

'ROLE OF CYCLOOXYGENASE IN PROGNOSTIC COMPLICATIONS OF DIABETES MELLITUS SUBJECTS'.

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ABSTRACT:

Background: Development of diabetes and its complications is associated with persistent inflammatory activity. It has been suggested that COX-mediated pathways may be involved in this inflammatory process in diabetes. For example, TXA₂ and PGE₂ released by platelets and monocytes due to the action of COX-1 and COX-2 respectively are important prostanoids involved in thrombosis and atherosclerosis development in diabetes.

Materials: Blood sample was collected from 25 diabetic subjects and 25 age and sex matched controls. Regular routine Biochemical & Pathological parameters were analyzed in diabetes. Both COX-1 and COX-2 were analyzed in type II diabetes and diabetic animal models (Wistar rats).

Results: Both COX-1 and COX-2 was increased in diabetic animal models (Wistar rats). The relationship between COX & Diabetes when studied in humans found that Type II diabetics showed slightly increased (but it is not significant) monocyte COX 2 & normal platelets COX1.

Conclusion: This suggests that changes in COX levels in these inflammatory cells may be species-dependent.

Key words: Cyclooxygenase (COX), Thromboxane A₂ (TX A₂),

Introduction:

Increasing evidence suggests that insulin resistance and progressive pancreatic β cell failure, key events in the development of Type 2 diabetes is associated with persistent inflammatory activity indicated by elevated inflammatory markers such as TNF- α , CRP and IL-6 (1). Further animal studies found that these inflammatory markers interfered with insulin signalling pathways. One

of the consequences of Type 2 diabetes is the development of chronic diabetic complications including atherosclerosis. Data derives from experimental and epidemiological studies have shown that low-grade inflammation plays an important role in the initiation and progression of atherosclerotic plaque development (2). It is clear that both Type 1 & Type 2 diabetes complications may share a common link, namely inflammation.

COX expression, particularly COX-2, can be elevated by inflammatory cytokines. COX isoforms are rate-limiting enzymes that catalyze arachidonic acid leading to the eventual production of various prostanoids, which can alter vascular reactivity, platelet aggregation and atherosclerotic plaque formation and stability (3). COX-1, expressed at constant levels throughout the cell cycle, is termed a constitutive isoform and has a "house-keeping" role (4).

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In contrast, inducible COX-2, whose expression under normal physiological conditions is not usually detected, is up-regulated in response to a variety of agents such as hormones, growth factors, pro-inflammatory stimuli and inflammation (5). Prostanoids produced by COX pathways are crucial to numerous physiological processes including platelet aggregation, vasoconstriction and vasodilatation. TXA2 released by platelet and PGE2 released by monocytes due to the action of COX-1 and COX-2, respectively are important prostanoids involved in thrombosis and atherosclerosis development. Given that inflammation is elevated in Type 2 diabetes and the ability of COX-mediated prostanoids to be involved in thrombosis and atherosclerosis, it was of interest to investigate if COX-mediated pathways.

Materials and Methods :

Treatment of Animals:

Male Wistar rats weighing 150-200 grams were used for this study. Animals were obtained from the institute central animal house and maintained in standard laboratory conditions at $22 \pm 2^{\circ}$ C with 12:12 hours dark-light cycle in the department animal room facility. Whole blood was collected. All experimental procedures used in this study were approved by the Institute Animal Experimentation Ethics Committee. Animals were allowed to acclimatize for one week to departmental animal room environment and then randomly assigned to the following two groups. Group 1. Control – Rats fed with rodent chow. Group 2. High fat diet – Rats fed with high fat diet.

Selection of Subjects

Twenty Five newly diagnosed type 2 diabetic patients (13 males and 12 females) in the age group of 35-50 years were taken as case from our OP.

As control, 25 age matched healthy volunteers (13 males and 12 females) were recruited. The present study did not collect samples from smokers and hypertensive, Type I DM, Type 2 diabetic subjects on NSAIDs treatment, subjects with Micro- Macro Vascular complications, with other known diseases. The diagnosis of diabetes was made based on plasma glucose levels of venous plasma using the diagnostic guideline provided by the American Diabetes association. All participants were informed of the study by an endocrinologist.

