaren, N. K., *et al.* (1996). Identification of a second trasmembrane protein tyrosine phosphase, IA-2B, as an autoantigen in insulin-dependent diabetes mellitus:precursor of the 37-kDa tryptic fragment. *Proc Natl Acad Sci USA.*93: 2307-2311.

Lu, Y. P., Chang, R. L., Lou, Y. R., Huang, M. T., Newmark, H. L., Reuhl, K. R., *et al.* (1994). Effect of curcumin on 12-O-tetradecanoylphorbol-13-acetate- and ultraviolet B light-induced expression of c-Jun and c-Fos in JB6 cells and in mouse epidermis. *Carcinogenesis.*15(10): 2363-70.

Mahesh, T., Sri Balasubashini, M. M. and Menon, V. P. (2004). Photo-irradiated curcumin supplementation in streptozotocin-induced diabetic rats: effect on lipid peroxidation. *Therapie.*59(6): 639-44.

Marnett, L. J., Rowlinson, S. W., Goodwin, D. C., Kalgutkar, A. S. and Lanzo, C. A. (1999). Arachidonic acid oxygenation by COX-1 and COX-2. Mechanisms of catalysis and inhibition. *J Biol Chem.*274(33): 22903-6.

Marnett, L. J., Wright, T. L., Crews, B. C., Tannenbaum, S. R. and Morrow, J. D. (2000). Regulation of prostaglandin biosynthesis by nitric oxide is revealed by targeted deletion of inducible nitric-oxide synthase. *J Biol Chem.*275(18): 13427-30.

Marsden, P. A., Heng, H. H., Scherer, S. W., Stewart, R. J., Hall, A. V., Shi, X. M., *et al.* (1993). Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. *J Biol Chem.*268(23): 17478-88.

Masaki, T. (1995). Possible role of endothelin in endothelial regulation of vascular tone. *Annu Rev Pharmacol Toxicol.*35: 235-55.

Masuda, T., Maekawa, T., Hidaka, K., Bando, H., Takeda, Y. and Yamaguchi, H. (2001). Chemical studies on antioxidant mechanism of curcumin: analysis of oxidative coupling products from curcumin and linoleate. *J Agric Food Chem.*49(5): 2539-47.

Masuda, T., Toi, Y., Bando, H., Maekawa, T., Takeda, Y. and Yamaguchi, H. (2002). Structural identification of new curcumin dimers and their contribution to the antioxidant mechanism of curcumin. *J Agric Food Chem.*50(9): 2524-30.

Matsumoto, H., Naraba, H., Murakami, M., Kudo, I., Yamaki, K., Ueno, A., *et al.* (1997). Concordant induction of prostaglandin E2 synthase with cyclooxygenase-2 leads to preferred production of prostaglandin E2 over thromboxane and prostaglandin D2 in lipopolysaccharide-stimulated rat peritoneal macrophages. *Biochem Biophys Res Commun.*230(1): 110-4.

Mayhan, W. G. and Patel, K. P. (1995). Acute effects of glucose on reactivity of cerebral microcirculation: role of activation of protein kinase C. *Am J Physiol.*269(4 Pt 2): H1297-302.

McLellan, A. C., Thornalley, P. J., Benn, J. and Sonksen, P. H. (1994). Glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications. *Clin Sci (Lond).*87(1): 21-9.

McVeigh, G. E., Brennan, G. M., Johnston, G. D., McDermott, B. J., McGrath, L. T., Henry, W. R., *et al.* (1992). Impaired endothelium-dependent and independent vasodilation in patients with type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia.*35(8): 771-6.

Meier, M. and King, G. L. (2000). Protein kinase C activation and its pharmacological inhibition in vascular disease. *Vasc Med.*5(3): 173-85.

Meininger, C. J., Marinos, R. S., Hatakeyama, K., Martinez-Zaguilan, R., Rojas, J. D., Kelly, K. A., *et al.* (2000). Impaired nitric oxide production in coronary endothelial cells of the spontaneously diabetic BB rat is due to tetrahydrobiopterin deficiency. *Biochem J.*349(Pt 1): 353-6.

Menon, V. P. and Sudheer, A. R. (2007). Antioxidant and anti-inflammatory properties of curcumin. *Adv Exp Med Biol.*595: 105-25.

