Endothelial nitric oxide synthase activity in aorta of normocholesterolemic rabbits: regional variation and the effect of estrogen

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Received 22 November 1999; accepted 25 February 2000

Abstract

Objective: Several animal studies suggest that nitric oxide (NO) produced by the endothelium attenuates arterial cholesterol accumulation. In the present study we have asked the following questions: (1) is the regional variation in aortic cholesterol accumulation in hypercholesterolemic rabbits preceded by a regional variation in endothelial NO synthase (eNOS) activity in normocholesterolemic rabbits, and (2) is the antiatherogenic effect of estrogen in hypercholesterolemic rabbits preceded by a higher eNOS activity in normocholesterolemic rabbits. Methods: The eNOS activity was determined by conversion of L-arginine to L-citrulline in freshly isolated endothelial cells of aorta in normocholesterolemic rabbits. In the regional variation study, 16 male and eight female rabbits were used. In the estrogen study, ovariectomized female rabbits were subcutaneously injected three times weekly with either 17β-estradiol (n=7) or vehicle (n=7) for 18 weeks. Results: In the regional variation study, the atherosclerosis prone aortic arch showed a significant lower eNOS activity than the more resistant abdominal aorta in both male (P<0.0001) and female (P<0.05) rabbits. In the estrogen study, the eNOS activity in the aortic arch and upper thoracic aorta was significantly higher in the estrogen than in the vehicle rabbits (P<0.05). In the lower thoracic aorta, however, the eNOS activity was the same. Conclusion: The findings suggest that a high NO production in the luminal endothelium of the arterial wall precedes a low cholesterol accumulation during a subsequent period of hypercholesterolemia.

Keywords: Arteries; Atherosclerosis; Cholesterol; Endothelial function; Hormones; Nitric oxide

1. Introduction

Several studies suggest that endothelial cells (ECs) covering the luminal side of arteries play an important role in the development of atherosclerosis by changes in the release of relaxation and constriction factors important for the dynamic properties of the arterial wall [1], the adhesion of leukocytes, monocytes, and platelets to the endothelium of the arterial wall [2–4], and permeation of cells [5] and lipoproteins into intima [6]. For instance, denudation of the endothelium by balloon catheter injury in the rabbit aorta increases the accumulation of cholesterol and furthermore eliminates the antiatherogenic effect of estrogen treatment [7–9].

Two nitric oxide synthase (NOS) isoforms are expressed in ECs: the constitutive endothelial NOS (eNOS) which is calcium-dependent and the inducible NOS which is calcium-independent. Via NOS the ECs catalyse the oxidation of L-arginine to L-citrulline and nitric oxide (NO) [10,11]. A number of investigators has shown that a basal level of NO is important to sustain the normal functions of the endothelium: (1) endothelium-dependent vasodilation by NO was impaired in humans and rabbits with hypercholesterolemia-induced atherosclerosis [12,13], (2) endothelial dysfunction and accelerated neointima formation were found after chronic inhibition of the NO production by Nω-nitro-L-arginine methyl ester (L-NAME) in the chole-
terol-fed rabbit [14,15], and (3) NO prevented leukocyte adhesion to the endothelium of rats [4]. These findings suggest that NO produced by the endothelium is an antiatherogenic molecule.

The endothelial production of NO can be determined indirectly by the conversion of L-arginine to L-citrulline because NOS catalyses the formation of equimolar quantities of NO and L-citrulline from L-arginine [16]. Fabricius et al. [17] used this method to determine the conversion of L-arginine in samples of freshly isolated ECs (FIECs) of aortas from rats. In order to determine measurable levels of eNOS activity it was necessary to pool samples from aorta of two identically treated rats.

