Expression of inducible nitric oxide synthase gene in diabetic and non-diabetic coronary artery disease patients

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Abstract:

Background: Little is known about the role of inducible nitric oxide synthase (iNOS) in atherosclerosis, coronary artery disease (CAD) and diabetes mellitus. The aim of this study was to evaluate serum level of iNOS and its mRNA expression in patients with CAD and diabetes mellitus.

Patients and Methods: The study was conducted on 42 CAD patients (28 with type II diabetes mellitus & 14 non-diabetics), mean age 58.35±5.38 years. Diagnosis of CAD was established by presence of characteristic chest pain, ECG changes, exercise tread mill test or dobutamine stress test echocardiography. Twenty healthy subjects of matched age were included as a control group. All subjected to through history taking, clinical examination, E.C.G., echocardiography, laboratory investigations as blood sugar, lipid profiles, measurement of iNOS serum level by enzyme immunosorbent assay and detection of iNOS mRNA expression using reverse transcription-polymerase chain reaction.

Results: The level of iNOS was significantly higher in patients group compared with control group (25.18±5.6 versus 7.61±1.55 IU/ml, P<0.0001) and in diabetics versus non diabetic patients (28.48±5.6 versus 16.45±5.62 IU/ml, P<0.0001). Also, iNOS mRNA expression was found to be positive in 54.8% of all patient group and 71.4% of diabetic patients compared to 21.4% of non-diabetics patients. The activity of iNOS and its expression increase significantly within the patients group with increase age and presence of dyslipidemia, but ejection fraction was lower in patients with iNOS mRNA expression than patient without. Positive correlation was detected between serum iNOS and total cholesterol, LDL cholesterol, triglycerides, systolic, diastolic and mean blood pressure, but negative correlation with ejection fraction was detected.

Conclusion: This study has demonstrated that, stimulated expression of iNOS gene and higher serum levels of iNOS is associated with CAD and/or diabetes mellitus. The use of iNOS gene transfer or antisense technology aiming at inhibiting the expression of iNOS may be beneficial therapeutic value in these conditions.

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Key words: Inducible nitric oxide synthase – Coronary artery disease – Diabetes mellitus.

Introduction:

Nitric oxide (NO) is an important cellular signaling molecule synthesized by enzyme nitric oxide synthase (NOS) [1]. There are three distinct isoforms of NOS: neural NOS-1 (nNOS), inducible NOS-2 (iNOS) and endothelial NOS-3 (eNOS) [2]. These three isoforms were discovered in the above order from 1991-1994 [3]. Each NOS isoform is transcribed from a separate gene. The genes encoding eNOS, nNOS and iNOS are located on chromosome 7, 12 and 17 respectively [4].

All three NOS isoforms play distinct roles in the regulation of vascular tone. Whereas, eNOS and nNOS are normal constituents of healthy cells, iNOS is not usually expressed in undiseased vascular tissue and its expression is seen mainly in conditions of infection or inflammation [2,5].

Also, the constitutive isoforms (nNOS & eNOS) are calcium dependant and regulated e.g. by shear stress, while inducible isoforms can be rapidly induced by microbial endotoxins or cytokines stimulation and its activity does not vary with intracellular calcium concentration [6].

Atherosclerosis accounts for half of the morbidity and mortality in western countries [7]. Also, cardiovascular disease accounts for at least 85 percent of deaths of patients with type II diabetes mellitus. Additionally, 75 percent of these deaths are due to ischemic heart disease [8]. Because NO inhibits many key steps in the atherogenic process (for example, Platelet adhesion and aggregation, adhesion molecules

and chemokine expression, inflammatory cell infiltration and smooth muscle cell migration and proliferation) [9,10]. The early NO deficit may facilitate atherogenic progression. Although, the endothelial NO pathway appears to be involved in atherosclerosis [11]. Little is known about the role of iNOS in human atherosclerosis, coronary heart disease and diabetes mellitus.

Aim of the Work:

This study was designed to detect iNOS mRNA expression and iNOS serum levels in patients with coronary heart disease and type II diabetes mellitus.

Patients and methods:

The present study was conducted on 42 patients with coronary heart disease (26 male and 16 female) selected from patients attending Cardiology Outpatient Clinic of Specialized Medical Hospital, Mansoura University, before admission for coronary angiography. Documentation of coronary heart disease (infarction or angina pectoris) was based on history of characteristic chest pain, characteristic E.C.G. changes, exercise tread mill test or dobutamine stress test echocardiography. Twenty eight patients had type II diabetes mellitus while, 14 patients were non diabetic. Their age ranged from 48 – 69 years.

