TARJA KUNNAS

Associations of Endothelial Nitric Oxide Synthase and Estrogen Receptor α Gene Polymorphisms with Coronary Artery Disease

ACADEMIC DISSERTATION

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Supervised by
Professor Seppo Nikkari
University of Tampere
Professor Pekka Karhunen
University of Tampere

Reviewed by
Docent Olavi Ukkola
University of Oulu
Professor Heikki Vapaatalo
University of Helsinki

Distribution

University of Tampere
Bookshop TAJU
P.O. Box 617
33014 University of Tampere
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Tel. +358 3 215 6055
Fax +358 3 215 7685
taju@uta.fi
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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original publications, referred to as I–V in the text.

I *Kunnas TA*, Ilveskoski E, Niskakangas T, Laippala P, Kajander OA, Mikkelsson J, Goebeler S, Penttilä A, Perola M, Nikkari ST, Karhunen PJ (2002): Association of the endothelial nitric oxide synthase gene polymorphism with risk of coronary artery disease and myocardial infarction in middle-aged men. J Mol Med 80:605–609

II *Kunnas TA*, Lehtimäki T, Laaksonen R, Ilveskoski E, Janatuinen T, Vesalainen R, Nuutila P, Karhunen PJ, Knuuti J, Nikkari ST (2002): Endothelial nitric oxide synthase genotype modulates the improvement of coronary blood flow by pravastatin: a placebo-controlled PET study. J Mol Med 80:802–807

III *Kunnas TA*, Holmberg-Marttila D, Karhunen PJ (1999): Analysis of estrogen receptor dinucleotide polymorphism by capillary gel electrophoresis with a population genetic study in 180 Finns. Human Heredity 49:142–145

IV *Kunnas TA*, Laippala P, Penttilä A, Lehtimäki T, Karhunen PJ (2000): Association of polymorphism of human α oestrogen receptor gene with coronary artery disease in men: a necropsy study. BMJ 29:273–274

V Lehtimäki T, *Kunnas TA*, Mattila KM, Perola M, Penttilä A, Koivula T, Karhunen PJ (2002): Coronary artery wall atherosclerosis in relation to the estrogen receptor 1 gene polymorphism: an autopsy study. J Mol Med 80:176–180

ABBREVIATIONS

ADP	adenosine diphosphate
ANCOVA	analysis of covariance
ATP	adenosine triphosphate
BMI	body mass index
CAD	coronary artery disease
CI	confidence interval
DNA	deoxyribonucleic acid
EDHF	endothelium-derived hyperpolarizing factor
eNOS	endothelial nitric oxide synthase
ER	estrogen receptor
ERα	estrogen receptor α
HSDS	Helsinki Sudden Death Study
LDL	low-density lipoprotein
MI	myocardial infarction
NO	nitric oxide
NOS	nitric oxide synthase
NOx	nitrite and nitrate
OR	odds ratio
PET	positron emission tomography
PGI_2	prostacyclin
mRNA	messenger ribonucleic acid
SD	standard deviation
SMC	smooth muscle cell

INTRODUCTION

Atherosclerosis is a multifactorial disease of large and medium-sized arteries characterized by smooth muscle cell migration and proliferation, accumulation of cholesterol, formation of connective tissue, presence of inflammatory cells, thrombus formation and calcification. This disease starts early in life, but it usually takes several decades before clinical symptoms appear. The ischemic complications of atherosclerosis, i.e. acute myocardial infarction and ischemic stroke, are the most common causes of morbidity and mortality in Western countries, representing around 40 % of all-causes mortality before the age of 74 years (for reviews, see Ross 1999 and Lusis 2000).

The major risk factors for atherosclerosis are high serum cholesterol, especially low-density lipoprotein (LDL) cholesterol, diabetes mellitus, arterial hypertension and cigarette smoking (Vartiainen et al. 1994, Wilson et al. 1998). In addition, also hereditary factors are strongly associated with the risk to develop atherosclerosis (Goldbourt and Neufeld 1986, Marenberg et al. 1994, for review, see Lusis 2000). The genes involved with human atherosclerosis can be identified by genome-wide random scans in family-based studies or by the candidate gene approach, if there is a possibility to utilize information from prior biochemical or physiological studies. The genome-wide scan is the only method to identify novel disease genes. The most frequently used study materials for genome scans are affected sib-pair collections. The candidate gene approach, which tests markers of selected loci speculated to be important for the atherosclerotic process, has been widely used in different study samples including case-control sets, affected sib-pairs and multiplex families (Pajukanta and Peltonen 1997).

Association studies are case-control studies, which compare allele frequencies of polymorphic candidate disease genes in unrelated individuals with and without the dis-

ease. A significant association between an allele and a disease suggests that the studied polymorphism is in some way involved in the disease (for reviews, see Cardon and Bell 2001, Daly and Day 2001).

The candidate genes for atherosclerosis are multiple. Most commonly studied polymorphic genes are involved in lipid metabolism, hemostasis, fibrinolysis and inflammation. Genes affecting the function and tone of the artery have not been in the focus so far. Endothelial nitric oxide synthase (eNOS) and estrogen receptor-α (ERα), are two strong candidate genes affecting vascular function. The products of these genes have been suggested to have important roles in the endothelium. It is thus possible that variation in these genes may affect the risk for coronary artery disease. This thesis aimed at studying the association between the polymorphisms of these two genes and coronary artery disease in Finnish males who had died suddenly out of hospital (the Helsinki Sudden Death Study) as well as measuring the genotype dependent effect of eNOS on coronary artery reactivity with positron emission tomography (PET) in a clinical series of healthy young men.

REVIEW OF LITERATURE

1. Structure of normal arterial wall

Three layers can be distinguished histologically in the walls of all larger arteries (Ross and Glomsed 1976). The three layers comprise an inner intima, an intermediary media, and an outer adventitia. A single layer of endothelial cells covers the luminal side of the intima. The intima has a narrow subendothelial space containing only a few smooth muscle cells (SMCs), and usually their number increases with age (Geer and Haust 1972). The intima is separated from the media by the internal lamina, consisting of elastin and collagen. In healthy arteries the media is the thickest layer composed of several layers of SMCs of the contractile phenotype. The outermost layer is the adventitia, which is connective tissue with fibroblasts, nerves and small blood and lymph vessels. The media and the adventitia are separated by the external elastic lamina (Geer and Haust 1972, Stary et al. 1992).

2. Atherosclerotic lesions

Atherosclerosis is a disease characterized by focal intimal thickenings of large and medium-sized arteries. Atherosclerotic lesions may be divided macroscopically into three types: the fatty streak, the fibrous plaque, and the complicated lesion (Guzman et al. 1968). According to this classification, the fatty streak is a flat lesion occurring already in childhood, causing no obstruction. Fatty streaks contain clusters of “foam cells”, which have a cytoplasm filled with lipid droplets giving the cells a foamy appearance. Fatty streaks are visible on the inner surfaces of arteries as relatively flat, yellow streaks or patches (Guzman et al. 1968). The fibrous plaque is the characteristic atherosclerotic advanced lesion. It usually protrudes into the lumen of the artery. Fibrous plaques are

made up of SMCs and connective tissue matrix, which cover a deeper accumulation of both intra- and extracellular lipid (Haust 1981). As a result of hemorrhage, calcification, cell necrosis, and mural thrombosis, fibrous plaques may develop into complicated lesions, responsible for the sudden complications of atherosclerosis.

The current classification of atherosclerotic lesions, as presented by the Committee on Vascular Lesions of the Council of Atherosclerosis, American Heart Association, divides the lesions into six types. This classification is based on microscopical examination of histological arterial samples (Stary et al. 1992, Stary et al. 1994, Stary et al. 1995).

Early atherosclerotic lesions are classified as types I, II and III. Type I lesions contain macrophages with intracellular lipid droplets (foam cells), but other histological changes are rare (Stary et al. 1994). Lesions known as fatty streaks are classified as Type II lesions. They consist primarily of macrophage foam cells, but intimal SMCs may also contain lipid droplets. Fatty streaks do not obstruct arterial blood flow to any degree. Type III lesions are the bridge between early and advanced lesions. They contain more extracellular lipid droplets than do fatty streaks, with the droplets fusing into small pools below the macrophage layer. These lesions are also called preatheromas (Stary et al. 1994).

Advanced atherosclerotic lesions are classified as types IV – VI. The type IV lesion is called an atheroma. These lesions have a dense accumulation of extracellular lipid, a “lipid core”, which occupies a well-defined region of the intima. The major cell types between the endothelium and the lipid core are macrophages and SMCs with or without intracellular lipid (Stary et al. 1995). Mast cells and lymphocytes may also be present (Kovanen et al. 1995, Kaartinen et al. 1998). The lesion narrows the lumen only minimally and may not be visible by angiography. Type V lesions are lesions in which a prominent layer of collagen, a “fibrous cap” has formed in the intima. When the fibrous

cap covers a lipid core the lesion is referred to as a fibroatheroma (type Va). A lesion in which the lipid core or other parts of the lesion are calcified is referred to as type Vb. A type V lesion in which the lipid core is absent is called type Vc. These lesions narrow the artery to various degrees. Type VI, or complicated lesions are formed from type IV and V lesions by disruption of the luminal surface resulting in a hematoma (type VIa), hemorrage (type VI b) or thrombosis (type VIc). These lesions account for most of the clinical manifestations of atherosclerosis (Stary et al. 1995).

The clinical picture and prognosis of coronary atherosclerosis depend both on the size and the type of an atherosclerotic lesions. The accumulation of lipid and connective tissue will narrow the arterial lumen, impede blood flow and lead to the clinical picture of angina pectoris or myocardial infarction. A more important cause of acute cardial events, however, is a rupture of an atheromatous lesion. Factors contributing to lesion rupture are those increasing the mechanical stress or weakening the integrity of the layer covering an atheromatous lesion. A thin fibrous cap and a large lipid core decrease resistance to physical stress (MacIsaac et al.1993, Schroeder and Falk 1995, Felton et al. 1997). The fibrous cap may be weakened by an increase in macrophages and T-cells, which are capable of degrading collagen. A low density of SMCs at critical locations may also weaken the fibrous cap, since these cells are primarily responsible for maintaining the mechanical integrity of the fibrous cap by collagen synthesis (Schroeder and Falk 1995, Lee and Libby 1997, Rogue et al. 1999).

It is likely that when the coronary atherosclerotic plaque is disrupted or eroded superficially, the thrombogenic stimulus is relatively limited and results in either superficial thrombosis with subsequent plaque growth or a transient thrombotic occlusion, as takes place in unstable angina pectoris. Deep plaque disruption or ulceration exposes collagen, tissue factor, and other elements of the vessel wall to circulating blood, leading

to a more persistent thrombotic occlusion, as in myocardial infarction (for review, see Fuster et al. 1992, Roque et al. 1999).

3. Endothelial function

The endothelium contributes to the physiological regulation of vasomotor activity. The endothelium-dependent control is mediated by endothelium-derived relaxing (EDRF) and contracting (endothelin-1 (ET-1)) factors. The main control of the tone of the underlying vascular smooth muscle is through the production of vasodilatator mediators.

If the endothelium is intact, several of the substances released from the platelets (ADP, ATP, serotonin (5-HT)) cause the release of EDRF from the endothelial cells. EDRF will relax the underlying vascular smooth muscle, which results in flushing of the microaggregate away from the endothelial surface. ERDF is also released towards the lumen of the blood vessel to prevent platelet adhesion to the endothelium and to inhibit platelet aggregation. Other factors causing the release of EDRF from the endothelial cell include shear stress, bradykinin, acetylcholine, thrombin and histamine (for review, see Vanhoutte 2003). The best characterized EDRFs are nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF).