The present study was designed to establish a method to measure the eNOS activity by conversion of $^{14}\text{C}$-L-arginine to $^{14}\text{C}$-L-citrulline in the presence of added cofactors in FIECs of different aortic segments from normocholesterolemic rabbits. The aim was to relate the eNOS activity in the aortic endothelium of normocholesterolemic rabbits to the aortic accumulation of cholesterol during a subsequent hypercholesterolemic period. However, since it was not possible to measure the eNOS activity during normocholesterolemia and the accumulation of cholesterol after hypercholesterolemia in the same rabbit aorta, it was decided to relate the eNOS activity before hypercholesterolemia to two situations in which ECs apparently make the difference between high and low subendothelial accumulations of cholesterol during exposure to the same degree of hypercholesterolemia: (1) the regional variation in aortic cholesterol accumulation [18], and (2) the antiatherogenic effect of estrogen [7–9].

2. Methods

2.1. Animals

A total number of 16 male and 22 female rabbits of the Danish Country strain were purchased from Statens Serum Institute, Copenhagen, Denmark. All rabbits were sexually mature, had a body weight of ~3–5 kg, and were used in three consecutive studies within a period of 10 months. Firstly, the male rabbits and one group of the female rabbits ($n=8$) were used to study the regional variation in aortic eNOS activity (the male rabbits were also used to study the effect of different cell preparation procedures). Secondly, the last group of female rabbits ($n=14$) were used to study the effect of estrogen treatment on aortic eNOS activity. The rabbits were housed individually at a temperature of 20±3°C, with a 12-h light/dark cycle (11 h with 100% light and 2×30 min with 50% light), and a humidity of 50±10%. The rabbits were given 130±5 g chow per day and had free access to drinking water unless otherwise stated. Experimental procedures were performed in accordance with Danish regulations for the care and use of experimental animals.

2.2. Treatment of female rabbits for the estrogen study

Ovariectomy was performed on the last group of female rabbits ($n=14$) as follows. After subcutaneous anaesthesia with about 6.6 mg midazolam (Dormicum®, F. Hoffmann-La Roche AG, Basel, Schweiz), 0.4 mg fentanyl citrate, and 13.2 mg fluanisone (Hypnorm™, Janssen-Cilag, Birkerød, Denmark) per kg body weight, abdomen was opened through a midline incision, and both ovaries were removed.

After 1 week of recovery the rabbits were divided into two treatment groups with similar baseline values of body weight. Every third day the estrogen rabbits ($n=7$) were injected subcutaneously with 50 μg 17β-estradiol cypionate (Sigma, St. Louis, MO, USA) dissolved in 0.3 ml maize oil (UniKem, Copenhagen) per kg body weight and the rabbits in the control group ($n=7$) were injected subcutaneously with the same amount of vehicle. The animals were treated for 18 weeks and every day throughout this period the rabbits were given 100 g of normocholesterolemic chow (Altromin from Chr. Petersen A/S, Ringsted, Denmark) supplemented with 10% maize oil.

After the endothelial cell preparation, uterus was removed and weighed.

2.3. Preparation of endothelial cells

The rabbits were killed with an intravenous injection of 100 mg pentobarbital (KVL apotek, Frederiksberg, Denmark) per kg body weight. The aorta was freed of the adventitia in situ, removed, and opened longitudinally. During the preparation the aorta was kept in saline (0.9%). The aortas of the male rabbits were divided into four consecutive segments: the aortic arch (from the heart to the first intercostal branches), the thoracic aorta (to the celiac orifice) which was subdivided into two segments of similar size, and the abdominal aorta (to the iliac bifurcation). The aortas of the first group of female rabbits ($n=8$) were divided into three segments: the aortic arch, the thoracic aorta, and the abdominal aorta. In the estrogen study, the aortas were divided into three segments: the aortic arch, and the upper and lower thoracic aorta which corresponds to the first three segments of the male aortas. Each segment was pinned to laboratory film on a cork board and the ECs were obtained by a single scrape of the luminal surface of the aorta with a razor blade. The cells were placed in Eppendorf tubes containing 70 μl of 50 mM Tris buffer (pH 7.4) and were immediately frozen in liquid nitrogen.

