Overexpression of Activated Nuclear Factor-κ B in Aorta of Patients With Coronary Atherosclerosis

Address for correspondence:
Qi Chong Xing, MD
Department of Cardiovascular Disease, Qianfoshan Hospital
66 Jingshi Road,
Jinan, 250014, PR China
xingqichong@163.com

Wei Zhang, PhD; Shan Shan Xing, PhD; Xue Lin Sun, MD; Qi Chong Xing, MD
Shandong University School of Medicine (Zhang, Xing), Jinan, PR China; Department of Cardiovascular Disease, Qianfoshan Hospital, Shandong University (Sun, Xing), Jinan, PR China

ABSTRACT

Background: Inflammation is an established risk factor for atherosclerosis. In an inflammatory state, nuclear factor-κ B (NF-κB) is frequently activated as a key transcription activator for the downstream responses.
Hypothesis: The aim of this study was to investigate the changes of NF-κB in the aorta of patients with coronary atherosclerosis and its association with atherosclerotic risk factors.
Methods: From 2004 to 2005, we collected a small piece of ascending aorta in the bypass procedure from patients (n = 31) undergoing coronary artery bypass graft (CABG) surgery. The expression of NF-κB was determined by immunohistochemistry, and its transcriptional activity was evaluated by electrophoretic mobility shift assay. Celiac aortic tissues from 4 subjects without known atherosclerosis through the kidney donation program were taken as control.
Results: NF-κB was detectable in aortas from CABG patients with the transcriptional activities significantly increased. The relative level of aortic NF-κB expression was elevated in patients who were smokers or with hypertension. Spearman correlation revealed that aortic NF-κB expression had significant correlation with coronary severity scores (Gensini score, $r = 0.608$, $P < .05$). The NF-κB expression was positively correlated with the levels of blood glucose, low-density lipoprotein cholesterol, lipoprotein(a), total cholesterol, and non-high-density lipoprotein cholesterol ($P < .05$); but negatively correlated with high-density lipoprotein cholesterol ($P < .05$).
Conclusions: Our study demonstrates a highly activated NF-κB in aortas from patients with coronary atherosclerosis, which may reflect overall arterial overinflammatory status. The findings of hyperactive NF-κB in aortas may provide a diagnostic parameter for the inflammation that is associated with and may cause atherosclerosis.

Introduction

Cardiovascular disease is the leading cause of morbidity and mortality in the Western world; its incidence has also increased lately in developing countries. Atherosclerosis is the underlying pathology of most cardiovascular disease. Recently, several lines of evidence support an important role for inflammation in atherogenesis.[1] As an inflammation process, several cellular and molecular events are known to be involved in the progression of atherosclerosis from an early fatty streak lesion to highly dangerous rupture-prone plaque. Inflammation at the cellular level can be described as an increase in the proinflammatory transcription factor nuclear factor-κB (NF-κB). NF-κB is a pivotal transcription factor that promotes the expression of a cascade of procoagulant and proinflammatory genes such as P-selectin, tumor necrosis factor (TNF)-α, intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), monocyte chemotactic protein-1 (MCP-1), interleukin 1 (IL-1), interleukin 8 (IL-8), and tissue factor (TF).[2] In the inactive state, the NF-κB bound to its inhibitory protein-κ B (IκB) in the cytoplasm. Activators—including cytokines and oxidants—release IκB from NF-κB, which then translocates to the nucleus and activates target genes. Although atherosclerosis may clinically and pathologically confine to localized arterial beds, for example, coronary arteries or carotid arteries, we hypothesize that the underlying inflammatory changes may be present in the entire vasculature. In anatomical locations where hemodynamic features are more turbulent, atherosclerotic changes may occur in these individuals with vascular inflammatory changes. In the current study, we investigated this hypothesis by measuring the changes in NF-κB in an aortic wall with normal appearance from patients with coronary atherosclerosis who underwent coronary artery bypass graft (CABG) surgery. We found that NF-κB was present and activated in aortas of patients with coronary atherosclerosis and its expression significantly

The first 2 authors contributed equally to this work.