Isolation of human platelets and monocytes

The isolation of human platelets and monocytes was performed using OptiPrep™ solution according to the manufacturer's standard protocol.

Protein assay

Determination of human sample protein concentration was performed using the Biorad microBCA.

Western blot: COX protein

The density of all Western blot bands was analyzed using the Chemi Doc™ XRS system (Biorad, CA, USA).

DISCUSSION :

The two questions of this current study were whether COX-1 and COX-2 expression was altered in Type 2 diabetes. The animal model used was obese Wistar rats, which has several of the elements of Type 2 diabetes including hyperglycemia, obesity, dyslipidemia and hypertension (6).

Obese Wistar rats have been extensively used as a model of Type 2 diabetes as they exhibit several characteristics of Type 2 diabetes including hyperglycemia, obesity, dyslipidemia and hypertension. In the present study, COX-1 levels in platelets were measured in obese Wistar rats and found to be elevated compared to lean Wistar rats. Similar finding has also been reported by Raju *ET AL.* (7)

in the platelets of obese Zucker rats. Hyperactive platelets have been reported by Schafer *ET AL.* (8) in the young Wistar rats with impaired glucose tolerance and increased COX-1 levels in platelets may potentially result in enhanced production of prostanoids such as TXA₂ and platelet aggregation (9). Potentially, changes in prostanoid production may result in an increased risk in thrombosis due to enhanced platelet activation and aggregation, a phenomenon that was not observed in COX-1 knock-out mice (10).

COX-2-mediated pathway in monocytes in Wistar rats

COX-2 is the rate-limiting enzyme in prostanoid production in monocytes. In diabetes, COX-2 up-regulation in monocytes is often associated with enhanced monocyte infiltration into the intima and macrophage-induced inflammation processes (11). In the present study, monocyte COX-2 levels were measured and found to be elevated in obese Wistar rats compared with the lean rats. In obese Wistar rats, enhanced COX-2 expression in the renal cortex and micro-vessels was found to contribute to renal damage (12). Increased monocyte COX-2 levels observed in the current study may further exacerbate the renal injury often seen in obese Wistar rats. In monocytes, elevated COX-2 levels can also lead to increased PGE₂ production. COX-2-mediated release of PGE₂ plays a role in inflammatory processes. For instance, PGE₂ increases blood flow to inflamed area by dilating blood vessels and stimulates the migration of leukocytes through capillary walls. Furthermore, COX-2-mediated release of PGE₂ has been suggested to play a role in destabilizing atherosclerotic plaque via MMP pathways. Therefore, an up-regulation of COX-2 in monocyte in obese Wistar rats indicates an elevated inflammatory activity and could account for the more severe degree of

atherosclerosis in obese Wistar rats compared to lean Wistar rats (13).

COX-1-mediated pathway in platelets in Type 2 diabetes

The present study showed that platelet COX-1 levels were unaltered in Type 2 diabetes. Our animal study showed that COX-1 in platelets was increased in diabetic Wistar rats, data from this current human study did not agree with data from the animal model. Several studies indicated that platelets are more hyperactive in diabetes and a good glycemic control has been shown to reduce platelet activation markers in human Type 2 diabetic subjects (14). Hyperglycemia has been proposed to be a causal factor for *IN VIVO* platelet activation. For instance, excess glucose contributes to non-enzymatic glycation of platelet lipid membrane, which may affect the activation of receptors on the surface of platelet lipid membrane. High levels of glucose can also increase oxidative stress via polyol pathway, which may be responsible for enhanced peroxidation of arachidonic acid and increased isoprostane production, which is known to cause persistent platelet activation (15). The current study, however, suggests that changes in platelet COX-1 is most likely not involved in hyperactivity of platelets in Type 2 diabetes.

The present study found that human subjects with hyperlipidemia had a significantly higher level of platelet COX-1 compared with human control subjects. Further sub-analysis showed that increased platelet COX-1 levels were clearly elevated with hyperlipidemia in control and diabetic groups, although this was not significant in human Type 2 diabetic subjects presumably due to the low numbers. A study by Davi *ET AL.* (16) found enhanced COX-1-mediated activity in platelets as indicated by an increased TXA₂ synthesis in hypercholesterolemic subjects. Belton *ET AL.* (17) further reported elevated COX-1 expression in the vascular lesions during atherosclerosis development was reported in apolipoprotein E-deficient mice.