Nitric oxide. In 1980 Furchgott and Zawadski showed that strips of rabbit aorta with intact endothelium relaxed in response to acetylcholine, but contracted in response to this same agonist when the endothelium had been rubbed of. The substance responsible for the acetylcholine-stimulated relaxation was initially called endothelium-derived relaxant factor (Furchgott and Zawadski 1980) and subsequently found to be NO or its derivative (Ignarro et al. 1987, Palmer et al. 1987). This substance has a wide range of biological properties that maintain vascular homeostasis, including modulation of vascular dilatator tone, regulation of local cell growth (Garg and Hassid 1989, McNamara et al

1993, Lee et al. 1996), protection of the vessels from platelet activation and aggregation (Mellion et al. 1983, Macdonald et al. 1988), and prevention of leukocyte adhesion to the vascular surface (Kubes et al. 1991). Once synthesized, NO diffuses to the SMCs and relaxes them mainly by activating the formation of cyclic GMP from GTP (Fig 1) (Ignarro et al. 1981, Rapoport and Murad 1983, Moncada and Higgs 1993; for review, see Vanhoutte 2003).

Prostacyclin was described by Moncada et al. in 1976. It is formed primarily in endothelial cells. PGI_2 is produced in response to shear stress and to substances that stimulate NO formation (Moncada et al. 1976). Although in most blood vessels the contribution of PGI_2 to endothelium-dependent relaxation is not of major importance, it acts synergistically with NO to inhibit platelet aggregation and promotes fibrinolysis (Macdonald et al. 1988). PGI_2 causes relaxation by binding to membrane receptors on the smooth muscle, which activates adenylate cyclase and subsequently increases the intracellular concentration of cAMP (Fig. 1) (Moncada et al. 1976, Busse et al. 1994, reviewed byVanhoutte 2003).

The name endothelium derived hyperpolarizing factor has been given to the substance that produces vascular smooth muscle hyperpolarization and relaxation which cannot be explained by NO or PGI_2 (Feletou and Vanhoutte 1988). In various animal and human arteries, acetylcholine and other endothelium-dependent vasodilatators cause endothelium-dependent hyperpolarizations, that contribute to endothelium-dependent relaxations. These effects have been attributed to diffusible EDHF, that causes hyperpolarization and relaxation by opening K^+ channels. The exact nature of EDHF is not known, but may be a short–lived metabolite of arachidonic acid, produced via the cytochrome P450 epoxygenase pathway (Fig. 1) (Feletou and Vanhoutte 1988, Cambell et al. 1996, Cannon 1998, Quillery and McGift 2000; for review, see Vanhoutte 2003).

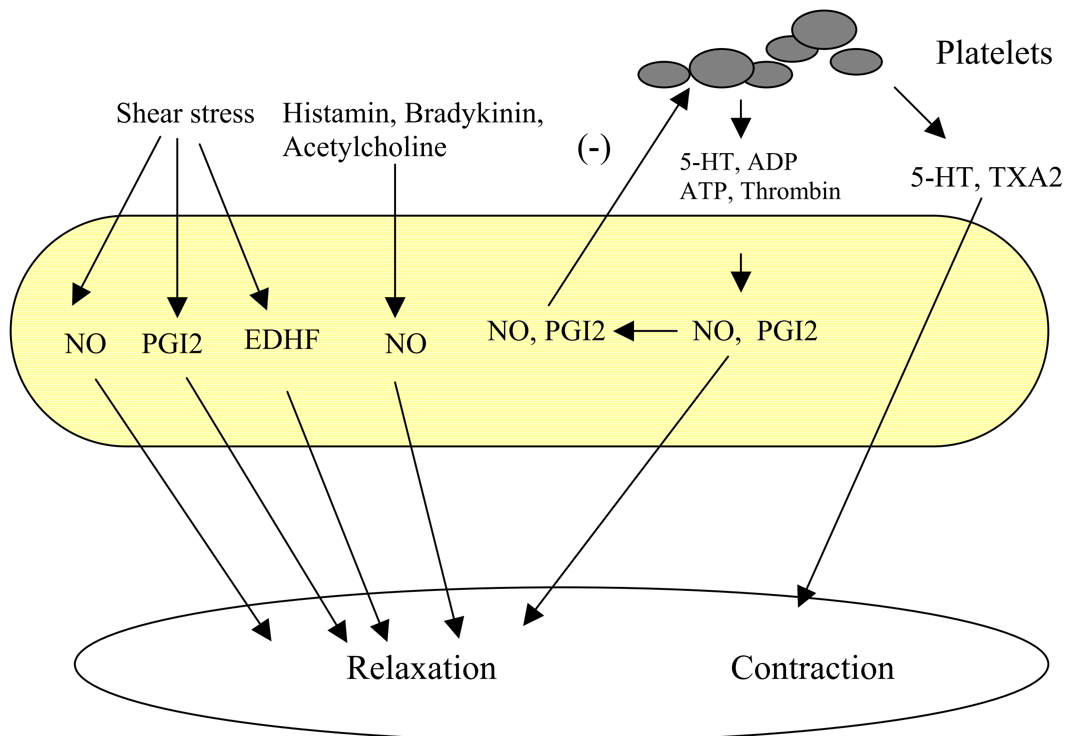

Fig 1. Simplified endothelial control of vasomotor function.

3.1. Nitric oxide synthases

The synthesis of NO is catalyzed by the nitric oxide synthase family of oxidoreductases. To date, three isoforms of nitric oxide synthase have been identified and named after the tissue from which they were first cloned: the neuronal isoform (nNOS, NOS1)(Bred et al. 1991), the macrophage or inducible isoform (iNOS, NOS2) (Lowenstein et al. 1992) and the endothelial isoform (eNOS, NOS3)(Marsden et al. 1992, Marsden et al. 1993). These enzymes share 50 % – 60 % homology of their amino acid sequence and, despite having similar biochemical properities, are encoded by separate genes on different chromosomes (Marsden et al. 1992, Marsden et al. 1993, Sessa, 1994). Whereas eNOS and nNOS are

normal constituents of healthy cells, iNOS is not usually expressed in vascular cells and its expression is seen mainly in conditions of infection or inflammation (MacNaul and Hutchinson 1993).

3.1.1. Endothelial nitric oxide synthase (eNOS)

The gene of endothelial nitric oxide synthase was cloned and sequenced in 1993 (Marsden et al. 1993) and localized to chromosome 7q35–7q36. The gene contains 26 exons spanning 21–22 kb of genomic DNA and encodes an mRNA of 4052 nucleotides. The promoter region of eNOS contains a number of binding domains, which suggests that it may be regulated by a variety of transcription factors (Marsden et al. 1992, Lamas et al. 1992, Marsden et al. 1993, Zhang et al. 1995, Karantzoulis-Fegaras et al. 1999).

The expression of eNOS is regulated in numerous ways to meet local demand. Under normal physiological conditions, synthesis of eNOS is regulated by intracellular Ca^{2+} and is activated by substances such as a acetylcholine or bradykinin (Luckhoff et al. 1988, Busse and Műlsch 1990). Factors that upregulate eNOS expression include also shear stress (Pohl et al. 1986, Sessa et al. 1994, Topper et al. 1996, Xiao et al. 1997), growth factors (Inoue et al. 1995, Kostyk et al. 1995), and estrogens (Hishikawa et al. 1995, Kleinert et al. 1998). Glucocorticoids, tumor necrosis factor α and bacterial lipopolysaccharides downregulate eNOS expression in most situations by decreasing the half-life of eNOS mRNA (Arriero et al. 2000, Xu et al. 2001, Lai et al. 2003). eNOS is myristoylated and palmitoylated, and can thus associate with intracellular membranes. Myristoylation (cotranslational) is required for eNOS targeting to the caveolae (Busconi and Michel 1993, Sakoda et al. 1995, Shaul et al. 1996), whereas palmitoylation (posttranslational) stabilizes

association of eNOS with the caveolar membrane (Garcia-Cardena et al. 1996). These membrane associations are required for the phosphorylation and activation of eNOS.

3.1.2. Localization of eNOS

The localization of eNOS within the cell determines its activity. Studies of cultured endothelial cells and cardiac myocytes (Feron et al. 1996, Shaul et al. 1996) have shown that the majority of functional eNOS is localized to caveolae that are specialized plasma membrane microdomains enriched in cholesterol, glycosphingolipids, and some structural proteins, such as caveolin (Anderson 1993, Parton 1996, Couet et al. 1997). The interaction of eNOS with caveolin-1 maintains the enzyme in its inactive state (Ju et al. 1998).

Typical eNOS stimulation is initated by ligand binding to plasma membrane receptors, which may reside in caveolae or are mobilized to caveolae upon activation. Receptor activation leads to the stimulation of multiple kinase pathways leading to phosphorylation of eNOS. Factors that activate eNOS phosphorylation of ser-1177 include estradiol (Haynes et al. 2000, Hisamoto et al. 2001), shear stress (Dimmeler et al. 1999, Gallis et al. 1999), and vascular endothelial growth factor (Fulton et al. 1999, Michell et al. 1999). In contrast, phosphorylation of the threonine at position 495 inactivates eNOS (Chen et al. 1999a). Thus, phosphorylation events result in either eNOS stimulation or inactivation depending on the kinase involved and the site of phosphorylation. Dephosphorylation events are also stimulatory or inhibitory (for review, see Fleming and Busse 2003). Accordingly, NO production in the endothelium is regulated through alterations in the expression or activity of the eNOS enzyme.

3.2. Synthesis of nitric oxide

Endothelium-derived NO is synthesized from the amino acid L-Arginine, yielding L-citrulline as a by-product (Moncada and Higgs 1993) (Fig 2).

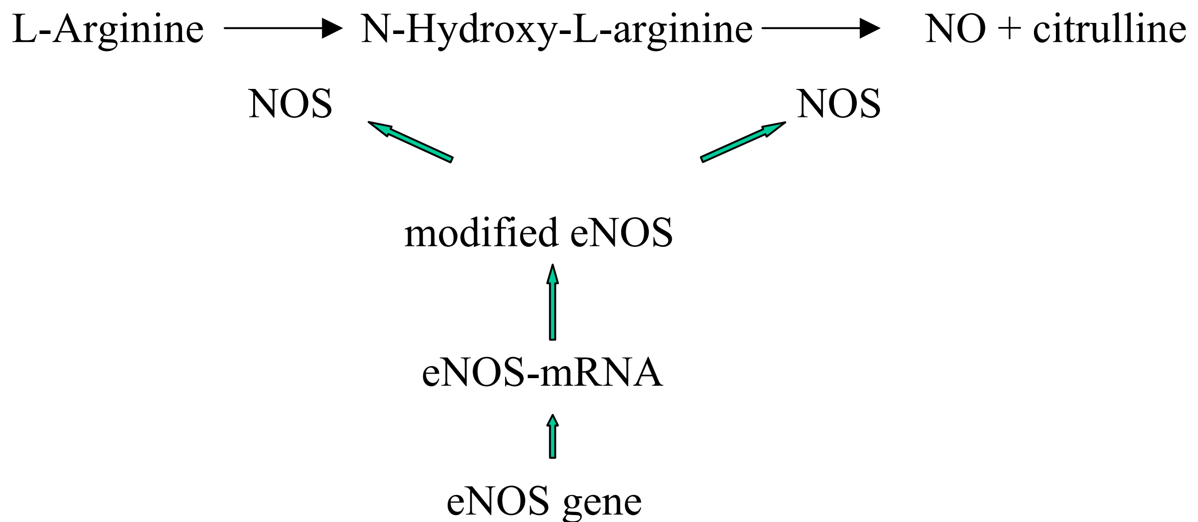

Figure 2. The pathway leading to the generation of NO in endothelial cells.

