THE USE OF ENOS MRNA EXPRESSION FROM ISOLATED ENDOTHELIAL CELLS FOR THE DETECTION OF CARDIOVASCULAR DISEASE RISK

by

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INTRODUCTION

Cardiovascular disease (CVD) is the primary cause of death and disability in all industrialized countries (1), and is a rising cause of death in developing countries. According to the American Heart Association, 2150 Americans die of CVD each day, which averages to 1 death every 40 seconds. A variety of factors have been linked to cardiovascular disease including tobacco use, physical inactivity, poor diet, obesity, family history and genetics, high blood cholesterol, and high blood pressure. Because of the high death rate of CVD, detecting risk factors that could lead to earlier detection and prevention of further progression is a high priority. The specific sequence of events leading to atherosclerosis has not yet been determined, however, clinically, atherosclerosis is known to be preceded by impaired vascular endothelium, the thin layer of cells that lines the lumen of blood vessels.

REVIEW OF LITERATURE

Dysfunctional endothelial cells do not synthesize and release adequate amounts of the powerful vasodilator substance, nitric oxide. As a result, critical vessels such as the coronary arteries that supply blood to the heart do not expand adequately during times of heavy demand; thus, heart tissue is starved for oxygen, leading to cell death in extreme situations (3). Measurement of endothelial function may provide a useful tool in CVD research for several reasons, one being that endothelial dysfunction often precedes CVD, and second, many interventions known to reduce CVD risk also improve endothelial function (3). The purpose of this research is to develop the technique of harvesting endothelial cells from human vasculature and measuring endothelial nitric oxide synthase

(eNOS) mRNA in these cells in order to determine their level of function and therefore CVD risk.

Role of Endothelium in Coronary Artery Regulation

For many years the endothelium was believed to be an inert barrier of cells that lined the blood vessels. This complex organ is difficult to study, because, unlike red blood cells, endothelial cells compose the inner lining of the blood vessels, making them hard to access. Researchers have discovered, however, that the endothelium is a highly reactive organ that synthesizes and releases various substances in response to hormones, drugs, physical, and chemical stimuli. These substances serve to maintain vascular tone and permeability, the inflammatory state, and hemostasis (12,13). The endothelium acts as a major regulator in local vasodilation and vasoconstriction, as well as prevention and stimulation of aggregation of blood platelets.

The endothelium reacts to mechanical stimuli, such as shear stress, by releasing substances that regulate vasodilation and vasoconstriction to maintain vascular equilibrium (4). One of these substances, NO, is synthesized in the endothelial cells from the amino acid L-arginine through endothelial nitric oxide synthase (eNOS) (5). The levels of eNOS in the endothelium vary among different people and can influence the effectiveness of the endothelium. Once synthesized, NO diffuses to the adjacent smooth muscle and interacts with guanylate cyclase (sGC), a receptor molecule that controls vessel tone and smooth muscle proliferation (6). Through a series of complex molecular reactions, cyclic guanylmonophosophate (cGMP) is produced and causes an influx of intracellular calcium, which causes smooth muscle relaxation and vasodilation of the vessel (7).

Dysfunction of Endothelial Cells

Endothelial dysfunction is characteristic of patients with atherosclerotic disease as well as patients who have had a cardiovascular event (6). Dysfunction of the NO-cGMP pathway occurs before the development of atherosclerosis and is believed to contribute to the development of the disease. There are several mechanisms underlying endothelial dysfunction; however, it is largely due to increased production of reactive oxygen species such as superoxide, which will decrease bioavailability of NO (6). In the absence of NO, platelets are more prone to adhere, aggregate, and release platelet-derived growth factor. Leukocytes also adhere, which promotes chronic inflammation and stimulates growth of underlying cells. When this happens, more superoxide is available to oxidize LDL, leaving the blood vessel prone to constriction (8).