Changes in lipid levels are known to promote thrombosis by affecting the activity of platelets (18), fibrinolytic factors (19) and coagulation proteins (20). Indeed, increased cholesterol levels increase the risk of arterial thrombotic events in patients with atherosclerosis (21). Potentially, alteration in COX-1 levels due to hyperlipidemia, a condition commonly observed in Type 2 diabetes may contribute to the development of macrovascular complications. The present results for the first time show a link between hyperlipidemia and COX-1, a factor which could promote platelet aggregation. It may also be an indication for the use of COX inhibitors in hyperlipidemia.

COX-2-mediated pathway in monocytes in Type 2 diabetes

Very little is data available about COX-2 in Type 2 diabetes. Increasing evidence has revealed increased inflammation in Type 2 diabetes and inflammation may be an important component in diabetic complication development (22), our study hence investigated if COX-2 expression in monocytes was elevated in Type 2 diabetes as elevated COX-2 occurs in inflammation. In contrast, COX-2 mRNA levels were increased in peripheral blood monocytes from human Type 2 diabetic subjects (23). This non age-matched study, however, did not measure the actual expression of COX-2 protein and only had a very small sample size (four human control and two human Type 2 diabetic subjects, respectively). Therefore, it is difficult to interpret the results and establish the relationship between increased COX-2 mRNA and actual functional protein expression. In another study involved age-matched human subjects, COX-2 up-regulation was observed in monocyte-derived macrophages at the site of atherosclerotic plaques in Type 2 diabetes (24).

Potentially, changes in COX-2 expression in these cells can lead to an activation of numerous signalling transduction pathways. For example, through PGE₂-mediated EP₄ signalling pathway, enhanced COX-2 is associated with the induction of MMPs, proteases that digest extracellular matrix and destabilize atherosclerotic plaques (25). The COX-2 expression in monocytes, which the present study observed is worth noting as it has been reported previously (26) and may be a potential factor in cardiovascular complications of aging. The present study found that plasma PGE₂ increased significantly in Type 2 diabetes in age-matched analysis. The exact source of enhanced plasma PGE₂ production is unclear as several sources of plasma PGE₂ have been identified. These sources include endothelial cells platelets, lung epithelial cells (27), alveolar macrophages and monocyte-derived macrophages at the site of atherosclerotic plaques. It has also been reported that stromal pre-adipocytes expressed high level of COX-2 protein and released PGE₂ after stimulation by adiponectin, a hormone secreted by adipocytes that regulates glucose and lipid metabolism (28). Regardless the source of the elevated plasma PGE₂, PGE₂ production has been associated with destabilizing atherosclerotic plaques (29), diabetic retinopathy and nephropathy possibly through increased MMP activity (30) and increased oxidative stress.

Conclusion:

The current study reported that COX levels were up-regulated in platelets and monocytes of Wistar rats, an observation that was not found in human Type 2 diabetic study. It means in humans Type II diabetics showed slightly increased (but it is not significant) monocyte COX 2 & normal platelets COX1. This suggests that changes in COX levels in these inflammatory cells may be species-dependent. Suggesting an overall increase in inflammatory activity in Type 2 diabetes.