Mercurio, F. and Manning, A. M. (1999). NF-kappaB as a primary regulator of the stress response. *Oncogene.*18(45): 6163-71.

Milstien, S. and Katusic, Z. (1999). Oxidation of tetrahydrobiopterin by peroxynitrite: implications for vascular endothelial function. *Biochem Biophys Res Commun.*263(3): 681-4.

Mochly-Rosen, D., Henrich, C. J., Cheever, L., Khaner, H. and Simpson, P. C. (1990). A protein kinase C isozyme is translocated to cytoskeletal elements on activation. *Cell Regul.*1(9): 693-706.

Mombouli, J. V. and Vanhoutte, P. M. (1999). Endothelial dysfunction: from physiology to therapy. *J Mol Cell Cardiol.*31(1): 61-74.

Moncada, S., Gryglewski, R., Bunting, S. and Vane, J. R. (1976). An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature*.263(5579): 663-5.

Moncada, S. and Vane, J. R. (1978). Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A2, and prostacyclin. *Pharmacol Rev*.30(3): 293-331.

Morigi, M., Angioletti, S., Imberti, B., Donadelli, R., Micheletti, G., Figliuzzi, M., *et al.* (1998). Leukocyte-endothelial interaction is augmented by high glucose concentrations and hyperglycemia in a NF-kB-dependent fashion. *J Clin Invest.*101(9): 1905-15.

Mugge, A., Elwell, J. H., Peterson, T. E., Hofmeyer, T. G., Heistad, D. D. and Harrison, D. G. (1991). Chronic treatment with polyethylene-glycolated superoxide dismutase partially restores endothelium-dependent vascular relaxations in cholesterol-fed rabbits. *Circ Res.*69(5): 1293-300.

Mullarkey, C. J., Edelstein, D. and Brownlee, M. (1990). Free radical generation by early glycation products: a mechanism for accelerated atherogenesis in diabetes. *Biochem Biophys Res Commun.*173(3): 932-9.

Muniyappa, R., Srinivas, P. R., Ram, J. L., Walsh, M. F. and Sowers, J. R. (1998). Calcium and protein kinase C mediate high-glucose-induced inhibition of inducible nitric oxide synthase in vascular smooth muscle cells. *Hypertension.*31(1 Pt 2): 289-95.

Murata, M., Takahashi, A., Saito, I. and Kawanishi, S. (1999). Site specific DNA methylation and apoptosis: induction by diabetogenic streptozotocin. *Biochem Pharmacol.*57: 881-887.

Myers, T. O., Messina, E. J., Rodrigues, A. M. and Gerritsen, M. E. (1985). Altered aortic and cremaster muscle prostaglandin synthesis in diabetic rats. *Am J Physiol.*249(4 Pt 1): E374-9.

Nakanishi, H., Brewer, K. A. and Exton, J. H. (1993). Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem.*268(1): 13-6.

Nanji, A. A., Jokelainen, K., Tipoe, G. L., Rahemtulla, A., Thomas, P. and Dannenberg, A. J. (2003). Curcumin prevents alcohol-induced liver disease in rats by inhibiting the expression of NF-kappa B-dependent genes. *Am J Physiol Gastrointest Liver Physiol.*284(2): G321-7.

Neeper, M., Schmidt, A. M., Brett, J., Yan, S. D., Wang, F., Pan, Y. C., *et al.* (1992). Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. *J Biol Chem.*267(21): 14998-5004.

Newton, A. C. (1995). Protein kinase C: structure, function, and regulation. *J Biol Chem.*270(48): 28495-8.

Newton, A. C. (1997). Regulation of protein kinase C. *Curr Opin Cell Biol.*9(2): 161-7.

Nishikawa, T., Edelstein, D. and Brownlee, M. (2000). The missing link: A single unifying mechanism for diabetic complications. *Kidney Int.*58(S77): S26-S30.

Nishikawa, T., Edelstein, D., Du, X. L., Yamagishi, S., Matsumura, T., Kaneda, Y., *et al.* (2000). Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature.*404(6779): 787-90.

Nishizuka, Y. (1988). The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature.*334(6184): 661-5.

Nishizuka, Y. (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science.*258(5082): 607-14.

Nishizuka, Y. (1995). Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J.*9(7): 484-96.

Nukatsuka, M., Yoshimura, Y., Nishida, M. and Kawada, J. (1990). Allopurinol protects pancreatic beta cells from the cytotoxic effect of streptozotocin: in vitro study. *J pharmacobiodyn.*13: 259-262.