The most important target of NO is the soluble guanylate cyclase in SMCs. Once released by the endothelial cells, NO diffuses across the SMC membrane and activates guanylate cyclase. This leads to increased SMC synthesis of cyclic guanosine monophosphate (cGMP). The resulting rise in cGMP concentration results in SMC relaxation, which contributes to vasodilatation (Knowles and Moncada 1992, Moncada and Higgs 1993). NO can also activate Ca^{2+} dependent K^{+} channels and increase the outward potassium current. The resulting hyperpolarization of the cell membrane also induces vasodilatation (Bolotina et al. 1994).

To study the role of eNOS in cardiovascular function, eNOS knockout mice have been generated. All these animals are hypertensive and lack NO-mediated, endothelium-dependent vasodilatation (Huang et al. 1995, Gödecke et al. 2001). This is strong evidence to indicate that endothelial NO is an important systemic vasodilatator. In addition to its effect on SMCs, NO can inhibit platelet aggregation (Radomski et al. 1987) and leukocyte adhesion to the endothelium by down-regulating the leukocyte adhesion glycoprotein complex CD11/CD18 (Bath et al. 1991, Kubes et al. 1991). All the above actions are together likely to repress the development of coronary thrombosis.

3.3. Nitric oxide and atherosclerosis

To investigate the role of eNOS in atherogenesis, a vascular injury model has been applied to eNOS knockout mice. Mice lacking the eNOS gene developed greater neointimal proliferation or showed diminished remodeling compared with control mice, suggesting that endothelial NO suppresses SMC proliferation (Moroi et al. 1998, Rudic et al. 1998). NO can also suppress oxidation of LDL, suggesting that NO production may protect the artery from cholesterol accumulation (Hogg et al. 1993). A deficiency in vascular NO production might thus contribute to the development of atherosclerosis (Moncada et al. 1991, Cooke and Dzau 1997). A recent study using transgenic mice overexpressing human eNOS suggested that elevation of eNOS activity decreased blood pressure and plasma levels of cholesterol, resulting in a reduction in atherosclerotic lesions by 40 % (van Haperen et al. 2002).

The effect of nitric oxide on atherogenesis has also been studied in other animal models of atherosclerosis. The development of an atheroma-like intimal thickening in rabbits is

associated with impairment of endothelium-dependent vasodilatation (Verbeuren et al. 1986, Cayatte et al. 1994), and it has been shown that impairment of endothelium-dependent vasodilatation actually precedes intimal thickening (Booth et al. 1989, Dusting et al. 1990, De Meyer et al. 1991). Also, in the arteries of pigs fed a high cholesterol diet (Stulak et al. 2001, Wilson et al. 2001) and in the hearts of hypercholesterolemic guinea pigs (Schwemmer et al. 2000), eNOS mRNA and protein have been found to be reduced.

In line with animal studies in advanced human atherosclerosis, vascular eNOS protein expression is usually diminished and NO production reduced (Buttery et al. 1996, Wilcox et al. 1997, Oemar et al. 1998). Release of NO from arteries of patients with coronary artery disease is significantly impaired (Chester et al. 1990), confirming similar data obtained from atherosclerotic rabbit aorta (Verbeuren et al. 1990). In isolated human coronary arteries, endothelium-dependent relaxation is reduced in atherosclerotic segments (Forstermann et al. 1988).

There is evidence that NO generation is depressed in blood vessels exposed to some risk factors that accelerate atherosclerosis (Celermajer et al. 1994, Quyumi et al. 1995). An impairment of endothelium-dependent vasodilation to flow or pharmacological stimuli has been demonstrated in hypercholesterolemic subjects (Creager et al. 1990, Chowienczyk et al. 1992), hypertensives (Panza et al. 1990, Calver et al.1992b, Cardillo et al. 1998), diabetics (Calver et al. 1992a, Williams et al. 1996), and active or passive smokers (Celermajer et al. 1993, Celermajer et al. 1996).

3.4. eNOS gene sequence variations

In 1995, it was hypothesized that eNOS gene would show polymorphisms causing inherited variation in NO synthesis and predisposing to the development of atheroma

(Hingorani et al. 1995). In general, polymorphisms in gene promoters have the potential to influence mRNA transcription, while an exon polymorphism could alter enzyme activity. Polymorphisms in introns are less likely to have a functional role in comparison to the promoter or coding region variants, but their role in gene regulation is, however, still obscure. To date, 11 polymorphic sites have been found in the eNOS gene (see Wang and Wang 2000) of which eight have been studied in respect to risk of coronary artery disease (CAD) (Fig 3).

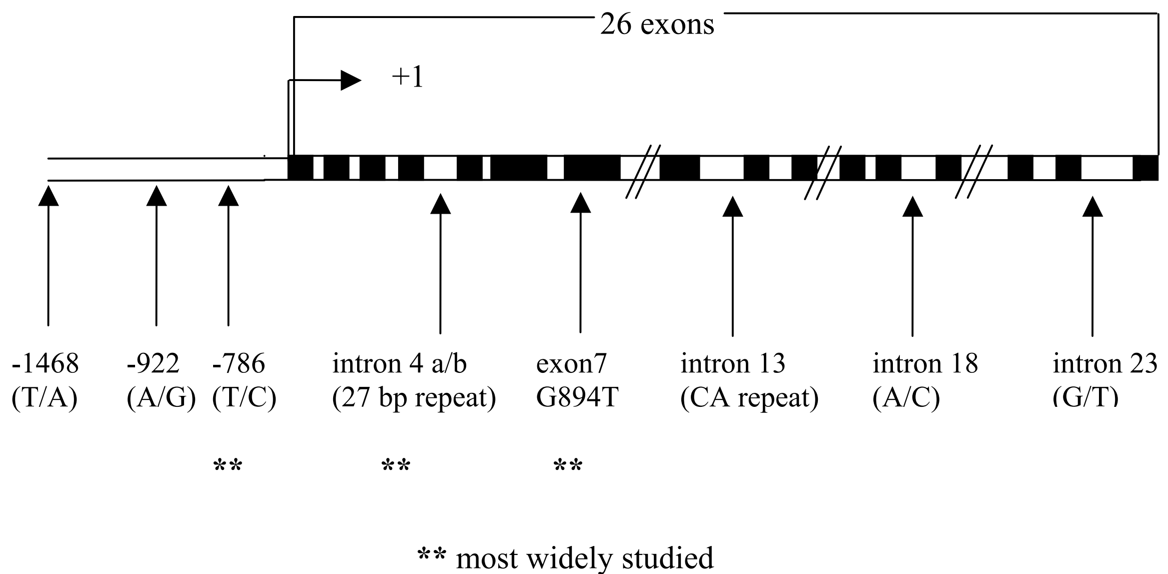

Figure 3. Polymorphic sites in eNOS that have been studied with respect to CAD risk.

3.4.1. Functional studies of eNOS gene variants

There are three widely analyzed polymorphisms in the eNOS gene, namely the eNOS 4a/b polymorphism in intron 4, the G894T (Glu298Asp) variation of exon 7, and the T-786C

replacement in the flanking region. However, a functional mechanism that would affect NO generation by these eNOS polymorphisms has not been defined.

eNOS 4a/b. The rare a-allele carriers of the intron 4a/b polymorphism have been proposed to have a reduced level of plasma NO metabolite compared with the b-allele carriers (the 4b is longer and consists of five tandem 27 bp repeats, and the shorter 4a allele consists of four tandem 27 bp repeats)(Tsukada et al. 1998). On the other hand, there is also a study which failed to find such an association (Wang et al. 1997).

eNOS G894T. The polymorphism in eNOS exon 7 (Glu298Asp) results in glutamate or aspartate at position 298. Because glutamate and aspartate are considered to be conservative replacements, the polymorphism was thought to be a marker for a functional locus elsewhere in the gene (Hingorani et al. 1995, Cai et al. 1999). It has been suggested that this polymorphism does not have a major direct functional effect on eNOS activity in human atherosclerosis (Guzik et al. 2001, Schneider et al. 2002). In accordance with the above findings, Golser and co-workers (2003) have demonstrated that the exchange of glutamate for aspartate does not alter the catalytic function of the protein. However, they also pointed out that their findings do not rule out that the polymorphism could affect endothelial NO synthesis through intracellular targeting and/or changes in the phosphorylation state of eNOS in vivo (Golser et al. 2003).

eNOS T-786C. Still another candidate functional polymorphic site is located –786 bp upstream of the transcription initiation site. Whereas in one study the T to C mutation strongly reduced eNOS gene promoter activity (Yoshimura et al. 2000), in another study the promoter activities were the same (Sim et al. 1998). One possible explanation of these conflicting results may be a link with the intron 4a/b (27 bp repeat) polymorphism. Wang and co-workers (2002) demonstrated a cis-acting role of the 27-bp repeats in eNOS T-

786C promoter function and a haplotype-specific expression pattern. The investigators showed that the wild-type T-promoter had a 1.6-fold higher transcription efficiency compared to the rare C-promoter when the 27-bp repeat was not present. However, the presence of the longer b-allele repressed the transcription efficiency of the T-promoter to 35 % of the original level. In contrast, the b-allele increased the transcription efficiency of the C-promoter to 288 % (or to 2.9-fold) of the original level (Wang et al. 2002).

3.4.2. eNOS gene variants and the risk for coronary artery disease

Many polymorphic variations of the eNOS gene have been investigated with respect to CAD risk. Only less than half of these studies have demonstrated significant associations between eNOS polymorphisms and CAD, and many reports are contradictory. Among the polymorphisms studied, the 4a/b at intron 4, the T-786C, and the G894T variants have so far received most attention (Table 1).

eNOS 4a/b. The first study of the associations between eNOS 4a/b polymorphism and CAD was reported by Wang et al. (1996), who found that homozygosity for the shorter 4a-allele predicted smoking-dependent risk of CAD. The rare 4a-allele has also been found to be associated with MI among African-Americans (Hooper et al. 1999). Moreover, Park and co-workers (2000) suggested that the link of eNOS polymorphism to the risk of MI is age-dependent. They claimed that young persons (less than 51 years) who have the rare eNOS aa-genotype might have increased risk of developing MI. Similarly, Ichihara et al. (1998) found that the frequency of the rare eNOS 4a-allele was significantly higher in Japanese MI patients than in controls in a subgroup of men who had no classical risk factors (defined as individuals with a BMI of less than 27 kg/m^2 and no history of

hypertension, diabetes mellitus or hypercholesterolemia). In contrast, in women and in the high-risk group, defined as individuals with 2 or more MI risk factors, the eNOS 4a-allele was not associated with MI. In another Japanese study, Hibi and coworkers (1998) conducted a case-control study of patients with MI and healthy gender- and age-matched control subjects. They did not find evidence of a significant increase in the risk of MI or the severity of coronary atherosclerosis among individuals with the eNOS 4aa-genotype. Similarly, the 4a-allele was not associated with the severity of CAD and the occurrence of MI or unstable angina in a hospital-based Taiwanese population (Hwang et al. 2002). Likewise, Sigush et al. (2000) and Pulkkinen et al. (2000) did not detect any association between this gene variation and cardiovascular events. Moreover, the allele frequencies of the eNOS 4a/b gene variation were also essentially the same in controls and in CAD and MI patients in a population of German subjects (Gardemann et al. 2002).

eNOS T-786C. The polymorphism in the promoter region (T-786C) of eNOS gene was reported to associate with the risk for CAD in an Italian population (Colombo et al. 2003). In Japanese patients, the C-allele was also significantly associated with MI, especially in patients without coronary arterial stenosis of more than 50 % (Nakayama et al. 2000). In contrast, the C-allele was not associated with CAD in white populations in Australia (Sim et al. 1998) and in France (Poirier et al. 1999).

eNOS G894T. Hibi et al. (1998) and Shimasaki et al. (1998) observed a significant relationship between the mutation G894T in exon 7 and a history of MI in the Japanese population, but no association was found between the mutation and the severity of coronary stenosis (Hibi et al. 1998). In the large ECTIM (Etude Cas-Temoins de l'Infarctus du Myocarde) study, the investigators found that homozygotes for the G-allele were more frequent among patients with MI than in control subjects in the French population, but that no such difference was observed in Northern Ireland (Poirier et al.