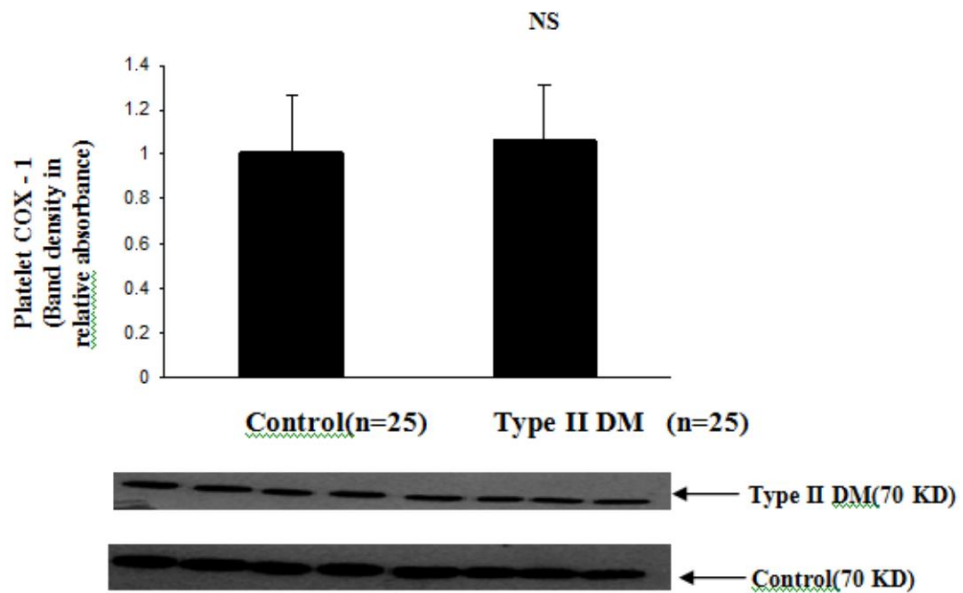


Figure I. Western blotting analysis of Platelet COX-1 in control and Type II DM subjects.

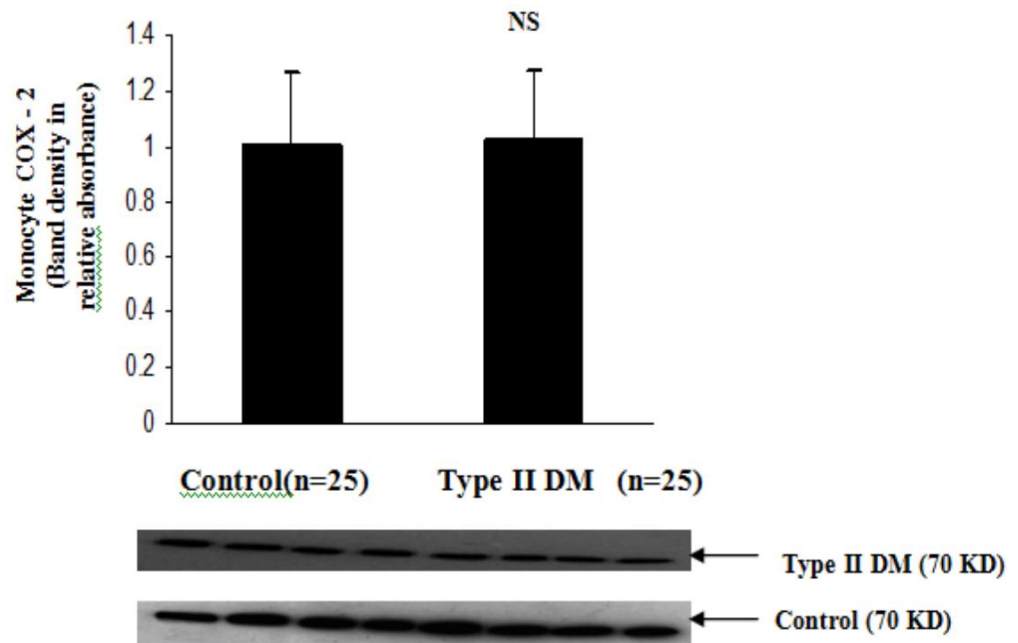
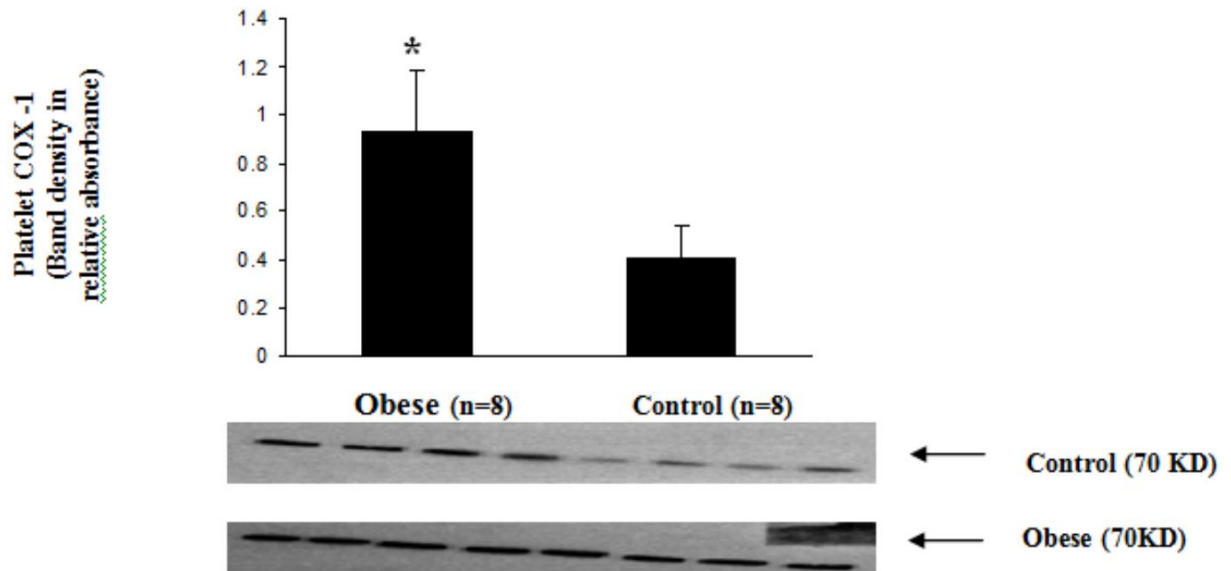