Clin. Cardiol. 32, 12, E42–E47 (2009)
Published online in Wiley InterScience. (www.interscience.wiley.com)
DOI:10.1002/clc.20482

Received: March 9, 2008
Accepted with revision: March 25, 2008

correlated with severity of coronary artery disease (CAD), which may suggest that the overall arteries were under overinflammatory status and aortic NF-κB expression was a possible marker to predict coronary artery severity.

Methods

Study Population

The study conforms to the principles of the Declaration of Helsinki[3] and was approved by the Ethics Committee of the Qianfoshan Hospital. From 2004 to 2005, 31 patients with CAD scheduled for CABG surgery were investigated and were defined as a research group. All CAD patients were confirmed for their disease status by coronary angiography and the Gensini score was used to assess the CAD severity.[4] Observers who were unaware of the clinical and laboratory data scored all the coronary angiograms. The Gensini score defines luminal narrowing of the lumen of the coronary arteries as 1 for 1% to 25% narrowing, 2 for 26% to 50% narrowing, 4 for 51% to 75% narrowing, 8 for 76% to 90% narrowing, 16 for 91% to 99% narrowing, and 32 for total occlusion. The severity of stenosis was evaluated preoperatively by a single angiographer who was also blinded to our laboratory assay. The score is then multiplied by a factor that incorporates the importance of the lesion location in the coronary arterial tree. For example, 5 was scored for the left main coronary artery, 2.5 for the proximal left anterior descending artery (LAD) or proximal left circumflex artery (LCX), 1.5 for the mid region of the LAD, and 1 for the distal LAD or mid distal region of the LCX. The exclusion criteria were acute infection, acute state of a chronic infectious or inflammatory disease, autoimmune disease, asthma, neoplasm, hematologic disorders, and acute or chronic liver or kidney disease. We also collected aortic tissue without atherosclerotic lesions from the celiac aorta of 4 subjects who donated kidneys as the control group.

Aortic Tissue Collection

Aortic specimens were obtained from the aorta that was routinely removed during CABG surgery as a button hole. Subsequently, part of the adventitia covered by the epicardium was removed from the ventral part of the ascending aorta, and 1 to 3 punch holes were made through the vessel wall for proximal aortocoronary anastomoses in the same area. The specimens were fixed in formalin and embedded in paraffin for immunohistological analysis. An aliquot of the aortic tissue was snap-frozen in liquid nitrogen and stored at −80 °C for nuclear protein collection.

Plasma Lipoprotein Profiles

Whole blood (3 ml) was drawn after an overnight fasting for measurement of routine laboratory items. Serum glucose, total cholesterol (TC), lipoprotein(a) [Lp(a)], high-density lipoprotein cholesterol (HDL-C), and triglycerides (TG) were analyzed by the Department of Clinical Chemistry, Qianfoshan Hospital, by an autoanalyzer (Technicon AXON Tarrytown, New York, USA). The low-density lipoprotein cholesterol (LDL-C) and non-high-density lipoprotein cholesterol (non-HDL-C) was calculated by the following formula respectively: LDL-C = TC − HDL-C −TG/2; non-HDL-C = TC − HDL-C.

Immunohistochemical Staining of NF-κB

Presence of NF-κB in aortic tissue was assessed using immunohistochemistry as previously reported.[5] Briefly, after formalin fixation, all specimens were sectioned to 3-μ m sections. After a serial rehydration process, sections were incubated with anti-p65 NF-κB (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) as the primary antibody before application of secondary goat anti-rabbit immunoglobulin (IgG); conjugated horseradish peroxidase. Sections were washed 3 times with phosphate buffered solution (PBS) between each step. For semiquantification, we counted the total number of cells with positive staining in 1000 smooth muscle cells from 5 high power fields (×400) per section and the mean percentage was calculated.