Ohara, Y., Peterson, T. E. and Harrison, D. G. (1993). Hypercholesterolemia increases endothelial superoxide anion production. *J Clin Invest.*91(6): 2546-51.

Okahara, K., Sun, B. and Kambayashi, J. (1998). Upregulation of prostacyclin synthesis-related gene expression by shear stress in vascular endothelial cells. *Arterioscler Thromb Vasc Biol.*18(12): 1922-6.

Onozato, M. L., Tojo, A., Goto, A., Fujita, T. and Wilcox, C. S. (2002). Oxidative stress and nitric oxide synthase in rat diabetic nephropathy: effects of ACEI and ARB. *Kidney Int.*61(1): 186-94.

Orr, J. W., Keranen, L. M. and Newton, A. C. (1992). Reversible exposure of the pseudosubstrate domain of protein kinase C by phosphatidylserine and diacylglycerol. *J Biol Chem.*267(22): 15263-6.

Pacher, P., Beckman, J. S. and Liaudet, L. (2007). Nitric oxide and peroxynitrite in health and disease. *Physiol Rev.*87(1): 315-424.

Pan, M. H., Huang, T. M. and Lin, J. K. (1999). Biotransformation of curcumin through reduction and glucuronidation in mice. *Drug Metab Dispos.*27(4): 486-94.

Parente, L. and Perretti, M. (2003). Advances in the pathophysiology of constitutive and inducible cyclooxygenases: two enzymes in the spotlight. *Biochem Pharmacol.*65(2): 153-9.

Patumraj, S., Wongeakin, N., Sridulyakul, P., Jariyapongskul, A., Futrakul, N. and Bunnag, S. (2006). Combined effects of curcumin and vitamin C to protect endothelial dysfunction in the iris tissue of STZ-induced diabetic rats. *Clin Hemorheol Microcirc.*35(4): 481-9.

Pieper, G. M. (1997). Acute amelioration of diabetic endothelial dysfunction with a derivative of the nitric oxide synthase cofactor, tetrahydrobiopterin. *J Cardiovasc Pharmacol.*29(1): 8-15.

Pieper, G. M. (1998). Review of alterations in endothelial nitric oxide production in diabetes: protective role of arginine on endothelial dysfunction. *Hypertension.*31(5): 1047-60.

Plummer, S. M., Holloway, K. A., Manson, M. M., Munks, R. J., Kaptein, A., Farrow, S., *et al.* (1999). Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-kappaB activation via

Pricci, F., Leto, G., Amadio, L., Iacobini, C., Cordone, S., Catalano, S., *et al.* (2003). Oxidative stress in diabetes-induced endothelial dysfunction involvement of nitric oxide and protein kinase C. *Free Radic Biol Med.*35(6): 683-94.

Priyadarsini, K. I., Maity, D. K., Naik, G. H., Kumar, M. S., Unnikrishnan, M. K., Satav, J. G., *et al.* (2003). Role of phenolic O-H and methylene hydrogen on the free radical reactions and antioxidant activity of curcumin. *Free Radic Biol Med.*35(5): 475-84.

Quilley, J. and Chen, Y. J. (2003). Role of COX-2 in the enhanced vasoconstrictor effect of arachidonic acid in the diabetic rat kidney. *Hypertension.*42(4): 837-43.

Rakieten, N., Rakieten, M. L. and Nadkarni, M. V. (1963). Studies on the diabetogenic action of streptozotocin (NSC-37917). *Cancer Chemother Rep.*29: 91-98.

Rao, C. V. (2007). *Regulation of COX and LOX by curcumin,* Springer.

Rasschaert, J., Eizirik, D. L. and Malaisse, W. J. (1992). Long term in vitro effects of streptozotocin, interleukin-1, and high glucose concentration on the activity of mitochondrial dehydrogenases and the secretion of insulin in pancreatic islets. . *Endocrinology.*130: 3522-3528.

Reddy, A. C. and Lokesh, B. R. (1994). Studies on the inhibitory effects of curcumin and eugenol on the formation of reactive oxygen species and the oxidation of ferrous iron. *Mol Cell Biochem.*137(1): 1-8.

Rees, D. A. and Alcolado, J. C. (2005). Animal models of diabetes mellitus. *Diabet Med.*22: 359-370.