Figure II. Western blotting analysis of Monocyte COX - 2 in control and type 2 DM subjects.



Figure(III). Western blotting analysis of Platelet COX -1 Protein in control and Obese Wistar Rats.

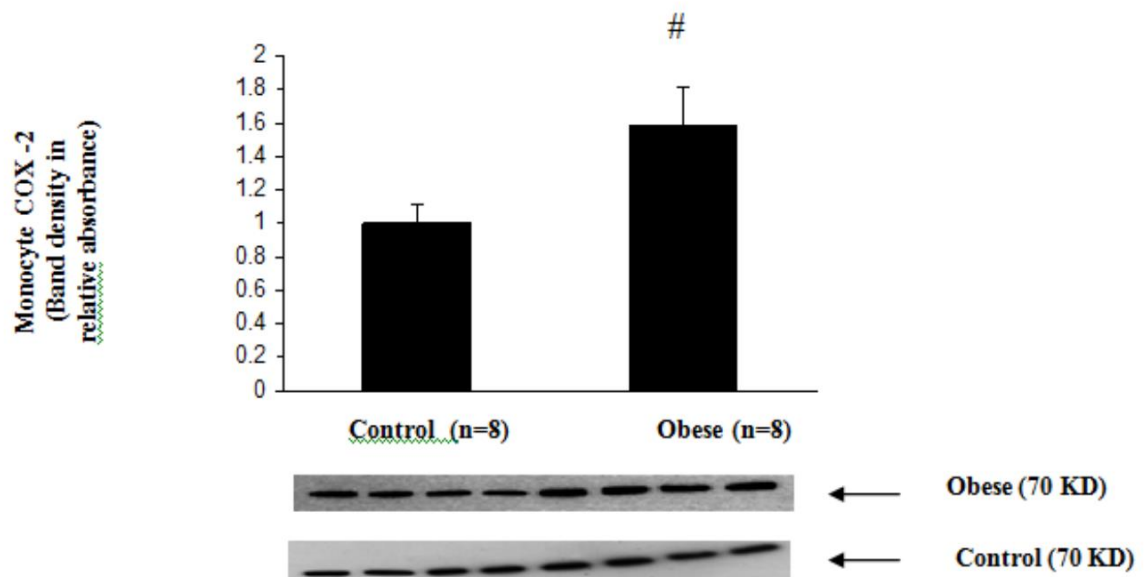


Figure (IV) Western blotting analysis of Monocyte COX - 2 in control and Obese Wistar Rats.

. * **significant** difference from lean values, $p < 0.001$, unpaired Student's t-test; # **significant** difference from lean values, $p < 0.05$, Mann Whitney test.