1999). In contrast, Gardemann et al. (2002) found in the German population that the T-allele carriers under 61 years old with various coronary high-risk profiles had an increased risk for CAD and/or MI. In support, Hingorani et al. (1999) also identified the G894T polymorphism as a major risk factor for CHD in individuals in the United Kingdom. They found that there was an excess of homozygotes for the T variant among patients with angiographically proven CAD and among patients with recent MI, when compared with their respective controls. The association of the G894T polymorphism of eNOS gene with CAD has been investigated in one Finnish study, in which no association was found (Pulkkinen et al. 2000).

Recently, Colombo et al. (2003) studied the interaction of G894T and T-786C polymorphisms in relation to CAD. They reported that both the T-allele of the G894T and C-allele of the T-786C polymorphic sites were associated with the prevalence and severity of angiographically defined CAD in an Italian population. They also found that individuals carrying both eNOS risk variants simultaneously might have a higher risk for developing CAD.

Table 1. Association of the three most widely studied eNOS polymorphisms with CAD/ MI.

Polymorphic site	Design	Allele/ Genotype	CAD	MI	Special	Ethnic population (case/control)	Study
Intron 4a/b	case-control	aa	+	+	Among smokers	W (549/153)	Wang et al. 1996
Intron 4a/b	case-control	a		+	In low risk individuals	J (455/550)	Ichihara et al. 1998
Intron 4a/b	case-control	aa	-	-		J (226/357)	Hibi et al. 1998
Intron 4a/b	case-control	a	-			J (40/34)	Nakagami et al. 1999
Intron 4a/b	case-control	a		+		A (110/185)	Hooper et al. 1999
Intron 4a/b	case-control		-			W (308/82)	Pulkkinen et al. 2000
Intron 4a/b	case-control	aa		+	< 51 years	J (121/206)	Park et al. 2000
Intron 4a/b	case-control	aa	-			W (630/413)	Sigusch et al. 2000
Intron 4a/b	case-control	a	+		In non-smokers	W (137/300)	Fowkes et al. 2000
Intron 4a/b	case-control		-	-	< 50 years	W (573/624)	Granath et al. 2001
Intron 4a/b	* angiography			-		J (219)	Hwang et al. 2002
Intron 4a/b	case-control		-	-		W (2717/533)	Gardemann et al. 2002
Exon 7 (G894T)	case-control	T		+		J (285/607)	Shimasaki et al. 1998
Exon 7 (G894T)	case-control	TT	-	+		J (226/357)	Hibi et al. 1998
Exon 7 (G894T)	* angiography		-	-	Cases:stenosis > 50%	W (605/158)	Cai et al. 1999
Exon 7 (G894T)	case-control	TT	+	+		W (547/321)	Hingorani et al. 1999
Exon 7 (G894T)	case-control	GG		+		W (631/610)	Poirier et al. 1999
Exon 7 (G894T)	case-control		-			W (308/82)	Pulkkinen et al. 2000
Exon 7 (G894T)			-			W (1918)	Hoffmann et al. 2000
Exon 7 (G894T)	MI/ CAD patients		-		< 50 years or > 65 years	W (149/150)	Nassar et al. 2001
Exon 7 (G894T)	case-control		-	-		W (573/624)	Granath et al. 2001
Exon 7 (G894T)	case-control	T	+	+	< 61 years	W (2717/533)	Gardemann et al. 2002
Exon 7 (G894T)	case-control	T	+			W (268/147)	Colombo et al. 2003
T-786C	case-control		-	-		W (633/160)	Sim et al. 1998
T-786C	case-control			-		W (631/610)	Poirier et al. 1999
T-786C	case-control	C	-	+		J (359/195)	Nakayama et al. 2000
T-786C	case-control		-	-		W (573/624)	Granath et al. 2001
T-786C	case-control	C	+			W (268/147)	Colombo et al. 2003

* = all cases underwent coronary angiography, MI= myocardial infarction, CAD= coronary artery disease, + indicates association, - indicates no association, W= white, J= Japanese, A= African American

4. Vascular effects of estrogens

The incidence of CHD is significantly lower in premenopausal women, in comparison with men of similar age, suggesting a fundamental role for estrogens as cardioprotective agents (Mendelssohn and Karas 1994, van der Schouw et al. 1996, Hu et al. 1999). The use of hormone replacement therapy (HRT) to prevent heart disease has been an area of controversy in recent years. An atheroprotective benefit of estrogen is strongly supported by research using animal models of atherosclerosis. Numerous studies using diet-induced hypercholesterolemic rabbits and monkeys have shown that estrogen treatment inhibits the development of the atherosclerotic lesion. The magnitude of protection varies from a 35 % to an 80 % reduction in lesion size or cholesterol content in the aorta and coronary arteries (for review, see Hodkin and Maeda 2002). Studies of estrogen treatment in atherosclerotic mice have also demonstrated a dramatic inhibitory effect of 17β-estradiol (E2) on lesion initiation and progression in ovariectomized females in a dose-dependent manner. When treated with E2, plaques rarely progressed beyond small and uncomplicated fatty streaks consisting almost entirely of macrophage-derived foam cells, suggesting that E2 targets atherogenesis at early stages of lesion development (Hodgin et al. 2001). E2 appears to be equally efficacious in males (Nathan et al. 2001). On the other hand, studies in animals have also shown that inhibitory effects of estrogen may be lost once atherosclerotic lesions are established (Williams et al. 1995, Hanke et al. 1999). This may partly explain why the latest randomised controlled trials of HRT in women have shown no cardioprotection if started late in menopause (Herrington et al. 2000, Rossouw et al. 2002).

4.1. Estrogen receptors (ER)

The effects of estrogens are mediated by estrogen receptors (ER). To date, two estrogen receptors, ERα (Green et al. 1986) and ERβ (Mosselman et al. 1996), are known in humans. Separate genes located in different chromosomes encode each of these ERs. Vascular endothelial cells (Kim-Schulze et al. 1996, Venkov et al. 1996) and SMCs (Karas et al. 1994, Lindner et al. 1998) express functional ERs (ERα and ERβ), which are transcription factors that alter gene expression when they are activated. ERs are activated by estrogen binding but may also be activated by growth factors in the absence of estrogen. The latter mechanism of activation may operate when local concentrations of growth factors are high or when serum estrogen concentrations are low (as in men and postmenopausal women) (Power et al. 1991, Karas et al. 1998). At present, however, the precise mechanism(s) by which ER mediates vascular effects in the presence of low levels of estrogen is unclear.

The vascular effects of estrogens appear to be mediated both by effects in lipoprotein/lipid profiles and by direct effects on the vessel wall (Mendelsohn 2002). The effect on lipoprotein metabolism includes an increase in HDL cholesterol and a reduction in both total cholesterol and LDL (Walsh et al. 1991, Stevenson et al. 1993, Davis et al. 1994, Tremollieres et al. 1999). Classically it has been thought that estrogen-induced alterations in serum lipids account for only approximately one third of the observed clinical benefits of estrogen on atherosclerosis (Bush et al. 1987, Mendelsohn and Karas 1994). Direct vascular effects of estrogen on the vessel wall can be divided to genomic and nongenomic effects. The primary genomic effects are believed to be caused by estrogen-induced changes in gene expression, leading finally to decrease in atherosclerosis (Vargas et al. 1993, Weiner et al. 1994, Farhat et al. 1996, Chen et al. 1999b). In vascular cells, ERα

alters the expression of a number of genes including eNOS, cyclo-oxygenase, prostacyclin synthase, iNOS, endothelin-1, collagens, matrix metalloproteinases, vascular cell adhesion molecule, vascular endothelial growth factor, elastin, c-fos, and progesterone receptor among others (reviewed by Mendelsohn and Karas 1999).

A nongenomic direct vascular pathway of estrogen effect has been discovered in humans. Studies of this new pathway have shown that estrogen can rapidly cause vaso-dilatation, an effect that is largely mediated by activation of eNOS (Hayashi et al. 1995, Caulin-Glaser et al. 1997, Chen et al. 1999b, Mendelsohn 2002) (Figure 4).

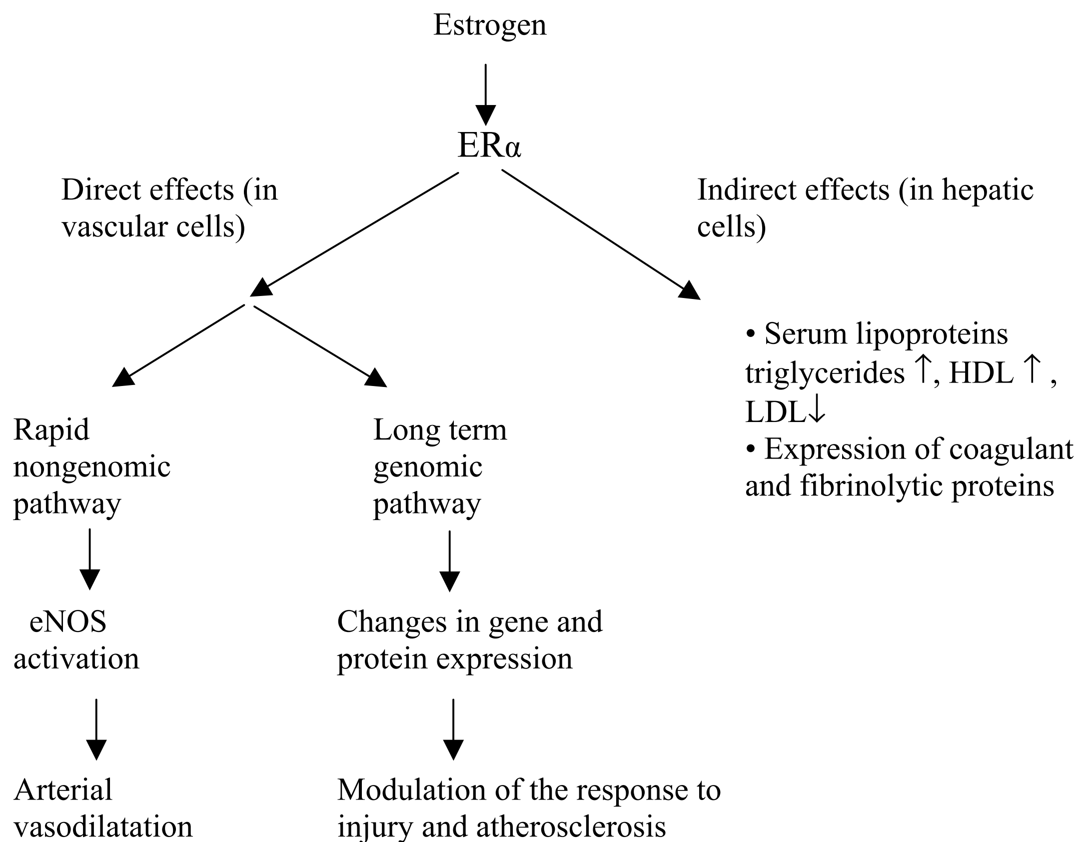

Figure 4. Proposed beneficial effects of ERα activation on the cardiovascular system.

In addition, endothelium-independent effects of E2 to vessel relaxation have been reported. Animal studies using rat mesenteric arteries have shown that E2 relaxes the vessel endothelium independently by acting on potassium channels and tyrosine kinase (Nevala et al. 2001, Nevala et al. 2002).