NF-κB DNA Binding Activity

Nuclear NF-κB DNA binding activity was measured by electrophoretic mobility shift assay (EMSA) as described previously.[6] Briefly, nuclear extracts from human aortic samples were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA). The supernatant fractions containing the cytoplasm and the nuclear extract were prepared separately. Protein concentrations were determined by bicin choninic acid (BCA method (Pierce, Rockford, IL, USA). Protein extracts (10 μg each) and biotin end-labeled oligonucleotide probe with the NF-κB binding site 5′-AGTTGAGGCGACTTTCCCAGGC-3′ (Sangon Biological Engineering Technology Services Co. Ltd. Shanghai, China) were incubated for 20 minutes at room temperature. The binding buffer contained 1 μg polydeoxy (Inosinate-Cytidylate) acid (dI-dC), 10 mM tris (hydroxymethyl) aminomethane-hydrogen chloride buffer (Tris-HCl), pH 7.5, 50 M sodium chloride (NaCl), 1 mM ethylenediamine tetraacetic acid (EDTA), 5% glycerol, 1 mM dithiothreitol (DTT) and 1 μg/μ L bovine serum albumin (BSA). DNA protein complexes were electrophoresed on a 6% polyacrylamide gel and electroblotted onto a positively-charged nylon membrane. After cross-link with UV light, the membrane was blocked, washed, and incubated with Light-shift TM Substrate Working Solution (Pierce, Rockford, IL, USA). Finally, the membrane was exposed to x-ray film for 2 to 5 minutes. Optical density was assessed with the use of the AlphaImager 2200 Densitometer (Alpha Innotech Corp., San Leandro, CA) and Molecular Analyst software (Bio-Rad Laboratories, Hercules, CA).

Published online in Wiley InterScience. (www.interscience.wiley.com)
DOI:10.1002/clc.20482

Table 1. Serum Lipid Levels of Patients in the Research Group

	n	Mean ± SD	Range
triglycerides (mmol/L)	31	2.03±0.33	1.63–3.89
total cholesterol (mmol/L)	31	5.40±0.55	4.16–6.72
low-density lipoprotein cholesterol (mmol/L)	31	3.56±0.58	2.18–5.08
high-density lipoprotein cholesterol (mmol/L)	31	1.16±0.13	0.78–1.41
lipoprotien(a) (mg/L)	31	43.49±11.21	14.50–78.00
non-high-density lipoprotein cholesterol (mmol/L)	31	4.21±0.66	1.83–5.78

Statistical Analysis

Statistical analysis was performed with Statistical Package for the Social Science (version 12.0; SPSS; Chicago, USA) SPSS 12.0. Quantitative variables are presented as mean ± SD. Student*t* test or Fisher's exact test were used to analyze continuous normally distributed variables or categorical variables, respectively. Correlation analysis was performed with linear correlation or Spearman rank order correlation where appropriate. A 2-tailed $P < .05$ was considered statistically significant.

Results

Patient Characteristics

During the period of 18 months, 31 patients (male: 28, female: 3) were enrolled in the study. The ages of the subjects ranged from 46 to 74 years. Of these patients, 9 patients were ex-smokers, 12 had a history of hypertension, 5 had diabetes, and 8 were treated for hypercholesterolemia. Serum lipid levels of these patients are presented in the Table.

Presence of Activated NF-κB in the Aorta of Patients who Underwent CABG

As shown in Figure 1 and Figure 2 NF-κB was present and activated in all ascending aorta tissues of CAD patients as evidenced by the presence of diffuse brown reaction product and the increased retardation of the DNA probe containing the NF-κB motif. The relative percentage of smooth muscle cells positive for NF-κB in these aortic samples ranged from 19.0% to 72.5%, with a mean 42.2% ± 12.8%. On the other hand, celiac aorta sections without atherosclerotic lesions obtained from kidney donors, had little or no expression of NF-κB according to immunohistological staining (Figure 1). As shown in Figure 2, the DNA binding