Ron, D. and Kazanietz, M. G. (1999). New insights into the regulation of protein kinase C and novel phorbol ester receptors. *FASEB J.*13(13): 1658-76.

Rothe, G. and Valet, G. (1990). Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescin. *J Leukoc Biol.*47(5): 440-8.

Rungseesantivanon, S., Thengchaisri, N. and Patumraj, S. (2006). PKC inhibition and vitamin C supplementation restore endothelial dysfunction of mesenteric arterioles in STZ-induced diabetic rats. 24th Conference of the European Society for Microcirculation. Amsterdam, The Natherlands, J Vasc Res. 43(suppl 1): 32.

Rungseesantivanon, S., Wongeakin, N., Thengchaisri, N. and Patumraj, S. (2007). Curcumin and vitamin C restore impaired acethylcholine-induced dilatation of mesenteric arterioles in STZ-diabetic rats. 8th World Congress for Microcirculation. Milwaukee, Wisconsin, USA, Microcirculation. 14: 499.

Sajithlal, G. B., Chithra, P. and Chandrakasan, G. (1998). Effect of curcumin on the advanced glycation and cross-linking of collagen in diabetic rats. *Biochem Pharmacol.*56(12): 1607-14.

Sakuma, S., Fujimoto, Y., Sakamoto, Y., Uchiyama, T., Yoshioka, K., Nishida, H., *et al.* (1997). Peroxynitrite induces the conversion of xanthine dehydrogenase to oxidase in rabbit liver. *Biochem Biophys Res Commun.*230(2): 476-9.

Sandler, S. and Swenne, I. (1983). Streptozotocin: depression of mouse liver pyridine nucleotides. *Cancer Res.*28: 1501-1506.

Sayeski, P. P. and Kudlow, J. E. (1996). Glucose metabolism to glucosamine is necessary for glucose stimulation of transforming growth factor-alpha gene transcription. *J Biol Chem.*271(25): 15237-43.

Schott, M., Schatz, D., Athkinson, M., Krischer, J., Mehta, H., Vold, B., *et al.* (1994). GAD_{65} autoantibodies increase the predictability but not the sensitivity of islet cell and insulin autoantibodies for developing insulin dependent diabetes mellitus. *J Autoimmunity.*7: 865-872.

Schreck, R., Albermann, K. and Baeuerle, P. A. (1992). Nuclear factor kappa B: an oxidative stress-responsive transcription factor of eukaryotic cells (a review). *Free Radic Res Commun.*17(4): 221-37.

Shaklai, N., Garlick, R. L. and Bunn, H. F. (1984). Nonenzymatic glycosylation of human serum albumin alters its conformation and function. *J Biol Chem.*259(6): 3812-7.

Shankar, T. N., Shantha, N. V., Ramesh, H. P., Murthy, I. A. and Murthy, V. S. (1980). Toxicity studies on turmeric (Curcuma longa): acute toxicity studies in rats, guineapigs & monkeys. *Indian J Exp Biol.*18(1): 73-5.

Sharma, R. A., Steward, W. P. and Gescher, A. J. (2007). *Pharmacokinetics and pharmacodynamics of curcumin*, Springer.

Sharma, S., Kulkarni, S. K. and Chopra, K. (2006). Curcumin, the active principle of turmeric (Curcuma longa), ameliorates diabetic nephropathy in rats. *Clin Exp Pharmacol Physiol.*33(10): 940-5.

Sharpe, P. C., Liu, W. H., Yue, K. K., McMaster, D., Catherwood, M. A., McGinty, A. M., *et al.* (1998). Glucose-induced oxidative stress in vascular contractile cells: comparison of aortic smooth muscle cells and retinal pericytes. *Diabetes.*47(5): 801-9.

Shiba, T., Inoguchi, T., Sportsman, J. R., Heath, W. F., Bursell, S. and King, G. L. (1993). Correlation of diacylglycerol level and protein kinase C activity in rat retina to retinal circulation. *Am J Physiol.*265(5 Pt 1): E783-93.

Shinohara, M., Thornalley, P. J., Giardino, I., Beisswenger, P., Thorpe, S. R., Onorato, J., *et al.* (1998). Overexpression of glyoxalase-I in bovine endothelial cells inhibits intracellular advanced glycation endproduct formation and prevents hyperglycemia-induced increases in macromolecular endocytosis. *J Clin Invest.*101(5): 1142-7.