ERα is expressed in vascular SMCs derived from both women and men (Karas et al. 1994, Losordo et al. 1994), but because of the higher estrogen production inducing the expression of ERs, the number of receptors are higher in females. However, Losordo et al. (1994) showed that premenopausal women who died of coronary disease had significantly fewer coronary intimal ERs than age-matched women who died of noncoronary causes. Thus, it is possible that the alterations in ER expression and its function might affect the atheroprotective roles of estrogens. In a study in mice, a significant positive association between the number of ERs and basal release of NO was found in the aorta, suggesting that decreased number of vascular ER may represent a risk factor for cardiovascular diseases (Rubanyi et al. 1997). Among others, it has been speculated that methylation of the ER gene may be responsible for the lower expression of the receptor (Post et al. 1999). Also the genetic variation of the gene may have an effect on the amount of the expressed receptor.

4.2. Polymorphisms of the ERα

The gene for the ERα has four common polymorphism, of which three are so called restriction fragment length polymorphisms, named after the corresponding restriction site, and one is a dinucleotide repeat. In addition, Lu and coworkers (2002) have recently identified six new polymorphisms in the ERα gene (Figure 5).

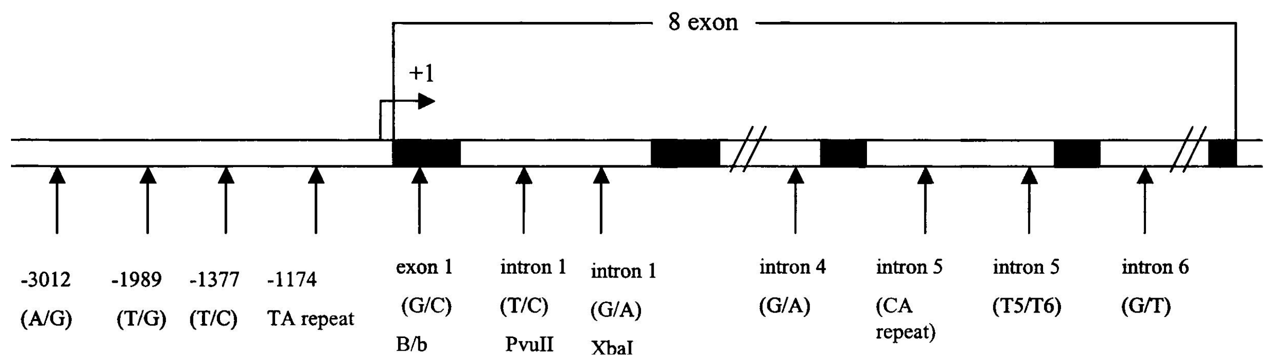

Figure 5. Polymorphic sites in the ERα gene that have been studied in respect to CAD risk

ERα PvuII. The most widely studied of the four common variants is the Pvu II polymorphism, which is caused by a T to C transition in intron 1, leading to genotypes P/P, P/p and pp, with capital letters signifying the absence of restriction sites (mutated) and small letters the presence of restriction sites (wild type)(Yaich et al. 1992). It has been speculated that the Pvu II polymorphism affects the splicing of ERα mRNA, resulting in the alteration of protein expression (Hill et al. 1989), or that the polymorphism is linked to some other polymorphism relevant for protein expression.

ERα XbaI and ERα G493C. The second polymorphism is the Xba I caused by a G- to- A transition in intron 1 (X1/X2) (Kobayashi et al. 1996). The third one is the B-variant polymorphism, caused by G/C transversion (B-wild type/ b-variant type) at nucleotide 493 in exon 1, resulting in a silent mutation at residue 87 of ERα (Taylor et al. 1992). The B-variant ERα has decreased binding affinity to estrogen (Garcia et al. 1988), and therefore it has been speculated that the presence of B-variant type might be a genetic risk factor for CAD (Matsubara et al. 1997).

ERα TA-repeat. The least studied but a promising common variant is the thymine-adenine (TA) repeat polymorphism lying –1174 bp upstream of the transcription initiation site in the regulatory region of the ERα gene, which may alter gene expression (del Senno et al. 1992). The number of the TA repeats varies from 10 to 27 between individuals, leading to numerous genotypes. This heterogeneity explains why only a small number of association studies have been done with this polymorphism.

4.3. Association of ERα polymorphisms with coronary artery disease

There are only few reports in which the association of ERα polymorphisms has been investigated with respect to CAD. Matsubara and co-workers (1997) have examined the association between CAD and three common polymorphisms of ERα (PvuII, XbaI and B-variant polymorphism) in subjects (n=87) with MI and angiographically proven CAD, and controls. They found no differences in the prevalence and severity of CAD between subjects with these ERα gene variants. In another study, Lu and co-workers (2002) studied subjects (n= 295) with heterozygous familial hypercholesterolemia. They found that the XbaI polymorphism in intron 1 was associated with CAD in men and in postmenopausal women, with a higher frequency of the X1/X1 genotype in the CAD group. Interestingly, the frequency of alleles of more than 17 TA repeats was found to be significantly higher in postmenopausal women with CAD than in those without CAD.

Despite the low circulating levels of estrogen found in men, a physiological role for ERα in males is supported by a study where a young man was shown to have endothelial dysfunction and early CAD caused by the C to T mutation at exon 2 of both alleles in the ERα gene. As a consequence of this mutation he had no functional estrogen receptor. The investigators concluded that some actions of estrogen, mediated via the ERα, are likely to

be protective against the development of premature vascular disease in men (Smith et al. 1994, Sudhir et al. 1997a,b).

5. The role of ERα in eNOS activation

The atheroprotective effects of estrogen in the vasculature have also been widely attributed to its ability to increase the amount of NO (Collins et al. 1994, Guetta et al. 1997, Hodkin and Maeda 2002). The mechanism by which estrogen regulates eNOS expression and activity is the focus of studies in many laboratories (Williams et al. 1992, Gilligan et al. 1994b, Mendelsohn and Karas 1994, Farhat et al. 1996, Guetta et al. 1997, reviewed by Fleming and Busse 2003).

Numerous investigations have assessed the effects of estrogen on eNOS expression. Although 17β-estradiol has been reported to upregulate eNOS mRNA and protein level in cultured endothelial cells, no convincing effect or even a decrease on eNOS expression have been found. The best demonstrations of a link between E_2 and eNOS expression have been made using animal models, but even here chronic changes in estrogen levels have been reported to increase as well as decrease eNOS levels (for review, see Fleming and Busse 2003).

The influence of ERα on eNOS has been suggested to take place at several levels. In 1999, Chen et al. showed that ERα could mediate the short-term effects of estrogen on eNOS activity within 5 minutes. They demonstrated that this activation of eNOS occurs in a nongenomic manner (Chen et al. 1999b). After that, it has been shown that different kinases are involved in this pathway leading finally to phosphorylation and activation of eNOS (Chambliss et al. 2000, Haynes et al. 2000, Simonchini et al. 2003, for review, see Fleming and Busse 2003). It has also been shown that 17β-estradiol promotes rapid heat

shock protein (Hsp90)-eNOS association, and this effect is blocked by an ER antagonist, suggesting ER-mediated modulation of eNOS activation (Russel et al. 2000). Recent data indicate that rapid eNOS responses occur in caveolae of endothelial cells, and it has been proposed that ER may activate caveolae membrane bound eNOS, thus leading to enhanced NO production (Chambliss et al. 2000).

The presence of both types of estrogen receptors in vascular cells has greatly increased the complexity of potential estrogen regulatory pathways in the cardiovascular system. Recently Chambliss and coworkers (2002) showed that also ERβ is localized to the endothelial cell caveolae and enhances eNOS stimulation by E2, indicating that ERβ has non-genomic action in caveolae (Chambliss et al. 2002). In addition, there is growing evidence that ERβ may also have an important function in the vasculature (for review, see Pettersson and Gustafsson 2001). ERβ expression is induced in VSMC after vascular injury (Lindner et al. 1998), and ERβ knockout mice exhibited hypertension and ion channel dysfunction in VSMCs (Zhu et al. 2002). It has been shown that both ERs can play redundant roles in at least three distinct regulatory pathways: inhibition of inflammatory cytokine-mediated endothelial cell activation, inhibition of basal ET-1 production, and induction of matrix-stabilizing enzymes (Evans et al. 2002).

After increasing our understanding of the ways in which estrogen achieves specificity in vascular cells, it is likely that vascular cell-specific selective ER modulators will be developed for the treatment of cardiovascular diseases in both women and men (Mendelsohn 2002).

AIMS OF THE STUDY

The advent of tools for studying individual variability in DNA has accelerated our capacity to investigate candidate genes for the risk of atherosclerosis. The objective of the present study was to examine genetic variations of two genes affecting vascular function, i.e. endothelial nitric oxide synthase (eNOS) and estrogen receptor-α (ERα), and their association with coronary artery disease and myocardial infarction in Finnish populations.

The detailed aims were:

1. To assess whether there is an association of the eNOS 4a/b polymorphism with coronary artery disease and myocardial infarction.

2. To determine whether the eNOS 4a/b polymorphism alone or combined with statin treatment has an effect on coronary reactivity among healthy men.

3. To develop a simplified method for the analysis of dinucleotide repeats of ERα by capillary gel electrophoresis.

4. To examine the possible association of two different ERα polymorphisms with coronary atherosclerosis and myocardial infarction.

SUBJECTS AND METHODS

1. Subjects

Table 2. Summary of subject distribution in different studies

	* HSDS A (1981-82)	HSDS B (1991-92)	Healthy women	Buccal swabs	Healthy men
Study I	X	X			
Study II					X
Study III		X	X	X	
Study IV		X			
Study V		X			

* HSDS, Helsinki Sudden Death Study

1.1. Autopsy series of middle-aged men (I, III-V)

The Helsinki Sudden Death Study (HSDS) was started in order to focus on the background and risk factors of sudden cardiac death and to follow changes in its epidemiology. The HSDS comprised two distinct series of Finnish men who died suddenly out-of-hospital and were subjected to a medico-legal autopsy at the Department of Forensic Medicine, University of Helsinki in 1980–1981 (n=400, A-series) and in 1991–1992 (n=300, B-series). Male sex and suitability for a complete autopsy were used as inclusion criteria. In addition, very young and very old victims were excluded, resulting in a final study series of males aged 33–69 years (mean 53.07, SD 9.58, median 54) from the area of Helsinki city and its surroundings. The causes of deaths were cardiovascular diseases in 41.1 % (n= 288), other diseases in 20.0 % (n=140), and suicides or accidents in 38.9 % (n=272).

A spouse, a relative or a close friend of the deceased was interviewed within 2 weeks post-mortem to collect data on CAD risk factors. Questions delineated past and

recent smoking, drinking habits, and medical history (Karhunen and Penttilä 1990). Interview data was available in 423 cases of the total of 700. On the basis of questions on previous illness, 107 men had hypertension and 113 men diabetes. Body mass index (BMI) and age were recorded as part of the routine autopsy protocol. The Ethics Committee of the Department of Forensic Medicine, University of Helsinki, approved the study protocol.

1.2. Clinical studies (II, III)

1.2.1. Positron emission tomography (PET) study (II)

Fifty-one men from the Archipelago Sea Naval Command, the Archipelago Coast Guard District, Säkylä Garrison, and from the Fire Department of Turku, all undergoing routine physical examination, were invited to participate in the study. The inclusion criteria were: 1) age 25 to 40 years, 2) clinically healthy, and 3) no continuous medication or use of antioxidant vitamins. The study was randomized, double blind and placebo-controlled (placebo, n = 26; pravastatin 40 mg/day for 6 months, n = 25). Pravastatin (Pravachol®) tablets and matching placebos were provided by Bristol-Myers Squibb (Espoo, Finland). Family history of CAD, alcohol and caffeine consumption, medications, smoking, and exercise habits were recorded using a questionnaire. Study participants were instructed to adhere to their normal diet during the study. The Joint Ethics Committee of the Turku University and the Turku University Central Hospital approved the study protocol. Each subject gave written informed consent.