For controls, thoracic pig aortas were purchased from Danish Crown (Odense, Denmark) and transported from the slaughterhouse to the laboratory in saline (~7°C). The ECs were prepared as described, however, all the cells were pooled in 20 ml of Tris buffer. Samples of 150 μl were immediately frozen in liquid nitrogen. The period from killing the pigs to freezing the samples was ~3 h.

In controls, the eNOS activity did not change during the
first 18 months of storage at −80°C. All samples were stored for less than 5 months.

2.4. Immunohistochemistry

In order to quantify the cell types in the sample, 3 μl of sample material were streaked onto glass slides, dried at room temperature (20 min), and stored at −80°C for immunostaining. Frozen sections of human muscular artery and rabbit aorta embedded in Tissue-Tek (Sakura, USA) were used as positive and negative controls.

Sections and sample smears were fixed with acetone (10 min), rinsed with 0.05 M Tris-buffered saline (TBS, pH 7.4), and preincubated with 2% bovine serum albumin (BSA, Sigma) in TBS (10 min). For incubation with primary antibody diluted with 1% BSA in TBS supplemented with 15 mM NaN₃ (30 min), three different primary antibodies were used: (1) mouse anti-human α-actin (Dako, Glostrup, Denmark) diluted 1:200, (2) mouse anti-human CD31 (Dako) diluted 1:20, and (3) mouse anti-human thrombomodulin (Serotec, Oxford, UK) diluted 1:5. After rinsing with TBS all sections and sample smears were incubated with biotinylated goat anti-mouse antibody (Dako) diluted 1:200 with 1% BSA in TBS supplemented with 15 mM NaN₃ (30 min). They were then rinsed with TBS, incubated with streptavidin–horseradish peroxidase (30 min, Dako), and rinsed with TBS, and water. The peroxidase activity was visualized with 3-amino-9-ethylcarbazol (20 min, Sigma). Sections and sample smears were rinsed in water, counter stained with Mayer’s haematoxylin (2 min, FAS centralapotek, Odense), rinsed with water again, and finally each slide was mounted with Aquamount (British Drug House, UK). Negative controls were immunostained under the same conditions, apart from omitting the primary antibody. All procedures took place at room temperature.

2.5. Cell counting

After the sample was thawed in a waterbath (37°C), three mixtures of 5 μl sample material and 5 μl of 0.025% methyl violet in 0.5% acetic acid (FAS centralapotek) were prepared. The remaining sample material was immediately frozen in liquid nitrogen and stored at −80°C until assayed. The number of cells in the mixtures was counted visually in a counting chamber at the light microscopic level, and the mean number of cells in the sample was calculated. The coefficient of variation (CV) was <5% on the number of counted cells in each sample.

2.6. Citrulline assay

Determination of the eNOS activity was carried out by a modification of methods previously described [17,19]. The samples were frozen in liquid nitrogen and thawed five times in a waterbath (37°C) to break up intracellular membranes. Duplicate determination of the conversion of l-arginine to l-citrulline was made on each sample. The cell homogenate (30 μl) was added to the reaction buffer: 0.4 μCi l-[U-¹⁴C]arginine monohydrochloride (specific activity: 296 mCi/mmol, Amersham, Buckinghamshire, UK), 15 μM 6R-tetrahydrobipterin (Sigma), 1 μM flavin adenine dinucleotide (Sigma), and 1 μM calmodulin (Sigma) in Tris buffer containing 1 mM Mn-dithiothreitol (Sigma), 1 mM ethylenediaminetetraacetic acid (Fluka, Buchs, Switzerland), and 1.25 mM CaCl₂ (Fluka). The reaction was initiated by adding the cofactor β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH, Sigma) to a final concentration of 1 mM (final volume of 150 μl). Samples were incubated for 30 min at 37°C and the reaction was terminated by adding 1 ml of ice-cold 100 mM HEPES buffer (pH 5.5) containing 10 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N' -tetraacetic acid (EGTA, Sigma). The total volume was applied to a 2-ml AG 50W-X8 column (Na form, Bio-Rad, Copenhagen) that had been equilibrated with HEPES buffer minus EGTA. Citrulline was eluted three times with 1 ml of HEPES buffer minus EGTA. The ¹⁴C-l-citrulline content in 0.5 ml of the eluate supplemented with 5 ml of liquid scintillation cocktail (BCS, Amersham) was quantified by liquid scintillation counting for 20 min leading to a CV of <5% on the number of counts in the sample. In each assay two baseline samples (A and B) and one control sample were placed as the first and the last samples in the rack. FIECs from slaughter pig aortas were added to these samples. Sample A was deprived of β-NADPH, giving the amount of surplus ¹⁴C-l-arginine that did not bind to the column resin. Sample B was deprived of β-NADPH and was not applied to a column, giving the total number of counts per minute in the sample corresponding to the amount of added ¹⁴C-l-arginine. Controls were used for the determination of inter-assay and intra-assay CV in eNOS activity. The activity of eNOS was calculated from the formation of ¹⁴C-l-citrulline/min (mean of the duplicate determination) and the mean number of ECs in the sample and expressed in pmol l-citrulline/min/10⁶ ECs.