Sikorski, B. W., Hodgson, W. C. and King, R. G. (1993). Effects of haemoglobin and N-nitro-L-arginine on constrictor and dilator responses of aortic rings from streptozotocin diabetic rats. *Eur J Pharmacol.*242(3): 275-82.

Sjoholm, A. and Nystrom, T. (2005). Endothelial inflammation in insulin resistance. *Lancet.*365(9459): 610-2.

Smith, D. W. and McFall, S. L. (2005). The relationship of diet and exercise for weight control and the quality of life gap associated with diabetes. *J Psychosom Res.*59(6): 385-92.

Soderling, T. R. (1990). Protein kinases. Regulation by autoinhibitory domains. *J Biol Chem.*265(4): 1823-6.

Somparn, P., Phisalaphong, C., Nakornchai, S., Unchern, S. and Morales, N. P. (2007). Comparative antioxidant activities of curcumin and its demethoxy and hydrogenated derivatives. *Biol Pharm Bull.*30(1): 74-8.

Sossin, W. S. and Schwartz, J. H. (1993). Ca(2+)-independent protein kinase Cs contain an amino-terminal domain similar to the C2 consensus sequence. *Trends Biochem Sci.*18(6): 207-8.

Spitaler, M. M. and Graier, W. F. (2002). Vascular targets of redox signalling in diabetes mellitus. *Diabetologia.*45(4): 476-94.

Sridulyakul, P., Chakraphan, D. and Patumraj, S. (2006). Vitamin C supplementation could reverse diabetes-induced endothelial cell dysfunction in mesenteric microcirculation in STZ-rats. *Clin Hemorheol Microcirc.*34(1-2): 315-21.

Srivastava, A. K. (2002). High glucose-induced activation of protein kinase signaling pathways in vascular smooth muscle cells: a potential role in the pathogenesis of vascular dysfunction in diabetes (review). *Int J Mol Med.*9(1): 85-9.

Srivastava, K. C. (1986). Evidence for the mechanism by which garlic inhibits platelet aggregation. *Prostaglainds Leukotr Med.*22: 313-321.

Stehouwer, C. D., Lambert, J., Donker, A. J. and van Hinsbergh, V. W. (1997). Endothelial dysfunction and pathogenesis of diabetic angiopathy. *Cardiovasc Res.*34(1): 55-68.

Sterin-Borda, L., Franchi, A. M., Borda, E. S., Del Castillo, E., Gimeno, M. F. and Gimeno, A. L. (1984). Augmented thromboxane generation by mesenteric arteries from pancreatectomized diabetic dogs is coincident with the vascular tone enhancement evoked by Na arachidonate and prostacyclin. *Eur J Pharmacol.*103(3-4): 211-21.

Strandell, E., Eizirik, D. L., Korsgren, O. and Sandler, S. (1988). Functional characteristics of cultured mouse pancreatic islets following exposure to different streptozotocin concentrations. *Mol Cell Endocrinol.*59: 83-91.

Strandell, E., Eizirik, D. L. and Sandler, S. (1989). Survival and B-cell function of mouse pancreatic islets maintained in culture after concomitant exposure to streptozotocin and nicotinamide. *Exp Clin Endocrinol.*93: 219-224.

Surh, Y. J., Chun, K. S., Cha, H. H., Han, S. S., Keum, Y. S., Park, K. K., *et al.* (2001). Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation. *Mutat Res.*480-481: 243-68.

Suryanarayana, P., Krishnaswamy, K. and Reddy, G. B. (2003). Effect of curcumin on galactose-induced cataractogenesis in rats. *Mol Vis.*9: 223-30.

Suzuki, H., Swei, A., Zweifach, B. W. and Schmid-Schonbein, G. W. (1995). In vivo evidence for microvascular oxidative stress in spontaneously hypertensive rats. Hydroethidine microfluorography. *Hypertension.*25(5): 1083-9.

Suzuki, K., Koh, Y. H., Mizuno, H., Hamaoka, R. and Taniguchi, N. (1998). Overexpression of aldehyde reductase protects PC12 cells from the cytotoxicity of methylglyoxal or 3-deoxyglucosone. *J Biochem.*123(2): 353-7.