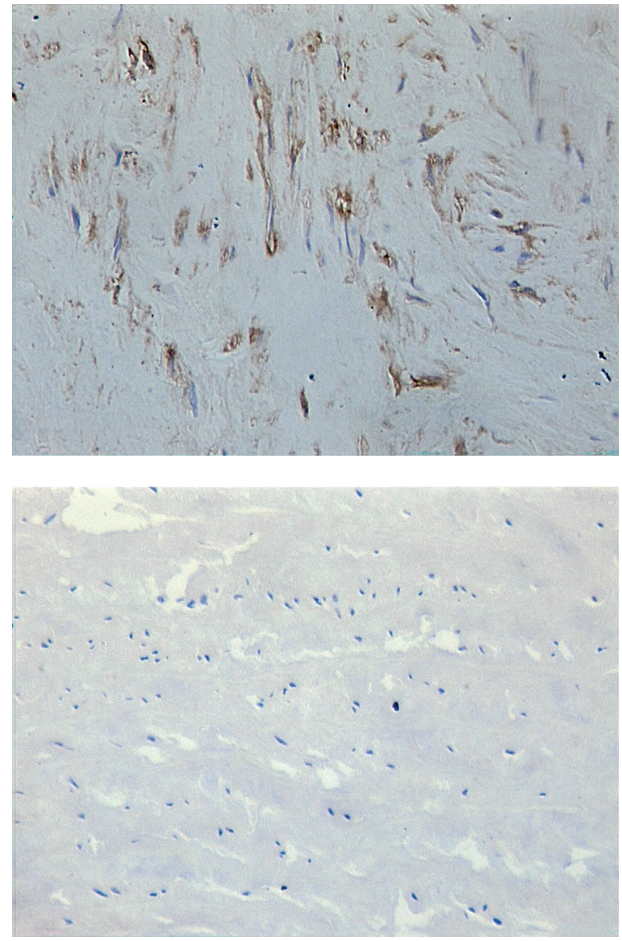

Figure 1. Immunostain of NF-κB in 2 groups. The upper: the diffuse brown reaction products indicate the presence of NF-κB in ascending aorta tissues of research group patients who underwent CABG surgery (×400). The subjacent: as control, little or no NF-κB is present in normal celiac aorta tissues from those who donated a kidney (×400).

of the transcriptionally active NF-κB in the control sample was significantly lower than the aortic tissues from CAD patients.

Relationship between Aortic NF-κB Expression and Clinical Risk Factors for Atherosclerosis

We next analyzed associations between the aortic NF-κB expression and clinical risk factors for atherosclerosis, including gender, status of smoking, hypertension, and diabetes. As shown in Figure 3, the aortic NF-κB expression was significantly elevated in smokers, patients with hypertension, or diabetes ($P < .05$), whereas gender had no significant effect. As shown in Figure 4, Spearman rank order correlation revealed that aortic NF-κB expression was significantly correlated with coronary severity scores, which ranged from 13 to 48 (29.7 ± 7.7).

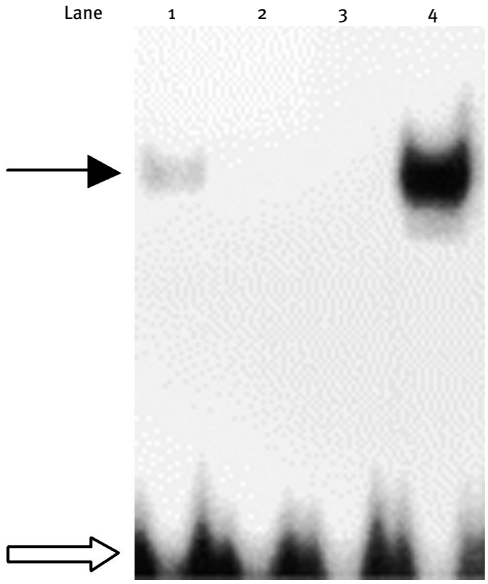

Figure 2. Detection of NF-κB DNA binding activity using EMSA. Equal amounts of nuclear protein extract prepared from normal celiac aorta tissues (lane 1) and ascending aorta tissues obtained from CABG surgery (lane 4) were determined by EMSA using labeled NF-κB oligonucleotide as a probe. The solid arrow and the open arrowhead indicate the bands of NF-κB/DNA complexes and free probe, respectively. A darker band reflects more NF-κB/DNA binding activity and the specific bands were assessed by using a competition experiment with a 200-fold excess of unlabeled probe (lane 2) with the same extracts. Negative control was only added free labeled probe (lane 3). Results showed that NF-κB/DNA binding activity is significantly higher in the ascending aorta from patients who underwent CABG surgery compared with normal celiac aorta tissues. This is representative of 3 separate experiments.