When controls were incubated with concentrations of 2.5, 5, 10, 20, and 40 μM l-NAME the eNOS activity was dose-dependently reduced by 60, 71, 83, 91, and 93%, respectively. This finding demonstrates that the conversion of l-arginine in this study occurs via the NOS pathway. The intra-assay CV in eNOS activity was 9% (n=6) and the inter-assay CV was 17% (n=41), when measured over a period of 10 months.

When the number of FIECs from slaughter pig aortas were below 10×10³ per 30 μl homogenate for the citrulline assay, the correlation between the cell number and the production of pmol l-citrulline/min was not linear, and the CV in eNOS activity was >10%. Thus, it was decided to reject samples with a cell number below this threshold value. The number of FIECs obtained per aortic segment ranged from ~30 to 1000×10³.
2.7. Statistical analysis

All the results are given as mean±S.E.M. In the endothelial cell preparation study, data were analyzed by two-way ANOVA. Repeated measures ANOVA, in which the regional values of each rabbit represent matched observations, followed by Newman–Keul’s multiple comparison test, was performed on the regional variation data of both male and female rabbits. In the estrogen study, interaction was found between treatment and aortic region by two-way ANOVA. Regional differences in eNOS activity between the estrogen rabbits and vehicle rabbits was therefore analyzed by Mann–Whitney U-test. Repeated measures ANOVA followed by Newman–Keul’s multiple comparison test, were performed on the data of the vehicle rabbits to test for a regional variation; however, since this test can not be used when data are missing, one-way ANOVA was performed on the data of the estrogen rabbits. Statistical calculations were made with the computer program GraphPad Prism version 2.01, and differences were considered significant when P<0.05.

3. Results

3.1. Identification and quantification of the cell types in the sample

Cells were identified by immunostaining against the smooth muscle cell marker α-actin and the endothelial cell markers CD31 and thrombomodulin. In human muscular arteries and rabbit aorta, the cells in media showed positive reaction against α-actin and the cells lining the lumen showed positive reaction when stained against CD31 or thrombomodulin. These observations suggest that the antibodies directed against human smooth muscle cells and ECs cross-react with the same cell types in rabbits. In all cases controls deprived of primary antibody showed no positive reaction. Sample smears from the arch (n=6), thoracic (n=6), and abdominal (n=5) aorta from three rabbits were immunostained and the % positive cells in the samples were determined by cell counting. In both the arch, thoracic, and abdominal aorta approximately 1% of the cells in the sample reacted positively against α-actin (range 0–3%), while ~98 and ~97% of the cells in the sample reacted positively when stained against CD31 (range 97–100%) and thrombomodulin (range 94–99%), respectively. In all cases negative controls showed no positive reaction. These findings demonstrate that the cell type in the samples is ECs nearly exclusively.