Role of Gender in Endothelium

Women tend to experience lower rates of CVD than age matched men. Researchers have linked this cardioprotective effect in premenopausal women to the higher levels of estrogen. Estrogen produces an increase in eNOS activity, which can cause greater arterial wall smooth muscle dilation (13). Grodstein *et al* (14) compared cardiovascular disease risk in postmenopausal women who took estrogen with progestin to those who took estrogen alone and those who did not use hormone therapy. They found a marked decrease in the risk of major coronary heart disease in women who took estrogen with progestin (14). This indicates that not only do young women have a protective effect, via greater eNOS, over men, but those women taking estrogen birth control or hormone replacement therapy may have an even greater advantage over women who do not.

Measurement of Endothelial Dysfunction

There are numerous ways to measure endothelial function. The most invasive method for testing endothelial function is intracoronary infusion of acetylcholine (ACH). In this method, ACH is injected into the coronary artery and the diameter of the coronary blood vessel and blood flow are subsequently measured (6). Ludmer *et al* (9) used this method in 18 patients with varying degrees of CVD. Eight subjects had advanced coronary stenosis, meaning greater than 50 percent narrowing, four subjects had normal coronary arteries, and six subjects had mild coronary atherosclerosis, or 20 percent narrowing. All four subjects with healthy coronary arteries experienced coronary vasodilation in response to ACH infusion. In contrast, 13 of the 14 subjects with mild to severe atherosclerotic coronary arteries experienced vasoconstriction. Ludmer *et al* (9) concluded that vasoconstriction in response to ACH occurs even in the early stages of coronary atherosclerosis. Intracoronary infusion of ACH is useful in determining normal or abnormal vascular responses. Abnormal vascular responses suggest a defect in the endothelial cells, possibly due to lack of available NO.

A Doppler device can also be used to quantify coronary blood flow and diameter in response to intracoronary infusion of ACH. In this method, a Doppler wire is placed inside the vessel, usually the left anterior descending coronary artery, where it can then analyze peak flow velocity and coronary diameter (7). Because of the invasiveness and potential for complications, the Doppler method is not used routinely.

One of the more common methods for measuring endothelial function is flow-mediated dilatation (FMD). In this method, a trained operator uses a high-resolution vascular ultrasound to measure mean basal arterial diameter after inducing shear stress.

Shear stress, such as increased blood flow in the vasculature, signals the endothelial cells to release NO, a vasodilatory substance (11). To induce shear stress, a sphygmomanometer cuff is inflated distal to the point of measurement and blood flow is occluded for a maximum of four minutes. When the cuff is released, the rush of blood acts as a mechanical stimulus to signal release of NO, causing vasodilation (7). Brachial artery diameters and velocities are measured using high-resolution ultrasound (10).

Padilla *et al* (10) used brachial artery FMD to test five different magnitudes of reactive hyperemia-induced shear stress on 20 subjects with moderate cardiovascular risk (MR) and 20 subjects with low cardiovascular risk (LR). They were interested in whether conventional FMD could distinguish between MR and LR. Their results showed that FMD alone did not distinguish between the two levels of disease; however, the dose response profile of FMD:shear stress ratio could be used to distinguish between the two levels of disease. They used their data to make a regression line to analyze the results, which showed greater distinction than FMD alone (10).

Cardiac positron emission tomography (PET) is another method used to measure the coronary flow reserve and coronary endothelial function in response to intravenous pharmacological injections. Magnetic resonance imaging (MRI) measures coronary flow reserve as well, without the radiation exposure of a PET. Although these methods are useful, they only provide evidence for the effects of NO, not quantifiable measurements on the amount of NO in each vessel, which is critical to understanding endothelial function.