1.2.2. Samples from healthy female subjects (III)

Fifty-four healthy adult women (mean age 30.9 ± 4.1 years) were drawn from a previous study of ER genotyping (Holmberg-Marttila et al. 2000). The Ethics Committee of Tampere University Hospital approved the study protocol, and written informed consent was obtained from the subjects.

1.2.3. Collection of buccal swab samples (III)

To study whether the analysis of ERα TA repeat polymorphism is suitable also from the DNA isolated from buccal swabs, samples were collected from five healthy volunteers using sterile cottonwood-tipped stick, which was rubbed on buccal mucosa back-and-forth for 10 - 20 sec.

2. Methods

2.1. Quantification of coronary artery stenosis and detection of myocardial infarction (I, IV, V)

At autopsy, an angiography was performed by compressing vulcanized liquid silicone rubber mixed with lead oxide into coronary arteries with physiological pressure (Weman et al. 1999). Stenosis at the proximal, middle, and distal parts of the main trunks of the three main epicardial coronary arteries (left anterior descending coronary artery (LAD), left circumflex coronary artery (LCx) and right coronary artery (RCA)) were measured from the rubber cast model. The percentage of stenosis was obtained by subtracting the

diameter of the rubber cast at the site of greatest stenosis of each part of the artery from the diameter of the rubber cast at the site representing the nearest proximal undamaged part of the artery. The most severe stenosis was used to define the extent of coronary narrowing for each individual. These measurements were available from 648 men for LAD, 602 for LCx, and 630 for RCA.

The presence of myocardial infarction was documented macroscopically by nitrobluetetrazolium (NTB) staining and confirmed by a histologic examination of the myocardium. Out of the 700 men, a total of 194 were found to have old or recent MI. The presence of neutrophilic granulocytes was considered diagnostic of an recent MI (n= 85) and the presence of fibrous scar diagnostic of a past MI (n=149). The presence of recent or organizing macroscopic coronary thrombosis was recorded after opening the coronary arteries (n=68).

2.2. Measurement of atherosclerosis at autopsy (I, IV, V)

In the Helsinki Sudden Death Study the definition of atherosclerosis was based on the macroscopic protocols used in the International Atherosclerosis Project (Guzman et al. 1968). Following angiography, coronary arteries were dissected free from the myocardial tissue, attached with a stapler onto a cardboard, and fixed in 10% buffered formalin. The arteries were radiographed to detect calcifications and then stained for fat by the Sudan IV staining method. The proximal parts of the RCA, LAD, and LCx were collected for the analysis. The following atherosclerotic changes were evaluated: fatty streak, fibrous plaque, complicated lesion, and an area of calcification. Any flat or slightly elevated intimal area that stained red with Sudan IV but did not show any other underlying changes

was classified as a fatty streak. Lesions protruding into the lumen of the artery were regarded as fibrous plaques. If ulceration, hemorrhage, necrosis or thrombosis was present the area was regarded as a complicated lesion. The part of the aorta which was strongly X-ray positive in the radiogram was classified as an area of calcification. The areas of atherosclerotic lesions and the total areas of coronary segments were assessed using computer-assisted planimetry, which measures the area in square millimetres. The area of different types of lesions was expressed in percentages (%). The data on the atherosclerotic changes of coronary arteries was available in 512 men from both series.

2.3. PET protocol and calculation of blood flow (II)

PET studies were done at baseline and at the end of the treatment period. All PET studies were performed after the subject had fasted for 6 hours. Alcohol and caffeine were not allowed for 12 hours before the study. Two catheters were inserted in the antecubital vein of the left hand for the injection of [^{15}O] H2O and for adenosine infusion, and another in the antecubital vein of the right hand for blood sampling. Myocardial perfusion was measured twice: once at rest and once after administration of adenosine. Heart rate, blood pressure, and the electrocardiogram were recorded throughout the studies to calculate the rate-pressure product.

The patients were positioned supine in a 15-slice ECAT 931/08-12 tomograph (Siemens/CTI Inc., Knoxville, TN, USA).

To determine blood flow, large regions of interest (ROI) were placed over representative transaxial images of the left ventricle, and values of regional myocardial blood flow (expressed as ml$\times$g$^{-1}\times$min^{-1}) were calculated using previously published method employing a single compartment model (Iida et al. 1988, Iida et al. 1995). Since there

were no differences in myocardial blood flow between different regions, global left ventricular blood flow was used for further analyses. Three men from the placebo group and four men from the pravastatin group were rejected because of technical problems with PET. Finally 44 men were included in the analysis.

2.3.3. Determination of serum lipids (II)

Blood samples for biochemical analyses were collected after an overnight fast. Plasma triglycerides, total cholesterol, and HDL cholesterol concentrations were analyzed with a Cobas Integra 700 automatic analyzer using reagents recommended by the manufacturer (Hoffmann-La Roche, Basel, Switzerland). LDL cholesterol concentration was calculated from the formula of Friedewald et al. (1972).

2.4. Genotyping (I-V)

2.4.1. Isolation of DNA (I-V)

Genomic DNA was prepared either from whole blood (II, III) or from frozen (–70 °C) pieces of myocardium taken during the collection of the B-series (1991–92) (I, IV, V) by the standard phenol-chloroform extraction. In the A-series (1981–82) DNA was isolated from paraffin embedded cardiac samples using the method of Isola et al. (1994) or the QIAamp DNA MiniKit (QIAGEN, Hilden, Germany) (I). DNA was released from buccal cell samples (III) using a rapid lysis method (Ilveskoski et al.1998). For the PET study (II), DNA was isolated from leukocytes using a commercial kit (QIAGEN Inc, Valencia, CA, USA).

2.4.2. Genotyping eNOS 4a/b polymorphism (I, II)

Primers for DNA amplification (5´-TTA TCA GGC CCT ATG GTA GT-3´; forward; 5´-AAC TCC GCT CAG CTG TCC T-3´; reverse) were designed according to the published sequence of the human NOS3 gene (NCBI/ D26607). The predicted length of the repeats was 167 bp for the aa genotype and 194 bp for the bb genotype. PCR conditions were: 5 min of denaturation at 94 °C followed by 41 cycles of 30s 94 °C, 30s of annealing at 50 °C and 1 min of extension at 72 °C, and a final extension time of 7 min at 72 °C. The PCR products were resolved in 3 % Metaphor agarose gel (FMC BioProducts, Rockland, ME, USA) using MspI digest of pUC19 DNA as base pair marker (MBI Fermentas, St. Leon-Rot, Germany).

2.4.3. Genotyping dinucleotide repeats of ERα by capillary gel electrophoresis and sequencing (III, IV)

Dinucleotide polymorphism of ERα was analyzed using PCR amplification with labeled primer. The primer sequences used were those described by Sano and co-workers (1995): 5´-6-FAM- GAC GCA TGA TAT ACT TCA CC-3´ (forward) and 5´- GCA GAA TCA AAT ATC CAG ATG-3´ (reverse). PCR was carried out with 25 cycles, consisting of two min at 94 °C, one min at 58 °C and one min at 72 °C, followed by 30 min at 60 °C after the last cycle to achieve a maximal A addition. The alleles were size-separated by capillary gel electrophoresis using Gene Scan™ Fragment Analysis Software 2.0.2. (*PE* Applied Biosystems, Foster City, CA, USA). Briefly, a 1µl of the product was diluted with 12 µl of deionized formamide containing 0.5 µl GeneScan-500 TAMRA internal lane

standard for sizing DNA fragments. Capillary electrophoresis was performed using ABI PRISM 310 Genetic Analyzer (*PE* Applied Biosystems) and Performance Optimised Polymer 4 (POP-4), which has been formulated for applications requiring high resolution under denaturing electrophoretic conditions. The capillary was from PE Applied Biosystems (Lt 47, Ld 36, 50µM ID).

Cycle sequencing of two of the PCR products (170 bp and 184 bp) was performed following the instructions of the Thermo Sequenase dye terminator cycle sequencing premix kit (Amersham Life Science, Cleveland, OH, USA). Electrophoresis of the reaction products was performed using ABI PRISM 310 Genetic Analyzer, performance optimised polymer 6 (POP-6) and the same short capillary as in fragment analysis described above. The data was analyzed using sequencing analysis software version 3.0.

2.4.4. Genotyping restriction fragment length polymorphism of ERα (IV)

ERα genotype was determined using a previously described method (Yaich et al. 1992, Kobayashi et al. 1996). Briefly, 1.3-kb fragment, including a part of intron 1 and exon 2 of the ERα gene, was amplified by PCR using the primers 5′-CTG CCA CCC TAT CTG TAT CTT TTC CTA TTC TCC-3′ and 5′-TCT TTC TCT GCC ACC CTG GCG CGA TTA TCT GA-3′. After amplification, the PCR products were incubated with PvuII restriction enzyme (New England Biolabs, Beverly, MA, USA) and the digested DNA was separated in 1 % agarose gel electrophoresis.

3. Statistical analyses

Differences in clinical characteristics between genotype groups were tested by the analysis of variance (ANOVA) or Pearson's χ^2 test (I, II,V).

The statistical analysis of morphometric data, stenosis measurements (I, IV, V), and differences in coronary blood flow (II) was based on the analysis of covariance using Statistica Version 5.0 (StatSoft Inc., Tulsa, OH, USA) (I, II, IV, V). Nonnormally distributed data were analyzed in square-root form (I, IV, V), but the results are displayed as crude data. In case of a significant interaction between the means of continuous variables and different genotype groups, Scheffe´(I, IV) or least significant difference´ (LSD)(II, V) post hoc tests were used to compare the differences between groups. In addition of ANCOVA, data analysis in the third work was based on linear regression analysis (mean area of complicated lesion). In study II and V, the power of the test was calculated using univariate analysis of variance. To analyze the change in serum lipids, ANCOVA for repeated measurements was performed separately for the pravastatin and placebo groups (II). Statistical calculations on the associations with myocardial infarction, coronary thrombosis and the number of diseased (>50% stenosis) vessels (I, IV, V) were studied by logistic regression analysis (Forward LR), supported by odds ratios (OR) with 95 % confidence intervals using SPSS 10.0 for Windows (SPSS INC., Chicago, IL, USA). The level of statistical significance was set at $p < 0.05$.

Linkage disequilibrium between ERα dinucleotide- and PvuII genotype variants was calculated using Arlequin software for population genetic data analysis (version 1.1, University of Geneva, Switzerland)(Schneider et al. 1997).

RESULTS

1. eNOS genotypes and coronary artery disease (I)

We studied associations of the eNOS 4a/b polymorphism with CAD and MI in the HSDS A and B series (1981-82 and 1991-92) comprising altogether 700 Finnish men (study I). In the subgroup of men with the risk factor interview data (n=355), there were no significant differences between genotypes and areas of atherosclerotic lesions or coronary stenosis percentages. Subjects with the rare a-allele had, however, significantly lower risk of MI (OR 0.44, 95% CI 0.25– 0.77, P=0.004) compared with those carrying the bb-genotype. Men with the a-allele also tended to have less often coronary thrombosis (OR 0.43, 95% CI 0.18 –1.01, P=0.055). The analyses were adjusted for the confounding effects of age, BMI, smoking, alcohol consumption, hypertension and diabetes. Due to the sudden death–nature of the series, all above mentioned risk factors were not available from all men in the HSDS. If the MI analysis was carried out in the whole series and adjusted only by age (n=649), the results did not change (unpublished data)(figure 6).