3.2. Number of scrapes of the luminal side of rabbit aorta

Different segments (n=6) of rabbit aorta were used to study whether the luminal side of the arterial wall should be scraped once or twice with a razor blade for the isolation of ECs. In the first scrape the cells in the sample were morphologically well and easily counted in a counting chamber, while the cells in the second scrape were disintegrated and almost impossible to count. The number of cells in the samples of scrape 2 was <5% of the number in scrape 1. Sample smears of scrape 2 (n=6) were immunostained against α-actin, CD31, and thrombomodulin. It was estimated visually that 20–50% of the cells in the samples of scrape 2 were smooth muscle cells. Based on these observations it was decided to scrape the luminal aorta only once.

3.3. Effect of different endothelial cell preparation procedures on eNOS activity

Eight of the male rabbits were used to study whether the eNOS activity was affected by the period from killing the rabbit to freezing the sample in liquid nitrogen. During the preparation, the aortic segments were kept in ice-cold oxygenated saline. The ECs from four rabbit aortas were isolated as follows: the aortic arch and lower thoracic aorta were scraped after ~25 min, while the upper thoracic and abdominal aorta were scraped after ~65 min. The aortic segments from the remaining four rabbits were scraped in reverse order with respect to time. The eNOS activity was the same when the preparation times were 23±1 min (range 18–29 min) and 66±1 min (range 56–72 min), respectively (P>0.3 by two-way ANOVA with no interaction, data not shown).

To study the effect on eNOS activity when the aorta was kept in oxygenated or non-oxygenated saline on ice during the cell preparation we used four male rabbits. The procedure of this study was the same as in the time study. The eNOS activity was not affected by oxygenation of the saline during the cell preparation (P>0.8 by two-way ANOVA with no interaction, data not shown).

Finally, four male rabbits were used to study the effect on eNOS activity when the aorta was kept in ice-cold saline or in saline at room temperature during the cell isolation. Again the procedure of the time study was used. The temperature of the saline during cell preparation did not affect the eNOS activity (P>0.5 by two-way ANOVA with no interaction, data not shown).

These findings suggest that the eNOS activity is relatively independent of the exogenous environment within the first hour. It was therefore decided to isolate the ECs within 1 h and to keep the aortas in non-oxygenated saline at room temperature during the preparation. However, it can not be excluded that the eNOS activity in vivo differs from that measured ex vivo.

3.4. Regional variation in aortic eNOS activity

Fig. 1 shows the regional variation in aortic eNOS activity in the male (n=16) and female (n=8) rabbits. The
Fig. 1. The regional variation in aortic eNOS activity of normocholesterolemic male (clear bars, n=16) and female (black bars, n=8) rabbits. The eNOS activity increased significantly with increasing distance from the heart in both male and female rabbits (P<0.0001 and P<0.05, respectively, by repeated measures ANOVA). All values are given as mean±S.E.M. *Thorax, upper thorax; **Thorax, lower thorax. * P<0.05, ** P<0.01, and *** P<0.001 by multiple comparison test.

male aortas were divided into four consecutive segments: the aortic arch, the upper thoracic aorta, the lower thoracic aorta, and the abdominal aorta. The female aortas, however, were only divided into three segments: the aortic arch, the thoracic aorta, and the abdominal aorta. The eNOS activity increased with ~50% from the aortic arch to the abdominal aorta in both male and female rabbits (P<0.0001 and P<0.05, respectively, by repeated measures ANOVA). In the male rabbits, the increase was significant by multiple comparison when the aortic arch was compared with the upper thoracic (P<0.01), lower thoracic (P<0.001), and abdominal (P<0.001) aorta, respectively, and when the upper thoracic aorta was compared with the abdominal aorta (P<0.05).

The aortic eNOS activity was higher in the female than in the male rabbits. However, since the male and the female rabbits were killed with an intervening period of 6 months, this sex difference in eNOS activity should be interpreted with due respect for the possibility of a time effect.