There is little literature on invasive methods of measuring endothelial function. Our current project is to develop a technique in which we harvest endothelial cells by

inserting a J-Hook wire into the brachial vein. We will then isolate the endothelial cells and quantify expression of mRNA for eNOS, the enzyme responsible for NO formation. The procedure upon which we are basing our methods was originally developed and Feng *et al* (16), Colombo *et al* (17) and Ezkurza *et al* (18). We will try to improve upon these methods in order to obtain a protocol for subsequent research on endothelial cells.

METHODS

Participants

Participants in this study consisted of 15 healthy male and female participants between the ages of 18 and 44. Based on a self-report questionnaire, participants were moderately active defined by regular exercise at least three times a week for the past six months prior to participation. Participants were free of disease, including cardiovascular disease, diabetes, inflammatory conditions and metabolic disorders. In addition, participants were non-smokers for a minimum of 6 months prior to their participation. Participants were recruited via fliers, word-of-mouth, and electronic postings. They were compensated monetarily for their voluntary participation. Each participant signed an informed consent form before his or her participation. The TCU Institutional Review Board (IRB) approval was obtained to protect the welfare and rights of the individuals who were participants of this research.

Endothelial Cell Harvesting Procedure

Participants came to the lab once for endothelial cell harvesting procedure. The participant lay supine on a training table for 5 minutes prior to beginning the procedure to calm nerves. To begin the procedure, the investigator sterilized the right antecubital space and surroundings with betadine wash and placed a sterile drape over the site, exposing

only the antecubital space. The investigator inserted a 20-guage angiocath in the brachial vein. Following insertion, the investigator inserted a .021-inch diameter J-shaped wire through the angiocath. The J-wire was moved in and out 10 times to extract a small amount of endothelial cells (approximately 100-200) from the vessel wall. This was repeated twice to maximize endothelial cells obtained. Each wire was transferred immediately into a sterile 15 ml conical tube containing dissociation buffer (0.5% bovine serum albumin, 2 mM EDTA and 100 ug/ml heparin in PBS, pH 7.4) kept at 4°C. Following each endothelial cell harvest, a two ml blood sample was taken.

Following rinses in the dissociation buffer, Invitrogen Dynabeads™ with CD31 endothelial cell specific antigen were added to the solution and the microtube was incubated for 20 minutes at 2-8 °C with gentle tilting and rotation to ensure the beads did not settle in the tube. The microtube was then placed on a magnet for 2 minutes to gather the bead-bound cells. While the tube was still in the magnet, the supernatant was carefully removed. Investigators continued by adding 1 ml Buffer 1, placing in the tube for 2 min, and removing the supernatant. This step was necessary to obtain a high purity of isolated cells. The whole blood was centrifuged and separated into RBC and plasma.

Isolation of RNA began with lysis of the beads and RBC using 0.25 ml of RBC added to 0.75 ml of TRI Reagent BD. The solution was mixed via shaking and placed at room temperature to allow complete dissociation of nucleoprotein complexes. Next, the lysate was supplemented by 0.2 ml of chloroform per 0.75 ml of TRI Regent BD. The resulting mixture was shaken vigorously for 15 seconds, stored at room temperature for 2-5 minutes, and then centrifuged at 12,000 g for 15 minutes at 4°C. The centrifugation separated the mixture into a lower brownish phenol-chloroform phase, and interphase,

and a clear upper aqueous phase. The sample was then transferred to Qiagen RNeasy column in a 2 ml tube and centrifuged for 15 seconds at 8,000 g and the flow-through discarded. Then, 700 ul Buffer RW1 was added to RNeasy column and the sample was centrifuged again at 8,000 g for 15 seconds and the flow-through discarded. 500 ul Buffer RPE was then added to the column and the sample was centrifuged for another 15 seconds at 8,000 g and the flow-through discarded. Once more, 500 ul Buffer RPE was added to the column, and this time it was centrifuged for 2 min at 8,000 g and the flow-through discarded. Next, the column was placed in a new 2 ml tube and centrifuged at full speed for 1 min. The column was then placed in a 1.5 ml tube and 50 ul RNase-free water was added and the sample was centrifuged for 1 min at 8,000 g. Upon the successful isolation the RNA was spec utilizizing a NanoDrop 1000 for determination of purity with 260/280 ratio and concentration in ng/ul.