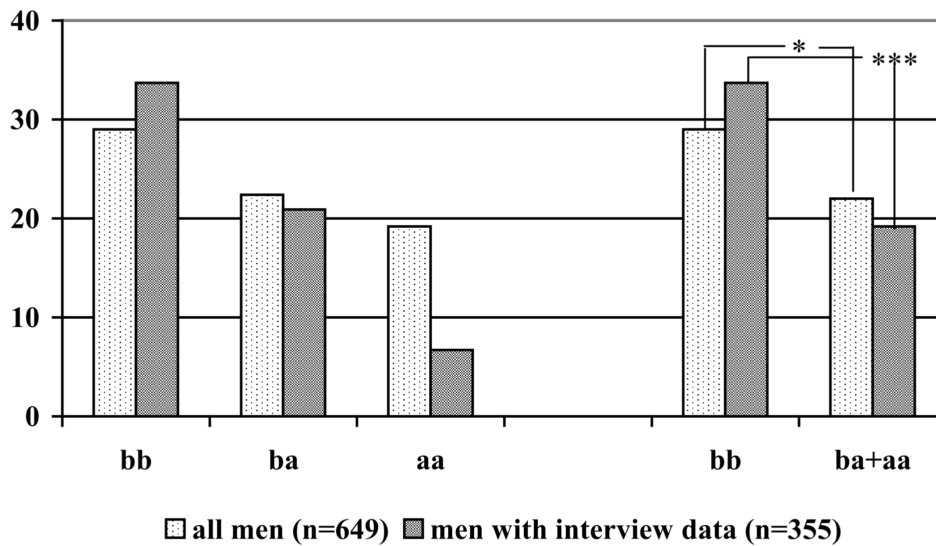

Figure 6. Percentages of men with MI among men with different eNOS 4a/b variants in the whole series and in the subgroup of men with available risk factor interview data. P values are from logistic regression analysis using age and eNOS genotype as variables in the whole series, and age, eNOS genotype, BMI, smoking, alcohol consumption, hypertension and diabetes in the subgroup of men with available risk factor data.

2. eNOS genotypes and coronary artery reactivity (II)

The aim of study II was to investigate whether the eNOS 4a/b polymorphism alone or combined with a six month statin or placebo treatment has an effect on coronary reactivity among healthy men. At baseline, there were no differences in basal or adenosine-stimulated coronary blood flow between subjects with eNOS bb or ba genotypes. At the end of

the study the two genotype groups reacted differently to pravastatin treatment with respect to the change in adenosine-stimulated flow (ANCOVA p=0.008). More specifically, after pravastatin treatment the adenosine-stimulated flow increased by 54.5 % in men with the eNOS ba genotype whereas in the men with the bb genotype no significant change in flow was observed (p=0.002 for ba versus bb). In the placebo group there were no significant changes in blood flow from the baseline values (p=0.916 for ba versus bb)(Figure 7). After pravastatin treatment, both genotype groups showed a similar decrease in serum total cholesterol and low-density lipoprotein cholesterol (p<0.00001 for both).

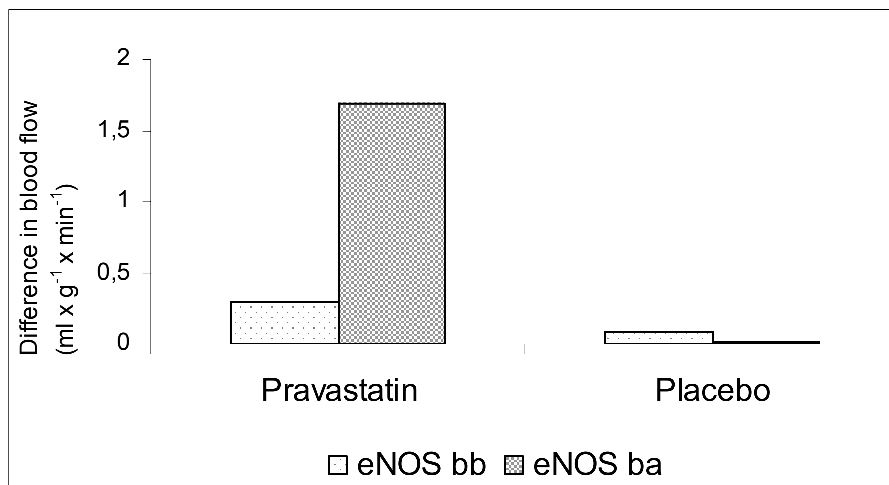

Figure 7. The difference between the start and the end of a 6 month treatment in adenosine-stimulated myocardial blood flow by eNOS genotype in the pravastatin and placebo groups.

3. Analysis of ERα genotypes (III)

In the third study, we developed a method for analysing dinucleotide repeats found in the upstream region of human alpha estrogen receptor gene by applying capillary electrophoresis. This method circumvents the need for polyacrylamide gel electrophoresis and radioactive chemicals. Using this method the frequency distribution of ERα alleles was determined from 180 Finnish individuals. The analysis showed two peaks at 13 and 14 repeats (166 bp, 168 bp) and also at 22 and 23 repeats (184 bp, 186 bp). The most common alleles in the Finnish population appear to be of 168 bp length (24%) (Table 3). The overall distribution of alleles in Finns seemed to be similar to those found among Italian and Japanese populations (del Senno et al. 1992, Sano et al. 1995).

Table 3. The frequency distribution estimated from 180 Finnish individuals.

Number of repeats	bp	Frequency	Number of repeats	bp	Frequency
12	164	0.01	19	178	0.03
13	166	0.16	20	180	0.01
14	168	0.24	21	182	0.05
15	170	0.09	22	184	0.12
16	172	0.03	23	186	0.11
17	174	0.06	24	188	0.04
18	176	0.00	25	190	0.04

4. ERα genotypes and coronary artery disease (IV, V)

To find out whether the length of the ERα repeat associates with coronary artery disease, the method of capillary electrophoresis was used to analyze the ERα repeats from the men in the HSDS B series (1991–92) (study IV). Of the 119 men (mean age 53.4 ± 8 years) in this autopsy series, 52 men had severe coronary atherosclerosis (mean coronary stenosis 65.9 % ± 10.8) and 67 men had only slightly narrowed coronary arteries (mean 22.1 % ± 13.7). The study population was divided into three groups according to median number of the repeat (=19), i.e. in the short allele genotype group both alleles were of < 19 repeats, whereas in the long allele group both alleles were ≥ 19 repeats. The third group comprised men who had one short and one long allele. We found that men with long alleles had significantly more severely narrowed coronaries (p=0.009), larger areas of complicated lesions (p=0.008), and more calcification (p=0.01) of the coronary arteries compared to men with short alleles. Atherosclerotic changes in the third group (one short and one long allele) did not significantly differ from either the short or the long genotype group. We also found that men carrying two long alleles had a higher risk for MI as compared to the short allele carriers (OR 4.4; 95% CI 1.21-15.70, p=0.025). The carriers of the long alleles also seemed to be more predisposed to coronary thrombosis (OR 11.4; 95% CI; 1.2-108.8, p=0.04).

The ERα gene has also a common two-allelic polymorphism (PvuII) in the first intron leading to genotypes P/P, P/p, and p/p. The aim of the study V was to investigate the relation of these ERα genotypes to the areas of the different types of atherosclerotic lesions and the presence of thrombosis in the coronary arteries of men included in the HSDS B series (1991–92) (study V). We found a significant association of ERα genotype with atherosclerotic area in men aged 53 years and older, but not in those less than 53

years. Men with the P/p and P/P genotypes had complicated lesion areas two to five times larger than those with the p/p genotype (P=0.001). ERα genotypes had no significant association with the percentages of coronary narrowing or areas of fatty streaks or fibrotic lesions. The presence of coronary thrombosis was significantly associated with the ERα genotype; the OR for coronary thrombosis were 10.6 for P/P (95% CI 1.08– 103.5, P= 0.043) and 6.2 for Pp (95% CI 0.74–52.9, P= 0.092) compared to men with the p/p genotype.

We also found a strong linkage between the ERα TA repeat and PvuII polymorphisms. Long TA repeat genotypes were linked to mutated Pvu genotypes and short TA repeat genotypes to normal Pvu genotypes (P<0.00001) (Table 4) (unpublished results).

Table 4. Reciprocal distribution of subjects with the ERα TA repeat and PvuII polymorphism.

Genotype	TA-repeat short/short	TA-repeat short/long	TA-repeat long/long	row totals
PvuII:PP (mutated)	0	12	14	26
PvuII: Pp	13	32	7	52
PvuII:pp (normal)	25	6	1	32
all groups	38	50	22	110

DISCUSSION

1. eNOS gene variants, myocardial infarction and coronary artery disease (I)

Study I showed that the less common a-allele of the eNOS 4a/b polymorphism is associated with a lowered incidence of MI among middle-aged Finnish men. As reviewed in Table 2, previous studies on this topic have shown conflicting results, and the rare a-allele has been reported to have either no effect or an association with increased risk for MI or CAD. Therefore, our finding that men carrying the a-allele had almost 50 % lower risk for MI than men with the bb-genotype was quite surprising. A possible explanation for the discrepancies between our study and the previous clinical studies may be due to differences in the distribution of eNOS genotypes in populations. The populations may differ even more with regard to haplotype heterogeneity (Cardon and Bell 2001, Ruse and Parker 2001). Furthermore, selection factors may be different, and CAD risk factors may differently be taken into account as confounders in the analyses. In our series, the association between genotypes and MI was based on logistic regression analysis in which all available confounding factors were taken into consideration, including age, BMI, hypertension, diabetes, alcohol consumption, and smoking.

The means of the percentages of coronary stenosis or the areas of atherosclerotic lesions did not have any statistically significant associations with the eNOS genotype. Thus, our results are in agreement with previous reports (Hibi et al. 1998, Sigush et al.2000, Pulkkinen et al. 2000, Fowkes et al. 2000, Grannath et al. 2001, Gardemann et al. 2002) in that eNOS genotype does not associate significantly with the severity of coronary artery stenosis.

If a polymorphism is located in an intron, it has not been thought to have any functional relevance. However, healthy male carriers of the eNOS 4a/b polymorphism a-allele have been reported to have increased concentration of plasma NO when measured as nitrite and nitrate (Wang et al. 1997). This result supports our findings of a protective role of the a-allele in vascular disease. There appears to be linkage disequilibrium between the rare 4a-allele and the T(-786)C mutation of the eNOS gene (Sim et al. 1998, Yoshimura et al. 2000). Recently, Wang and co-workers (2002) demonstrated a cis-acting role of the 27-bp repeats in the eNOS T-786C promoter function and a haplotype-specific expression pattern *in vitro*. This suggests that also the eNOS 4a/b polymorphism might have a direct role in gene transcription. Haplotype specific transcription efficiency may also explain in part the conflicting results observed in this field. Therefore, haplotyping of a large study population would be needed to provide further insight into the scenario of combined roles of the different genotypes.

2. Effect of pravastatin on eNOS genotypes and coronary function (II)

Because of contradictory findings on the role of eNOS 4a/b polymorphism in CAD and MI, we aimed at elucidating functionalities of the different alleles. We hypothesized that eNOS polymorphism might be influenced with coronary function and reactivity as measured by PET. Before the present study (II), PET has not been used to investigate the role of eNOS polymorphism in coronary function. Study II showed that the effect of pravastatin on coronary artery reactivity in young men is at least partly eNOS genotype-dependent, because adenosine-stimulated myocardial blood flow increased with treatment significantly in men with the ba genotype but not in men with the bb genotype. The validity of

the present findings is strengthened by the fact that the study population was homogeneous. All subjects were males with age range of 26 to 40 years and had no clinical symptoms of any disease.

The cardiovascular benefits of statin therapy have mainly been attributed to reductions in LDL cholesterol (Holme 1990, Klag et al. 1993, Libby and Aikawa 2003). However, subgroup analysis of large clinical trials, such as West of Scotland Coronary Prevention Study (WOSCOPS) (Shephard et al. 1995), The Cholesterol and Recurrent Events study (CARE) (Goldberg et al. 1998), and Scandinavian Simvastatin Survival Study (4S) (Ballantyne et al. 2001), suggest that the clinical benefits of statins are not solely related to baseline cholesterol levels or the degree of cholesterol reduction. In our study, changes in lipid levels did not explain the differences in coronary reactivity, because both genotypes responded equally to the lipid lowering effects of pravastatin. Furthermore, the differences in the coronary reactivity between genotypes were adjusted for the change in LDL. Therefore the differences may be mediated by other mechanisms, e.g. by effects of pravastatin on eNOS availability.