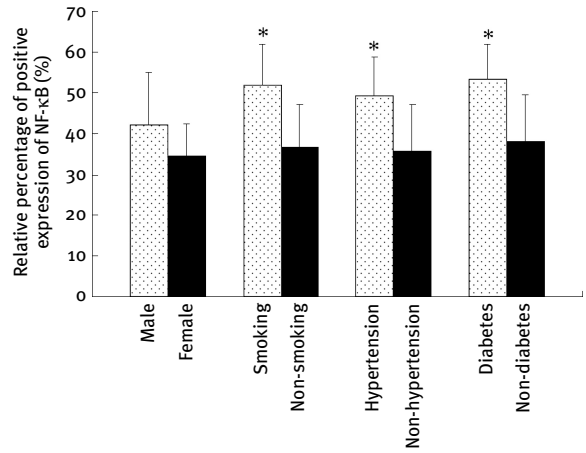

Figure 3. Quantitative analysis of aortic NF-κB expression in research group patients according to gender, smoking, hypertension, and diabetes. * $P < .05$.

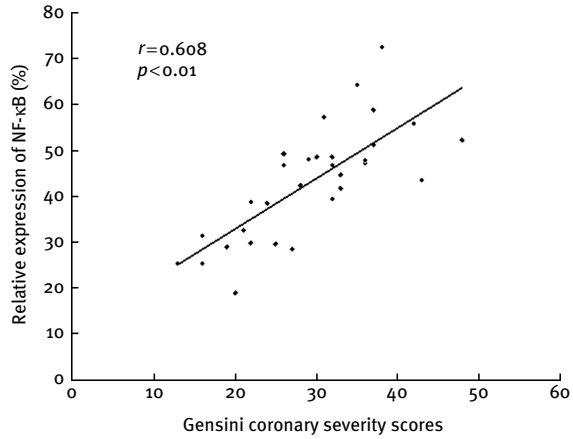

Figure 4. Relationship between aortic NF-κB expression and coronary severity scores.

Using linear correlation analysis, the aortic NF-κB expression were positively correlated with LDL-C ($r = 0.545$, $P = .01$), Lp(a) ($r = 0.799$, $P < .01$), TC ($r = 0.381$, $P = .03$), and non-HDL-C ($r = 0.449$, $P = .01$). On the other hand, there was a strong negative correlation between aortic NF-κB expression and serum HDL-C levels ($r = 0.685$, $P < .01$). However, no direct correlation between aortic NF-κB expression and age ($r = -0.020$, $P = .45$) or triglycerides ($r = 0.151$, $P = .70$) was found.

Discussion

This study shows that NF-κB is expressed and activated in aortas of patients with CAD; the NF-κB activation was also correlated with atherosclerotic risk profiles and the severity of CAD.

It is well established that inflammation plays a crucial role in all steps characterizing the atherosclerotic process.[1] A variety of genes mostly regulated by NF-κB, including TNF-α, IL-1β, MCP-1, ICAM-1, VCAM-1, and TF are overproduced in human vascular wall cells, act in concert and may have the potential of initiating or perpetuating inflammation reactions that lead to atherosclerosis.[2] Meanwhile, several cytokines found in the atherosclerotic lesion, such as TNF-α and IL-1, are able to activate NF-κB in vitro.[7] These cytokines can be produced by monocyte/macrophages, endothelial cells, smooth muscle cells, and lymphocytes, which are key cellular components in atherogenesis.[8] Activated NF-κB has been identified in these cells as well as human coronary plaque.[5,9–12] Although the lesions of atherosclerosis are distributed irregularly throughout the vasculature, they do not occur at random sites.[13] Unlike those systemic factors such as obesity, smoking, and LDL-C, hemodynamic factors (eg, shear stress and cyclic circumferential strain) have a widely accepted role in the localization of atherosclerosis.[14] Since the ascending aorta is where hemodynamic features are