Szerafin, T., Erdei, N., Fulop, T., Pasztor, E. T., Edes, I., Koller, A., *et al.* (2006). Increased cyclooxygenase-2 expression and prostaglandin-mediated dilation in coronary arterioles of patients with diabetes mellitus. *Circ Res.*99(5): e12-7.

Tahara, Y. and Shima, K. (1995). Kinetics of HbA1c, glycated albumin, and fructosamine and analysis of their weight functions against preceding plasma glucose level. *Diabetes Care.*18(4): 440-7.

Tak, P. P. and Firestein, G. S. (2001). NF-kappaB: a key role in inflammatory diseases. *J Clin Invest.*107(1): 7-11.

Takai, Y., Kishimoto, A., Inoue, M. and Nishizuka, Y. (1977). Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. I. Purification and characterization of an active enzyme from bovine cerebellum. *J Biol Chem.*252(21): 7603-9.

Takeishi, Y., Chu, G., Kirkpatrick, D. M., Li, Z., Wakasaki, H., Kranias, E. G., *et al.* (1998). In vivo phosphorylation of cardiac troponin I by protein kinase Cbeta2 decreases cardiomyocyte calcium responsiveness and contractility in transgenic mouse hearts. *J Clin Invest.*102(1): 72-8.

Tanaka, S., Avigad, G., Brodsky, B. and Eikenberry, E. F. (1988). Glycation induces expansion of the molecular packing of collagen. *J Mol Biol.*203(2): 495-505.

Taniyama, Y. and Griendling, K. K. (2003). Reactive oxygen species in the vasculature: molecular and cellular mechanisms. *Hypertension.*42(6): 1075-81.

Tesfamariam, B. (1994). Free radicals in diabetic endothelial cell dysfunction. *Free Radic Biol Med.*16(3): 383-91.

Tesfamariam, B., Brown, M. L. and Cohen, R. A. (1991). Elevated glucose impairs endothelium-dependent relaxation by activating protein kinase C. *J Clin Invest.*87(5): 1643-8.

Tesfamariam, B., Brown, M. L., Deykin, D. and Cohen, R. A. (1990). Elevated glucose promotes generation of endothelium-derived vasoconstrictor prostanoids in rabbit aorta. *J Clin Invest.*85(3): 929-32.

The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (2003). Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care.*26: S5-S20.

The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (2004). Diagnosis and classification of diabete mellitus. *Diabetes Care*.27 (supplement 1): S5-S10.

The National Research Council of Thailand (NRCT) (1999). The ethical principles for the use of animal for scientific purpose.

Thomas, G. and Roques, B. (1972). Proton magnetic resonance studies of ethidium bromide and its sodium borohydride reduced derivative. *FEBS Letters*.26(1-2): 169-175.

Thornalley, P. J. (1990). The glyoxalase system: new developments towards functional characterization of a metabolic pathway fundamental to biological life. *Biochem J*.269(1): 1-11.

Timimi, F. K., Ting, H. H., Haley, E. A., Roddy, M. A., Ganz, P. and Creager, M. A. (1998). Vitamin C improves endothelium-dependent vasodilation in patients with insulin-dependent diabetes mellitus. *J Am Coll Cardiol*.31(3): 552-7.

Toda, S., Miyase, T., Arichi, H., Tanizawa, H. and Takino, Y. (1985). Natural antioxidants. III. Antioxidative components isolated from rhizome of Curcuma longa L. *Chem Pharm Bull (Tokyo)*.33(4): 1725-8.

Touyz, R. M., Chen, X., Tabet, F., Yao, G., He, G., Quinn, M. T., *et al.* (2002). Expression of a functionally active gp91phox-containing neutrophil-type NAD(P)H oxidase in smooth muscle cells from human resistance arteries: regulation by angiotensin II. *Circ Res*.90(11): 1205-13.

Tsilibary, E. C., Charonis, A. S., Reger, L. A., Wohlhueter, R. M. and Furcht, L. T. (1988). The effect of nonenzymatic glucosylation on the binding of the main noncollagenous NC1 domain to type IV collagen. *J Biol Chem*.263(9): 4302-8.

Turk, J., Cirbett, J. A., Ramanadham, S., Bohrer, A. and McDaniel, M. L. (1993). Biochemical evidence for nitric oxide fromation from streptozotocin in isolated pancreatic islets. *Biochem Biophys Res Commun*.197: 1458-1464.