The SuperScript VILO cDNA Synthesis Kit was used to generate first-strand cDNA for use in real-time quantitative RT-PCR. First 4 ul of 5X VILO Reaction Mix was combined with 2 ul of 10X SuperScript Enzyme Mix and 14 ul of RNA sample was added to the tube for a total of 20 ul. The tube was incubated in an Eppendorf Mastercyler for cDNA generation with the following cycle parameter beginning at 25°C for 10 minutes. The tube continued to incubate for another 60 minutes at 42°C. To terminate the reaction, the tube was incubated for another 5 minutes at 85°C.

Then, the sample was ready to perform Quantitative PCR using the ABI 7500 Real Time PCR system to detect eNOS mRNA and ACTA2, the endogenous control. We were able to add to same amount of master mix and sample, since some of the samples were diluted prior to the RT reaction. To prepare the master mix, we used Taqman 2X

Universal PCR Master Mix and Taqman 20X Probe/Primer mixes. The master mix and sample were loaded into a 96 well plate. In order to spin down contents and remove any air bubbles, the well plate was centrifuged. The well plate ran in the thermal cycler for 2 minutes at 50°C for AmpErase UNG activation, 10 minutes at 95°C for AmpliTaq Gold Enzyme Activation, 15 seconds at 95°C for PCR (40 cycles) and 1 minute at 60°C for PCR (40 cycles).

RT-PCR, real-time quantitative reverse-transcription polymerase chain reaction, allowed us to examine gene expression, or how much mRNA was in a sample. The target samples showed eNOS expression and our endogenous control showed ACTA2 expression. RT-PCR uses fluorescence readings in each well by the specialized thermal cycler unit. It takes multiple measures during the exponential portion of the DNA amplification curve, instead of only one final measurement, to increase accuracy. To analyze the readings from the RT-PCR, we looked at the cycle in which expression reaches the threshold. Samples with more mRNA hit the threshold at an earlier cycle.

In order to make comparisons about our data, we used the 2^ -ΔΔCt method of analysis of real-time PCR Data. This method uses relative quantification to describe the change in expression from the target genes to some reference group. In this study, the gene expression was reported using the relative change rather than the absolute transcript copy number. For example, when making comparisons, the fold-change difference was reported between populations. In order for the ΔΔC calculation to be valid, the amplification efficiencies of the target, eNOS, and control, ACTA2, must be approximately equal. The purpose of the internal control is to normalize the PCRs for the amount of RNA added to the reverse transcription reactions.

RESULTS

Due to methodological difficulties, data on endothelial cells (EC) are not available. The data reported below are based on analysis of red blood cells (RBC). The results of the RT-PCR threshold cycles for all subjects for both the target (eNOS) and endogenous control (actin) mRNA are shown in Table 1.

Table 1. Threshold cycle values for all subjects for eNOS and actin mRNA.

Subject	Target eNOS	Endogenous Control actin	Subject Mean	Control Mean
1R1	Undetected	38.286		
1R2	Undetected	38.703	-	-
2R1	Undetected	34.024		
2R1	Undetected	33.794	-	-
3R1	37.126	28.578		
3R2	36.919	27.776	37.023	28.177
4R1	34.228	29.299		
4R2	34.273	29.511	34.251	29.405
5R1	34.750	27.436		
5R2	34.186	27.147	34.468	27.292
6R1	37.490	28.505		
6R2	37.149	28.689	37.320	28.597
8R1	33.708	28.164		
8R2	33.745	28.164	33.727	28.164
11R1	36.745	30.576		
11R2	38.153	31.002	37.449	30.789
12R1	38.626	29.521		
12R2	37.980	28.776	38.303	29.149
13R1	35.539	28.533		
13R2	35.406	32.378	35.473	30.456
14R1	33.814	27.216		
14R2	34.081	27.120	33.948	27.168
15R1	34.474	27.046		
15R2	34.090	27.113	34.282	27.080
Mean			35.624 +/- 1.73	28.627 +/- 1.32
CONT1	29.543	29.689		
CONT2	29.248	undetected		

The results displayed in Table 2 show a comparison of eNOS mRNA expression in males (n=4) versus females (n=6). The females had an expression 3.12 times greater than the expression in males.