Published online in Wiley InterScience. (www.interscience.wiley.com)
DOI:10.1002/clc.20482

more turbulent, atherosclerotic changes may be sensitive to individuals with vascular inflammatory changes. Using a specific antibody (α-p65mAb), Brand et al[15] first demonstrated the presence of activated NF-κB in human aortic atherosclerotic lesions. Comparing with the advanced atherosclerotic lesions in their report, our study further suggests that patients with coronary atherosclerosis also show arterial inflammation in other arteries including the aorta, thus the underlying inflammatory changes need to be present in the entire vasculature and these vessels are prone to atherosclerosis as well.

To identify the potential role of NF-κB in atherogenesis, we further investigated the relationship between NF-κB activation and other cardiovascular risk factors. Our results show that aortic NF-κB expression has a positive correlation with smoking, high blood glucose, hypertension, LDL-C, Lp(a), total cholesterol, and non-HDL-C ($P < .05$), but a negative correlation with HDL-C ($P < .05$); whereas there was no direct association with age, gender, and triglycerides. Smoking, hypertension, diabetes, and hyperlipidemia are all risk factors that contribute to the development of atherosclerosis, for which the NF-κB activation pathway has been implicated directly or indirectly in atherogenesis[16–19]: activated NF-κB cause the dysfunction of endothelial cells,[20] aggregation of monocytes,[21] and migration of smooth muscle cells[22] through its regulated gene products to induce atherosclerosis. In accordance with previous studies, our results confirm that NF-κB expression is associated with the clinical risk factors for atherosclerosis. Thus the clinical risk factors linked to the onset of atherosclerosis maybe at least partly through activation of NF-κB.

The significant correlation between aortic NF-κB expression and coronary artery severity found in our study is in accordance with the hypothesis that atherosclerotic process is a generalized pathology that may involve the entire vasculature. Using transesophageal echocardiography, Fazio et al[23] demonstrated a high sensitivity (90%) and positive predictive value (90%) for atherosclerotic aortic plaque to identify the obstructive CAD. Together with these studies, we suggest that NF-κB activation may be a molecular target in treating atherosclerosis.

A limitation of the present study is the small size of the study population. However, our data drawn from these samples indicate a highly activated NF-κB in those aortas from patients with coronary atherosclerosis. As for the control specimens, since healthy human aorta tissues can rarely be ethically obtained for obvious reasons, we selected celiac aorta from subjects who donated kidneys as the control group. To our knowledge, there are no data to show any difference in NF-κB expression between human normal ascending aorta and celiac aorta.

In summary, our present study shows that NF-κB is overactivated in aortas of patients with severe CAD, which may reflect overall arterial inflammatory status. The significant correlations between aortic NF-κB expression and other cardiovascular risk factors support the hypothesis that NF-κB participates in the atherogenesis induced by numerous clinical risk factors and may serve as a marker for the inflammation associated with atherosclerosis.