References :

References:

1. Yudkin JS, Stehouwer CD, Emeis JJ & Coppack SW. (1999) C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue? *ArterioscleroThromb Vasc Biol.* 19: 972-8.
2. Pasceri V, Chang J, Willerson JT & Yeh ET. (2001) Modulation of C-reactive protein-mediated monocyte chemoattractant protein-I induction in human endothelial cells by anti-atherosclerosis drugs. *Circulation.* 103: 2531-4.
3. Zwaka TP, Hombach V & Torzewski J. (2001) C-reactive protein mediated low density lipoprotein uptake by macrophages: implications for atherosclerosis. *Circulation.*103:1194-7.
4. DeWitt DL & Meade EA. (1993) Serum and glucocorticoid regulation of gene transcription and expression of the prostaglandin H synthase-1 and prostaglandin H synthase-2 isozymes. *Arch Biochem Biophys.* 306(1):94-102
5. Garavito RM & DeWitt DL. (1999) The cyclooxygenase isoforms: structural insights into the conversion of arachidonic acid to prostaglandins. *Biochim Biophys Acta.*1441(2-3):278-87
6. Khan AM, Lally KP, Larsen GL & Colasurdo GN. (2002) Enhanced release of thromboxane A(2) after exposure of human airway epithelial cells to meconium. *Pediatr Pulmonol.* 33(2):111-6.
7. Raju J, Bajaj G, Chrusch J & Bird RP. (2006) Obese state leads to elevated levels of TGF-beta and COX isoforms in platelets of Zucker rats. *Mol Cell Biochem.* Feb 14:1-6
8. Schafer A, Widder J, Eigenthaler M, Bischoff H, Ertl G & Bauersachs J. (2004) Increased platelet activation in young Zucker rats with impaired glucose tolerance is improved by acarbose. *Thromb Haemost.* 92(1):97-103
9. Liu J, Pestina TI, Berndt MC, Steward SA, Jackson CW & Gartner TK. (2004) The roles of ADP and TXA in botrocetin/VWF-induced aggregation of washed platelets. *J Thromb Haemost.* 2(12):2213-22
10. Yu Y, Cheng Y, Fan J, Chen XS, Klein-Szanto A, Fitzgerald GA & Funk CD. (2005) Differential impact of prostaglandin H synthase 1 knockdown on platelets and parturition. *J Clin Invest.* 115(4):986-95
11. Martin-Ventura JL, Blanco-Colio LM, Gomez-Hernandez A, Munoz-Garcia B, Vega M, Serrano J, Ortega L, Hernandez G, Tunon J & Egido J. (2005) Intensive treatment with atorvastatin reduces inflammation in mononuclear cells and human atherosclerotic lesions in one month. *Stroke.* 36(8):1796-800
12. Komers R, Zdychova J, Cahova M, Kazdova L, Lindsley JN & Anderson S. (2005) Renal cyclooxygenase-2 in obese Zucker (fatty) rats. *Kidney Int.* 67(6):2151-8.
13. Kasiske BL, O'Donnell MP & Keane WF. (1992) The Zucker rat model of obesity, insulin resistance, hyperlipidemia, and renal injury. *Hypertension.* 19(1 Suppl):I110-5
14. Eibl N, Krugluger W, Streit G, Schratlbauer K, Hopmeier P & Schernthaner G. (2004) Improved metabolic control decreases platelet activation markers in patients with type-2 diabetes. *Eur J Clin Invest.* 34(3):205-9
15. Ferroni P, Basili S, Falco A & Davi G. (2004) Platelet activation in type 2 diabetes mellitus. *J Thromb Haemost.* 2(8):1282-91