Table 2. **Comparison of eNOS mRNA expression in males (blue) and females (pink).**					
Subject	Target (eNOS CT)	ACTA2 (actin CT)	Δ CT	ΔΔCT	Normalized
3	37.023	28.177			
5	34.468	27.292			
6	37.320	28.597			
15	34.282	27.080			
Male Mean	35.773	27.78625	7.98675	0	1
4	34.251	29.405			
8	33.727	28.164			
11	37.449	30.789			
12	38.303	29.149			
13	35.473	30.456			
14	33.948	27.168			
Female Mean	35.5248	29.1806	6.3442	-1.6426	**3.122**

The results displayed in Table 3 are a comparison of eNOS mRNA expression levels in females not taking estrogen-based oral contraceptives (n=2) vs. females taking ethinyl estradiol and norgestimate (n = 4). Women taking the estrogen-based birth control had 3.5 times greater eNOS mRNA expression than those women not taking birth control.

Table 3. **Comparison of eNOS mRNA expression in females on birth control versus not on birth control**					
Subject	**Target (eNOS CT)**	**ACTA2 (actin CT)**	**Δ CT**	**ΔΔCT**	**Normalized**
12	38.303	29.149			
8	33.727	28.164			
No BC Mean	36.01475	28.63352	7.3815	0	1
4	34.251	29.405			
13	35.473	30.456			
14	33.948	27.168			
BC Mean	34.55683	29.0095	5.54733	-1.83417	**3.5656**

The results displayed in Table 4 show a comparison of eNOS mRNA expression in males (n = 4) vs. females not taking birth control (n = 2). This comparison produced an eNOS mRNA expression 1.52 times greater in this group of females compared to the males.

Table 4. **Comparison of eNOS mRNA expression in males (n=4) versus females not taking BC (n=2)**

Subject	Target (eNOS CT)	ACTA2 (actin CT)	Δ CT	ΔΔCT	Normalized
3	37.023	28.177			
5	34.468	27.292			
6	37.320	28.597			
15	34.282	27.080			
Male Mean	35.773	27.78625	7.98675	0	1
12	38.303	29.149			
8	33.727	28.164			
Female No BC Mean	36.01475	28.63352	7.3815	-0.60525	**1.52124**

DISCUSSION

The developed protocol yielded results showing quantification of eNOS mRNA from RBC and EC; however, the results from EC were unusable. The first problem was with the isolation and purification of EC. Since the J-wire was in constant contact with blood, it is possible that RBCs were not fully washed off the J-wire. The purification process is still being developed; thus, future procedures will consist of isolating EC directly from the J-wire after washing RBC away. The second problem was the fact that RBC expresses the same eNOS as EC and we were unable to confirm whether the eNOS mRNA expression from the EC samples were solely from EC. To overcome this problem we will test for hemoglobin-beta (HBB) expression, which is only found in RBC. When we run RT-PCR, we will target eNOS and ACTA2, which are both found in RBC and EC, and HBB, which is only found in RBC. If we see amplification of eNOS and ACTA2, but not in HBB, it will confirm that our sample is EC. On the other hand, if HBB expression appears, further methodology will have to be developed, possibly using an antibody other than one specific to the CD31 antigen to detect EC.