UK Prospective Diabetes Study (UKPDS) Group (1998). Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with T2-diabetes (UKPDS 33). *Lancet* 352: 837-853.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M. and Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol*.39(1): 44-84.

Vasquez-Vivar, J., Kalyanaraman, B., Martasek, P., Hogg, N., Masters, B. S., Karoui, H., *et al.* (1998). Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci U S A*.95(16): 9220-5.

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

Wang, Z. and Gleichmann, H. (1998). GLUT2 in pancreatic islets: crucial target molecule in diabetes induced with multiple low doses of streptozotocin in mice. *Diabetes*.47: 50-56.

Wellen, K. E. and Hotamisligil, G. S. (2005). Inflammation, stress, and diabetes. *J Clin Invest*.115(5): 1111-9.

Wells-Knecht, K. J., Zyzak, D. V., Litchfield, J. E., Thorpe, S. R. and Baynes, J. W. (1995). Mechanism of autoxidative glycosylation: identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. *Biochemistry*.34(11): 3702-9.

Wells, L. and Hart, G. W. (2003). O-GlcNAc turns twenty: functional implications for post-translational modification of nuclear and cytosolic proteins with a sugar. *FEBS Lett.*546(1): 154-8.

WHO Expert Committee on Diabetes Mellitus (1980). Second report. Technical report series 646. Geneva, World Health Org.

Whorton, A. R., Smigel, M., Oates, J. A. and Frolich, J. C. (1978). Regional differences in prostacyclin formation by the kidney. Prostacyclin is a major prostaglandin of renal cortex. *Biochim Biophys Acta.*529(1): 176-80.

Wild, S., Roglic, G., Green, A., Sicree, R. and King, H. (2004). Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care.*27(5): 1047-53.

Williams, C. S. and DuBois, R. N. (1996). Prostaglandin endoperoxide synthase: why two isoforms? *Am J Physiol.*270(3 Pt 1): G393-400.

Williamson, J. R., Chang, K., Frangos, M., Hasan, K. S., Ido, Y., Kawamura, T., *et al.* (1993). Hyperglycemic pseudohypoxia and diabetic complications. *Diabetes.*42: 801-813.

Williamson, J. R., Chang, K., Frangos, M., Hasan, K. S., Ido, Y., Kawamura, T., *et al.* (1993). Hyperglycemic pseudohypoxia and diabetic complications. *Diabetes.*42(6): 801-13.

Wilson, D. K., Bohren, K. M., Gabbay, K. H. and Quiocho, F. A. (1992). An unlikely sugar substrate site in the 1.65 A structure of the human aldose reductase holoenzyme implicated in diabetic complications. *Science.*257: 81-4.

Wilson, G. L., Hartig, P. C., Patton, N. J. and LeDoux, S. P. (1988). Mechanisms of nitrosourea-induced beta-cell damage. Activation of poly(ADP-ribose) synthetase and cellular distribution. *Diabetes.*37: 213-216.

Wink, D. A. and Mitchell, J. B. (1998). Chemical biology of nitric oxide: Insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. *Free Radic Biol Med.*25(4-5): 434-56.

Wohaieb, S. A. and Godin, D. V. (1987). Alterations in free radical tissue-defense mechanisms in streptozocin-induced diabetes in rat. Effects of insulin treatment. *Diabetes.*36(9): 1014-8.

Wolin, M. S. (2000). Interactions of oxidants with vascular signaling systems. *Arterioscler Thromb Vasc Biol.*20(6): 1430-42.

Wolin, M. S., Davidson, C. A., Kaminski, P. M., Fayngersh, R. P. and Mohazzab, H. K. (1998). Oxidant--nitric oxide signalling mechanisms in vascular tissue. *Biochemistry (Mosc).*63(7): 810-6.

Wolin, M. S., Gupte, S. A. and Oeckler, R. A. (2002). Superoxide in the vascular system. *J Vasc Res.*39(3): 191-207.

World Health Organization (1985). Diabetes mellitus: Report of a WHO study group. Technical report series 727. Geneva, World Health Org.

World Health Organization (1999). Definition, diagnosis and classification of diabetes mellitus and its complications: Report of a WHO Consultation. Part 1: Diagnosis and classification of diabetes mellitus. Geneva, World Health Org.

World Health Organization (2006). Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia: report of a WHO/IDF consultation. Geneva, World Health Org.