16. Davi G, Chiarelli F, Santilli F, Pomilio M, Vigneri S, Falco A, Basili S, Ciabattini G & Patrono C. (2003) Enhanced lipid peroxidation and platelet activation in the early phase of type 1 diabetes mellitus: role of interleukin-6 and disease duration. *Circulation*. 107(25):3199-203
17. Belton OA, Duffy A, Toomey S & Fitzgerald DJ. (2003) Cyclooxygenase isoforms and platelet vessel wall interactions in the apolipoprotein E knockout mouse model of atherosclerosis. *Circulation*. 108(24):3017-23.
18. Di Minno G, Silver MJ, Cerbone AM, Rainone A, Postiglione A & Mancini M. (1986) Increased fibrinogen binding to platelets from patients with familial hypercholesterolemia. *Arteriosclerosis*. 6:203.211
19. Lowe GDO, Wood DA, Douglas JT, Riemersma RA, Macintyre CC & Takase T. (1991) Relationships of plasma viscosity, coagulation and fibrinolysis to coronary risk factors and angina. *Thromb Haemost*. 65:339.343
20. Hoffman CJ, Lawson WE, Miller RH & Hultin MB. (1994) Correlation of vitamin K-dependent clotting factors with cholesterol and triglycerides in healthy young adults. *Arterioscler Thromb*. 14:1737.1740
21. Pitt B, Mancini GB, Ellis SG, Rosman HS, Park JS & McGovern ME. (1995) Pravastatin limitation of atherosclerosis in the coronary arteries (PLAC I): reduction in atherosclerosis progression and clinical events: PLAC I investigation. *J Am Coll Cardiol*. 26:1133-1139
22. Bluher M, Fasshauer M, Tonjes A, Kratzsch J, Schon MR & Paschke R. (2005) Association of interleukin-6, C-reactive protein, interleukin-10 and adiponectin plasma concentrations with measures of obesity, insulin sensitivity and glucose metabolism. *Exp Clin Endocrinol Diabetes*. 113(9):534-7
23. Shanmugam N, Gaw Gonzalo IT & Natarajan R. (2004) Molecular mechanisms of high glucose-induced cyclooxygenase-2 expression in monocytes. *Diabetes*. 53(3):795-802
24. Cipollone F, Fazio ML, Iezzi A, Cucurullo C, De Cesare D, Uchino S, Spigonardo F, Marchetti A, Buttitta F, Paloscia L, Mascellanti M, Cucurullo F & Mezzetti A. (2005). Association between prostaglandin E receptor subtype EP4 overexpression and unstable phenotype in atherosclerotic plaques in human. *Arterioscler Thromb Vasc Biol*.25(9):1925-31
25. Lu Y & Wahl LM. (2005) Oxidative stress augments the production of matrix metalloproteinase-1, cyclooxygenase-2, and prostaglandin E2 through enhancement of NF-kappa B activity in lipopolysaccharide-activated human primary monocytes. *J Immunol*. 175(8):5423-9
26. Cipollone F, Fazio ML, Iezzi A, Cucurullo C, De Cesare D, Uchino S, Spigonardo F, Marchetti A, Buttitta F, Paloscia L, Mascellanti M, Cucurullo F & Mezzetti A. (2005) Association between prostaglandin E receptor subtype EP4 overexpression and unstable phenotype in atherosclerotic plaques in human. *Arterioscler Thromb Vasc Biol*. 25(9):1925-31
27. King KM. (2003) A history of insulin: from discovery to modern alternatives. *Br J Nurs*. 12(19):1137-41.
28. N'guessan PD, Hippenstiel S, Etouem MO, Zahlten J, Beermann W, Lindner D, Opitz B, Witzernath M, Rosseau S, Suttrop N & Schmeck B. (2006) Streptococcus pneumoniae-induced p38 MAPK- and NF-

{kappa}B-dependent COX-2 expression in human lung epithelium. *Am J Physiol Lung Cell Mol Physiol.* 35(5):17-23.

29. Yokota T, Meka CS, Medina KL, Igarashi H, Comp PC, Takahashi M, Nishida M, Oritani K, Miyagawa J, Funahashi T, Tomiyama Y, Matsuzawa Y & Kincade PW. (2002) Paracrine regulation of fat cell formation in bone marrow cultures via adiponectin and prostaglandins. *J Clin Invest.* 109(10):1303-10
30. Gomez-Hernandez A, Sanchez-Galan E, Martin-Ventura JL, Vidal C, Blanco-Colio LM, Ortego M, Vega M, Serrano J, Ortega L, Hernandez G, Tunon J & Egido J. (2006) Atorvastatin reduces the expression of prostaglandin E2 receptors in human carotid atherosclerotic plaques and monocytic cells: potential implications for plaque stabilization. *J Cardiovasc Pharmacol.* 47(1):60-9.

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