There are several ways in which statins may regulate eNOS. Kaesemayer and colleagues (1999) showed that pravastatin sodium activates eNOS independently of its cholesterol-lowering actions. Also other experimental studies have revealed that statins can directly upregulate eNOS expression and activity *in vitro* and *in vivo* by stabilizing eNOS mRNA (Endres et al. 1998, Laufs et al. 1998, Kano et al, 1999, Hattori et al. 2003). Statins may also decrease cellular caveolin levels and decrease the inhibition of eNOS by caveolin, resulting in increased NO production in endothelial cells (Feron et al. 2001)(Figure 7).

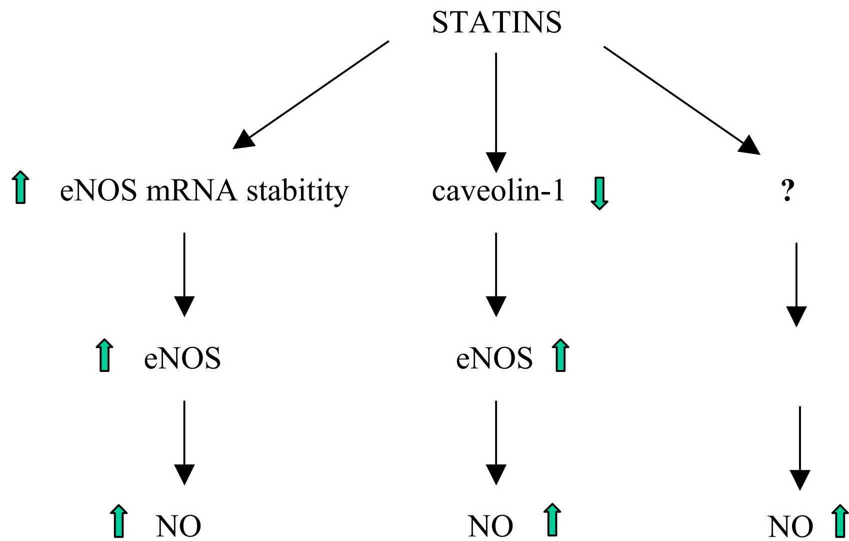

Figure 7. Possible effects of statins on NO production

The precise mechanisms of how statins regulate eNOS are likely to be even more complex. However, we did not observe a direct effect of pravastatin on coronary flow, but adenosine stimulation was needed for the men with the eNOS 4a allele to benefit from the statin treatment.

The adenosine-induced coronary flow response reflects a combined effect of endothelial-mediated vasodilatory function (Mayhan 1992) and vascular smooth muscle relaxation (Wilson et al. 1990) and has been used as an integrating measure of coronary reactivity (Dayanikli et al. 1994, Uren et al. 1994).

In the myocardium, adenosine is released in small amounts at constant basal rate during normoxia.During ischemia the production of endogenous adenosine increases several fold, due to breakdown of adenosine triphosphate, and leads to coronary vasodilatation (Sommerschild and Kirkeboen 2000). We reported that subjects with the eNOS ba-genotype that have been treated with pravastatin showed significantly improved coronary

vasodilation by exogenously administered adenosine as compared to subjects with the bb genotype. This potentiating effect on vascular relaxation may also have clinical significance in subjects with myocardial ischemia and increased endogenous adenosine. Recently Wolfrum et al. (2003) showed that statins reduce the extent of myocardial necrosis in normocholesterolemic rats after acute ischemia/reperfusion injury by increasing myocardial eNOS activity. According to their results and ours, statins may protect the heart not only by reducing the incidence of ischemic events, but also by increasing coronary vasodilatation. This effect may depend on individuals´ eNOS genotype. The mechanism by which pravastatin enhances adenosine-stimulated vasodilatation remains unclear.

3. Development of a capillary electrophoresis method (III)

It is known that alleles differing by only 2 bp in size are difficult to separate in conventional gel electrophoresis. Therefore, in the third study, we have created a system by capillary electrophoresis for analyzing the dinucleotide (TA) repeat polymorphism found in the upstream region of the human ERα gene. This method allows the user to avoid neurotoxins associated with polyacrylamide. Also the use of radioisotopes can be avoided, because the analysis is based on fluorescence. The analysis of fragment length is reproducible, since an internal size standard is loaded with every sample to normalize for differences in electophoretic mobility between different injections. Nevertheless, we observed a potential problem in our method. Although the difference between the shortest (160bp) and longest (194bp) alleles was only 24 bp, these alleles did not amplify with the same efficiency. The short alleles were more efficiently amplified than the longer ones. This kind of preferential amplification might lead to an incorrect genetic typing of the

sample, and has been also previously described in a polymorphic locus with several hundred bp differences between shortest and longest allele (Walsh et al. 1992, Sham et al. 2002). However, we did not regard the observed preferential amplification as a problem, since there were no other extra peaks in the amplification product. In addition, the shorter the distance in base pairs between different alleles, the smaller was the preferential amplification observed. We also found that the amplification of DNA for the analysis can be done from blood or tissue samples as well as from buccal mucosa cell lysates.

In 180 Finnish individuals the number of the TA repeats varied between 12 (164 bp) and 25 (190 bp), and the overall distribution of different genotypes seemed to be similar to that published from an Italian population (del Senno et al. 1992) as well as from Japanese individuals (Sano et al. 1995).

4. ERα genotypes and coronary artery disease (IV, V)

There are only few reports in which the inherited genotypic effects of ERα have been investigated with respect to CAD (Matsubara et al. 1997, Lu et al. 2002). Moreover, no previous study has investigated the association of TA repeat variation of ERα gene with CAD, and no previous studies have investigated the impact of the ERα gene variations regarding the degree of specific atherosclerosis lesion phenotype, measured precisely from the artery wall after autopsy.

We first analyzed these ERα gene variants from the subpopulation of 119 men included in the HSDS B series (1991-92) (IV). We found that men with long alleles had significantly larger areas of complicated lesions and calcification of the coronary arteries compared to men with short alleles. Also men carrying long alleles had significantly

higher risk for coronary thrombosis and MI compared to the short allele carriers. Thus, our results suggest that the length of the TA repeat of the ERα gene may partly explain differences between individuals in the development of coronary artery disease. Although the biochemical evidence is lacking at the moment, we speculate that, as compared to carriers of the short alleles, carriers of the long repeat variants may have lower expression of the ERα gene and less cardiovascular protective effects from ERs. There is at present only a limited amount of information known about how ERα transcription is regulated. It has been suggested that as differential expression of ERα transcripts occurs between different cell types, regulation of the level of individual promoters must be a key event in ERα mRNA formation (Reid et al. 2002). Because the TA repeat is in the regulatory region of the ERα gene, there is a possibility that this polymorphism affects gene transcription and thus affects ER production.

Although Losordo and co-workers (1994) showed a positive correlation between the presence of the ERs and the absence of atherosclerosis in coronary arteries from premenopausal women already a decade ago in 1994, it is still unclear how ERs influence cardiovascular function in men. Sudhir and co-workers (1997b) reported early CAD in a 31-year-old man lacking functional ERα. They concluded that some actions of estrogens, mediated via the ERα, are likely to be protective against premature vascular disease in men. Studies using the male ER knockout mouse showed a significant association between the number of ERs and basal release of NO in the aorta of mice, suggesting that decreased vascular ER number may represent a novel risk factor for cardiovascular diseases by this mechanism (Rubanyi et al. 1997).

Of the three other polymorphisms in this gene, the most extensively studied is the PvuII polymorphism (Matsubara et al. 1997). We analyzed also this polymorphism from the men included in the HSDS B series (1991-92) (V). There was an age-dependent asso-

ciation between the ERα PvuII genotype and the area of complicated coronary lesions and the presence of coronary thrombosis in subjects who suffered sudden death. These results demonstrated at the level of the vessel wall that the area of complicated lesions in coronary arteries and the presence of coronary thrombosis increase with allele dose (p/p<p/P<P/P) in men aged 53 years or over.

It has been speculated that the PvuII polymorphism affects the splicing of ERα mRNA, resulting in alteration of protein expression (Hill et al. 1989, Matsubara et al. 1997), or that the polymorphism is linked to some other polymorphism relevant to protein expression (Matsubara et al. 1997). We found that there is strong linkage disequilibrium with PvuII and TA repeats of ERα in our study population. This is in accordance with previously published data where ERα gene variants and their relationship to bone mass variation in postmenopausal Italian women have been analyzed (Becherini et al. 2000). Although the mechanisms by which these gene variants affect ERα production has not been adequately explained, they are strongly associated with the area of advanced atherosclerotic lesions, coronary thrombosis, and MI, supporting the hypothesis that at least some variation in the ERα gene affects the way in which the atheroprotective action of estrogen is mediated to artery wall cells.

5. Strengths and limitations of the studies

The strengths of the present autopsy studies (I, IV, V) as compared with those carried out in living CAD patients, are that the quantitation atherosclerotis lesions and the percentage of coronary stenosis could be measured directly from the arteries and myocardium. Also the presence of recent or old MI and acute coronary thrombosis could be verified. The

weakness of the HSDS series includes the fact that study subjects may have had more severe atherosclerosis than individuals selected randomly, leading to selection bias. Moreover, cholesterol levels of the deceased were not available, as ante mortem measurements had only been performed in a few individuals. In addition, alcohol consumption was very high in the HSDS series (mean 7–8 drinks per day, median 60 g/day) and smoking was common (proportions of current and ex-smokers were 69 % and 13 % respectively), suggesting that risk factors may have been unfavorable in these subjects. In contrast, because of their occupation, the participants of the PET study (II) may have been healthier and in better physical condition than average people. The strength of the study II is that it had a randomized, placebo-controlled, and double blind study design, which makes the findings more reliable. The limitation of the study II was the small number of subjects, because the PET technique is expensive and laborious. However, power analysis of the differences in blood flow between eNOS genotype groups was adequate, suggesting that the differences observed are statistically valid. Increasing the number of study subjects would have enable haplotype-specific analyses. Finally, it should be noted that we studied only men, and therefore these results cannot be directly extrapolated to women.

Limitations of the association studies include that they are case-control studies, which suggest that the given genotype occurs more frequently in one group of individuals than in another group. Moreover, association studies make no use of family data. It should also been pointed out that, because of the complexity and multifactorial nature of CAD, it is unlikely that any single gene will explain more than a few percent of the CAD risk in the general population (Hamsten 1996, Gambaro et al. 2000).

SUMMARY AND CONCLUSIONS

The present study examined associations of ERα and eNOS gene polymorphisms with coronary artery disease and myocardial infarction in a Finnish male population.

The major findings and conclusions are:

1. The a-allele of the eNOS 4a/b polymorphism may be associated with protection from myocardial infarction in a Finnish male population.

2. Pravastatin treatment improves coronary reactivity by a mechanism that is eNOS genotype and adenosine -dependent. Pravastatin in subjects with eNOS a-allele may modulate coronary reactivity by increasing NO production by the endothelium or by affecting some other mechanisms in the coronary artery wall itself. This could have important consequences also in the course of myocardial infarction.

3. Capillary gel electrophoresis is a reliable method for studying dinucleotide repeat variants of the ERα gene.

4. The ERα gene may provide a candidate gene for coronary artery disease. Men in the HSDS with a long variant of the ERα gene and/or a mutated allele in exon 1 had a significantly greater severity of coronary artery disease than did men with other forms of the gene. They were also more susceptible to coronary thrombosis and myocardial infarction.

To conclude, these studies suggest that ERα and eNOS genetic variations are associated in the mechanisms of coronary artery disease pathogenesis in men.

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Tarja Kunnas

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