References

1. Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med*. 1999; 340(2):115–126.
2. de Winther MP, Kanters E, Kraal G, Hofker MH. Nuclear factor kappaB signaling in atherogenesis. *Arterioscler Thromb Vasc Biol*. 2005; 25(5):904–914.
3. World Medical Association declaration of Helsinki: Recommendations guiding physicians in biomedical research involving human subjects. *JAMA*. 1997;277(11):925–926.
4. Gensini GG. A more meaningful scoring system for determining the severity of coronary heart disease. *Am J Cardiol*. 1983;51(3): 606.
5. Wilson SH, Best PJ, Edwards WD, et al Nuclear factor-kappa B immunoreactivity is present in human coronary plaque and enhanced in patients with unstable angina pectoris. *Atherosclerosis*. 2002;160(1):147–153.
6. Kranzhöfer R, Schmidt J, Pfeiffer CA, Hagl S, Libby P, Kübler W. Angiotensin induces inflammatory activation of human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 1999;19(7):1623–1629.
7. Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. *Annu Rev Immunol*. 1994;12:141–179.
8. Stoll G, Bendszus M. Inflammation and atherosclerosis: novel insights into plaque formation and destabilization. *Stroke*. 2006; 37(7):1923–1932.
9. Kastl SP, Speidl WS, Kaun C, et al The complement component C5a induces the expression of plasminogen activator inhibitor-1 in human macrophages via NF-kappaB activation. *J Thromb Haemost*. 2006;4(8):1790–1797.
10. Collins T. Endothelial nuclear factor-kappaB and the initiation of the atherosclerotic lesion. *Lab Invest*. 1993;68(5):499–508.
11. Viedt C, Hansch GM, Brandes RP, Kubler W, Kreuzer J. The terminal complement complex C5b-9 stimulates interleukin-6 production in human smooth muscle cells through activation of transcription factors NF-kappaB and AP-1. *FASEB J*. 2000;14(15): 2370–2372.
12. Ginn-Pease ME, Whisler RL. Redox signals and NF-kappaB activation in T cells. *Free Radic Biol Med*. 1998;25(3):346–361.
13. Strong JP. Atherosclerotic lesions. Natural history, risk factors, and topography. *Arch Pathol Lab Med*. 1992;116(12):1268–1275.
14. Frangos SG, Gahtan V, Sumpio B. Localization of atherosclerosis: role of hemodynamics. *Arch Surg*. 1999;134(10):1142–1149.
15. Brand K, Page S, Rogler G, et al Activated transcription factor nuclear factor-kappaB is present in the atherosclerotic lesion. *J Clin Invest*. 1996;97(7):1715–1722.
16. Zhang S, Day IN, Ye S. Microarray analysis of nicotine-induced changes in gene expression in endothelial cells. *Physiol Genomics*. 2001;5(4):187–192.
17. Browatzki M, Larsen D, Pfeiffer CA, Gehrke SG, Schmidt J. Angiotensin II stimulates matrix metalloproteinase secretion in human vascular smooth muscle cells via nuclear factor-kappaB and activator protein 1 in a redox-sensitive manner. *J Vasc Res*. 2005;42(5):415–423.
18. Schwartz EA, Reaven PD. Molecular and signaling mechanisms of atherosclerosis in insulin resistance. *Endocrinol Metab Clin North Am*. 2006;35(3):525–549, viii.
19. Cominacini L, Anselmi M, Garbin U, Fratta Pasini A, Stranieri C. Enhanced plasma levels of oxidized low-density lipoprotein increase circulating nuclear factor-kappaB activation in patients with unstable angina. *J Am Coll Cardiol*. 2005;46(5):799–806.

Published online in Wiley InterScience. (www.interscience.wiley.com)
DOI:10.1002/clc.20482

20. Oitzinger W, Hofer-Warbinek R, Schmid JA, Koshelnick Y, Binder BR, de Martin R. Adenovirus-mediated expression of a mutant IkappaB kinase 2 inhibits the response of endothelial cells to inflammatory stimuli. *Blood*. 2001;97(6):1611–1617.
21. Ping D, Boekhoudt GH, Rogers EM, Boss JM. Nuclear factor-kappa B p65 mediates the assembly and activation of the TNF-responsive element of the murine monocyte chemoattractant-1 gene. *J Immunol*. 1999;162(2):727–734.
22. Bäck M, Bu DX, Bränström R, Sheikine Y, Yan ZQ, Hansson GK. Leukotriene B4 signaling through NF-κB-dependent BLT1 receptors on vascular smooth muscle cells in atherosclerosis and intimal hyperplasia. *Proc Natl Acad Sci USA*. 2005;102(48):17501–17506.
23. Fazio GP, Redberg RF, Winslow T, Schiller NB. Transesophageal echocardiographically detected atherosclerotic aortic plaque is a marker for coronary artery disease. *J Am Coll Cardiol*. 1993;21(1):144–150.

Published online in Wiley InterScience. (www.interscience.wiley.com)
DOI:10.1002/clc.20482