3.5. Effect on aortic eNOS activity after long-term estrogen treatment

Two weeks after ovariectomy all rabbits thrived well and were divided into one estrogen group (n=7) and one vehicle group (n=7) with similar baseline values of body weight. No difference in final body weight was found between the treatment groups, however, the estrogen treated animals had a ~10-fold increase in uteri weight (P<0.001 by Mann–Whitney U-test, data not shown). Two samples from the upper thoracic aorta of the estrogen rabbits were rejected due to a cell number below 10×10³ for the citrulline assay.

The eNOS activity in the aortic arch, and the upper and lower thoracic aorta of rabbits treated with either estrogen or vehicle is shown in Fig. 2 (corresponds to the first three segments of the male rabbits). Interaction was found and thus treatment does not have the same effect on eNOS activity in all aortic regions (P<0.05 by two-way ANOVA). The eNOS activity was higher in the aortic arch and the upper thoracic aorta of the estrogen rabbits than in the corresponding regions of the vehicle rabbits (P<0.05 by Mann–Whitney U-test), however, in the lower thoracic aorta it was the same.

In the vehicle rabbits, the eNOS activity increased with increasing distance from the heart (P<0.05 by repeated measures ANOVA). By multiple comparison the increase was significant when the aortic arch was compared with the lower thoracic aorta (P<0.05). In the estrogen rabbits, however, the eNOS activity decreased between the upper and lower thoracic aorta to the same level as in the aortic arch (P<0.05 by multiple comparison between consecutive regions).
in rabbits before cholesterol feeding [18]. In the present study, three rabbit groups out of four, studied consecutively within 10 months, demonstrated that the eNOS activity in normocholesterolemic rabbits is significantly higher in the downstream regions of aorta compared with the regions located more upstream. The results support the hypothesis that a high endothelial NO production precedes resistance to aortic cholesterol accumulation during a subsequent hypercholesterolemic period.

Since the eNOS activity of the present study was measured under optimal conditions, the regional variation in eNOS activity in our study is probably due to a regional variation in the amount of active enzyme. Shear stress is directly linked to blood flow and blood vessel diameter; it is high when the blood flow is high and can be reduced by increasing the vessel diameter [20]. Interestingly, rat aortas with chronic high blood flow and cultured ECs exposed to arterial levels of shear stress had elevated levels of eNOS mRNA and protein expression [21,22]. Thus, the regional variation in aortic eNOS activity of the present study may in part be due to changes in eNOS mRNA and protein expression caused by the continuous activation of the endothelium by the regional variation in shear stress. In support of this notion, Tsao et al. [23] proposed that the preferential distribution of atherosclerotic plaque in areas of low shear stress is partly explained by reduced NO production at these sites.

The pattern of aortic cholesterol deposition may reflect variations in the uptake of plasma macromolecules by the aortic wall. This notion is supported by a rabbit study, demonstrating a close positive association between the permeability to low density lipoprotein (LDL) of a given aortic segment in normocholesterolemic rabbits and the cholesterol accumulation in that same aortic segment after cholesterol feeding [18]. The regional eNOS activity pattern in the present study was the opposite of the regional variation pattern in aortic permeability to LDL in normocholesterolemic rabbits found in the previous study [18]. This suggests that increased eNOS activity in the endothelium is present in areas with low macromolecular permeability, and that low eNOS activity is present in areas with high permeability. A connection between NO production and macromolecular permeability is supported by a study of cultured aortic ECs in which inhibition of NOS activity increased the macromolecular permeability [24]. One plausible explanation for the association between low macromolecular permeability and high endothelial production of NO is that high NO production by an autocrine dilatory effect tightens the endothelial cell layer followed by a decrease in endothelial permeability compared with a situation in which the NO production is low leading to a relative cell contraction with a widening of the intercellular macromolecular pathways [25].