Another consideration in this study is that we harvested EC and RBC from the venous circulation, when arterial endothelium is really where we want to assess endothelial function because dysfunction in arteries carrying oxygenated blood to the tissues is the basis for increased risk of CVD. Had we harvested from arterial circulation, expression of eNOS mRNA may have been greater than expression of eNOS mRNA in the venous circulation. Cicinelli *et al* (19) compared the different plasma levels of NO metabolites, the more stable form of NO after it has broken down, in arterial and venous blood plasma. Results showed nearly twice the NO metabolite level in arterial plasma compared to venous plasma. This suggests that endothelial release of NO is different in *in vivo* arteries and veins (19), which justifies reasoning that there may be higher eNOS mRNA levels in arterial vessels than venous vessels.

The comparisons that we were able to make regarding gender and birth control remain consistent with previous research on the topic. Estrogen produces an increase in eNOS activity, which can cause greater arterial wall smooth muscle dilation (13). Our results showed women to have a 3-fold greater expression of eNOS mRNA than men. Our results showed women taking estrogen-based birth control to have a 3.5-fold increase in expression of eNOS mRNA compared to women not taking estrogen-based oral contraceptives. Another comparison showed that females not taking oral contraceptives had a 1.52-fold increase in expression of eNOS mRNA compared to men. This comparison highlights the difference in estrogen levels in men and women. We had a very low sample of women not taking birth control, so these results are not definitive. We did not collect data from the women indicating the date of their last menstrual cycle, which would indicate which phase of the menstrual cycle they were in at the time of

harvesting. Women's estrogen levels are highest during ovulation. If our participants were in the ovulation phase of the cycle, the fold difference would likely have been higher. The cardioprotective effect of estrogen is greater than the short-term vasodilating effects. Pre-menopausal women have a reduced risk for mortality from cardiovascular disease. One of the changes menopause causes is a significant decrease in estrogen levels. Post-menopausal women have similar or even increased risk for cardiovascular disease compared to men.

Assuming successful outcomes from the previous mentioned changes to the protocol, this methodological research allows for future comparisons of different interventions and the impact on eNOS mRNA expression. Interventions include exercise, diet, medicines, etc. The J-hook technique could be used before and after a bout of exercise to determine what impact and the magnitude of the impact exercise had on eNOS expression. This could be repeated using different types of exercise such as high-intensity vs. low-intensity or endurance-based vs. strength-based exercise. Exercise training has been shown in animal studies to increase endothelial vasodilation via NO. We would expect that after a single bout of exercise, eNOS mRNA expression would increase. In fact, studies have shown that endothelial function improves after one bout of exercise and can even counteract the negative effects that a high fat meal have on endothelial function (20). These findings suggest that exercise could be used as a daily prescription in people with cardiovascular disease to improve their endothelial function as well as offset short-term damage to endothelial function from dietary intake.

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ABSTRACT

Cardiovascular disease is associated with endothelial dysfunction, a condition that results in the reduction of the vasodilator, nitric oxide (NO). Due to the difficulty in conducting *in vivo* analysis of EC function, previous research has focused on measuring the vascular endothelial (EC) and red blood cell (RBC) eNOS (or its mRNA) responsible for NO production primarily using animal models; however, harvesting of EC from human vasculature is possible. The purpose of this study was to determine if eNOS mRNA could be measured in EC taken from human veins. METHOD: Fifteen healthy males (n=8) and females (n=7) between the ages of 18 and 30 were cannulated in an antecubital vein, EC were harvested, and whole blood was drawn for RBC. The EC and RBC were placed into Trizol and mRNA isolated, followed by two step RT-PCR and analysis using the ΔΔCt method. RESULTS: Expression of eNOS mRNA in RBCs was detectable and was 3-fold greater in females compared to the males. The expression of eNOS mRNA in RBCs of the females using estrogen-based birth control (n = 3) was 3.5 fold greater than females not taking birth control. CONCLUSION: eNOS mRNA can be measured in EC taken from human veins.