World Health Organization (2006). "Mortality and country Fact Sheet 2006." Death and DALY estimates by causes, 2002. From www.who.int /entity/healthinfo /statistic/ bodgbddeathdalyestimates.

Xia, P., Inoguchi, T., Kern, T. S., Engerman, R. L., Oates, P. J. and King, G. L. (1994). Characterization of the mechanism for the chronic activation of diacylglycerol-protein kinase C pathway in diabetes and hypergalactosemia. *Diabetes.*43(9): 1122-9.

Xia, P., Kramer, R. M. and King, G. L. (1995). Identification of the mechanism for the inhibition of Na,K-adenosine triphosphase by hyperglycemia involving activation of protein kinase C and cytosolic phospholipase A2. *J Clin Invest.*96: 733-740.

Yakubu, M. A., Sofola, O. A., Igbo, I. and Oyekan, A. O. (2004). Link between free radicals and protein kinase C in glucose-induced alteration of vascular dilation. *Life Sci.*75(24): 2921-32.

Yamamoto, H., Uchigata, Y. and Okamoto, H. (1981). Streptozotocin and alloxan induce DNA strand breaks and poly(ADP-ribose) synthetase in pancreatic islets. *Nature.*294: 284-286.

Yamauchi, T., Kamon, J., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., *et al.* (2002). Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med.*8(11): 1288-95.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., *et al.* (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature.*332(6163): 411-5.

Yerneni, K. K., Bai, W., Khan, B. V., Medford, R. M. and Natarajan, R. (1999). Hyperglycemia-induced activation of nuclear transcription factor kappaB in v ascular smooth muscle cells. *Diabetes.*48(4): 855-64.

Zandi, E., Rothwarf, D. M., Delhase, M., Hayakawa, M. and Karin, M. (1997). The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. *Cell.*91(2): 243-52.

Zou, M., Martin, C. and Ullrich, V. (1997). Tyrosine nitration as a mechanism of selective inactivation of prostacyclin synthase by peroxynitrite. *Biol Chem.*378(7): 707-13.

Zou, M. H. and Bachschmid, M. (1999). Hypoxia-reoxygenation triggers coronary vasospasm in isolated bovine coronary arteries via tyrosine nitration of prostacyclin synthase. *J Exp Med.*190(1): 135-9.

APPENDIX

PUBLICATIONS

- Prortein kinase C inhibition and vitamin C supplementation can improve endothelial dysfunction of mesenteric arterioles in STZ-induced diabetic rats.
  Rungseesantivanon S., Thengchaisri N. and Patumraj S.
  24th Conference of the European Society for Microcirculation.
  Amsterdam, The Natherlands. August 30- September 2, 2006.
  J Vasc Res. 2006; **43** (suppl 1):32

- PKC inhibition and vitamin C supplementation restore endothelial dysfunction of mesenteric arteriole in STZ-induced diabetic rats.
  Rungseesantivanon S., Thengchaisri N. and Patumraj S.
  36th Annual Scientific Meeting the Physiological Society of Thailand.
  Ayudhaya, Thailand. April 25-27, 2007.

- Curcumin and vitamin C restore impaired acetylcholine-induced dilatation of mesenteric arterioles in STZ-induced diabetic rats.
  Rungseesantivanon S., Wongeakin N., Thengchaisri N. and Patumra S.
  8th World Congress for Microcirculation.
  Milwaukee, Wisconsin, USA. August 15-19, 2007.
  Microcirculation. 2007; **14**: 499

- Anti-oxidant effect of curcumin on diabetes-induced endothelial dysfunction: an *in vivo* detection using hydroethidine.
  Rungseesantivanon S., Thengchaisri N. and Patumraj S.
  25th Conference of the European Society for Microcirculation.
  Budapest, Hungary. August 26-29, 2008.

- Anti-oxidant effect of curcumin on diabetes-induced endothelial dysfunction: in vivo detection using hydroethidine.
  Rungseesantivanon S., Thengchaisri N. and Patumraj S.
  Joint Conference in Medical Science 2009.
  Bangkok, Thailand. May 29, 2009

PUBLICATIONS

- Rungseesantivanon S., Thenchaisri N. and Patumraj S.
  Curcumin supplementation could improve diabetes-induced endothelial dysfunction associated with decreased vascular superoxide production and PKC inhibition. (*BMC comp alt med*. Submission).

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