In the present study, treatment with the female sex hormone estrogen significantly increased the eNOS activity in the aortic arch and the upper, but not in lower, thoracic aorta of the normocholesterolemic, ovariectomized rabbits treated with estrogen (chequered bars, *n* = 7) or vehicle (striped bars, *n* = 7). In the estrogen rabbits, the eNOS activity was higher in the aortic arch and upper thoracic aorta when compared with the corresponding regions of the vehicle rabbits (*P* < 0.05 by Mann–Whitney *U*-test), however, in the lower thoracic aorta it was the same. The eNOS activity increased with increasing distance from the heart in the vehicle rabbits (*P* < 0.05 by repeated measures ANOVA). However, in the estrogen rabbits the eNOS activity increased between the aortic arch and the upper thoracic aorta and then surprisingly decreased between the upper and lower thoracic aorta. All values are given as mean±S.E.M. uThorax, upper thorax; lThorax, lower thorax. *P* < 0.05 by multiple comparison test.

4. Discussion

We have established a method sensitive enough to measure the eNOS activity in FIECs removed from the surface of different segments of rabbit aorta. With this method we demonstrate: (1) that relatively atherosclerosis-resistant regions of thoracic and abdominal aorta in normocholesterolemic rabbits have a significantly higher eNOS activity than the more atherosclerosis-prone aortic arch before atherosclerosis develops, and (2) that the eNOS activity in the aortic arch and upper, but not in lower, thoracic aorta is significantly increased by long-term treatment with 17β-estradiol compared with vehicle in normocholesterolemic, ovariectomized rabbits.

It has previously been shown that aortic cholesterol accumulation decreases with increasing distance from the heart after a period of hypercholesterolemia, whereas there is no regional variation in aortic cholesterol accumulation
aorta of normocholesterolemic, ovariectomized rabbits. The effect of estrogen on eNOS activity is in accordance with previous findings that the eNOS activity was enhanced in several tissues (including the heart and the uterine artery) and in the aorta of estrogen treated and pregnant guinea pigs and rats, respectively [26,27]. Several studies in hypercholesterolemic rabbits have shown a direct antiatherogenic effect of estrogen not mediated by the plasma cholesterol level [7,8]. We propose that the increased eNOS activity in aorta of normocholesterolemic, ovariectomized rabbits treated with estrogen attenuates the cholesterol accumulation in the arterial wall during a subsequent hypercholesterolemic period. This hypothesis is supported by the observation that treatment with L-NAME attenuated the antiatherogenic effect of estrogen in cholesterol-clamped rabbits maintained at the same hypercholesterolemic level for 13 weeks [8].

Surprisingly, the eNOS activity in the estrogen rabbits decreased significantly between the upper and lower thoracic aorta to the same level as that found in the aortic arch of the vehicle rabbits. This finding does not agree with the regional variation in aortic cholesterol accumulation in cholesterol-clamped, ovariectomized rabbits treated with estrogen [7,8]. In that study, the cholesterol accumulation was high in the aortic arch and lower in both upper and lower thoracic aorta.

In conclusion, we have established a method sensitive enough to measure the eNOS activity in FIECs from different segments of the rabbit aorta. With this method we demonstrate that in normocholesterolemic rabbits: (1) the eNOS activity in the atherosclerosis resistant thoracic and abdominal regions of the aorta was significantly higher than in the more atherosclerosis prone aortic arch and (2) the eNOS activity was significantly higher in the aortic arch and upper thoracic aorta of estrogen-treated rabbits compared with rabbits given vehicle alone. The findings suggest that a high endothelial production of NO in the arterial wall precedes a low cholesterol accumulation during a subsequent period of hypercholesterolemia, and thus add support to the notion that low endothelial NO production facilitates the development of atherosclerosis.

Acknowledgements

This study was supported by grants from The Danish Heart Foundation (97-1-2-18-22452), The Clinical Institute (Odense University), The King Christian X Foundation, The Danish Medical Association Research Fund, The A.P. Møller and Chastine McKinney Møller Foundation, and The Carpenter Alfred Andersen and Wife’s Foundation. S. Stender was a Research Professor of The Danish Heart Foundation. We thank B. Nielsen and D.L. Bartlien for technical assistance, the Biomedical Laboratory (Odense University) for housing and handling the rabbits, and the veterinarians at Danish Crown for providing slaughter pig aortas.

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