The study also, included 20 healthy subjects (12 male and 8 female) of matched age as a control group. Their age ranged from 48-63 years.

Exclusion criteria include the following:

Patients with heart failure, previous stroke, liver, renal or other systemic diseases, collagen disease, transplant patients and smoking.

Written informed consents were taken from all selected subjects (patients and controls). The stydy protocol was approved by local ethics committee of the hospital.

All selected subjects in the study were subjected to:

- Careful history taking.
- Through clinical examination.
- Resting 12 leads E.C.G. at speed 25mm/sec.
- Echocardigraphic evaluation of heart especially left ventricular function.
- Laboratory investigations e.g. blood sugar, lipid profile, liver and renal function tests, and serum level of iNOS as well as detection of iNOS mRNA expression.

Peripheral venous blood samples were obtained from all patients and controls in the morning after 12 hours overnight fasting. Each blood sample was divided into 2 tubes, one tube is left for coagulation for 30 minutes then centrifuged at 3000 rpm for 15minutes to separate serum which was immediately aliquoted and stored at -20°C until performing the biochemical tests. The other part was put in EDTA-K3 tubes and stored at -20 °C until RNA extraction.

Determination of inducible nitric oxide synthase levels:

Serum concentrations of iNOS were measured using a commercially available enzymes linked immunosorbent assay kit (Biozol Diagnostica Vertrieb GmbH, Loxo GmbH, Germany). The method of measurement was carried out according to the manufacturer instructions [12]. The data were expressed as IU/ml. Detection range, 0.156-60 IU/ml, while, sensitivity was 0.057 IU/ml.

Determination of iNOS mRNA:

RNA extraction:

- Total RNA was extracted from leukocytes of whole blood sample after lysis of red blood cells, using Promega Corporation SV total RNA isolation kit (Madison, USA), according to the method of Otto [13]. RNA was quantified spectrophotometrically at 260 nm and stored at - 70°C until assay. Two microliters of the extracted RNA was revers transcribed to complementary DNA (cDNA).

RT-PCR and analysis of the products:

- Access RT-PCR kit supplied by Promega , USA, was used according to method of Miller and Storts [14].
- RT-PCR was carried out on total RNA isolated from leukocytes of different groups studied.
- All primers were synthesized at the BioSource Europe laboratories, Belgium. The sequences of the two primers used, as designed by Bonmann et al., [15] are shown as follow:

Sense primer: 5'- ATTCAGGTACGCTGTGTTTGG-3'.

Anti-sense primer: 5'- CATGGTGAACACGTTCTTG G-3.

- In a total reaction volume 50µl, containing 0.5 umol/ of each primer, AMV/TFI reaction buffer, 2 mmol/l $Mgcl_2$, 0.2 mmol/l dNTPs, 0.1 unit AMV reverse transcriptase and 0.1 unit TFI DNA polymerase. The mixture was overlayed with 50 µl, on of mineral oil and then RT-PCR was performed in a programmable thermal minicycler.
- The cycle conditions were as follow: 45 min at 48°C and 2 min at 95°C for RT-reaction followed by 40 cycles of 30 sec at 94°C (denaturation), 1 min at 62°C (annealing) and lastly 2 min at 72°C (extension). Then one cycle 7 min at 68°C for final extension. Then the samples were rapidly cooled to 40°C.

- The solution containing the PCR product (9µl) was mixed with 1 µl, of loading dye (0.1 Bromophenol blue and 30% glycerol in water) and loaded into agarose gel containing 0.5 ug/ml ethidium bromide in 1 X TBE buffer. The samplers were run in 1 X TBE buffer for 30 min at 140V in a mini-gel apparatus. A DNA marker VIII (Boehringer manheim) was run in parallel as size marker. The specificity of the amplified bands was validated by their predicted size. The resulting bands were visualized by UV-light and photographed **(Figure 1).**

Statistical analysis:

Statistical analysis was done by using SPSS statistical pachage for social science program version 10, 1999. The data were parametric by using kolmogrov smirnov test. The quantitative data were presented in the form of mean and standard deviation. Student t test was used as a test of significance for two groups. The qualitative data were presented in the form of number and percentage. Chi-square with Yates correction was used as a test of significance for qualitative data. Overall predictability was calculated for prediction of coronary heart disease. Significance was considered when *P* value less than 0.05.

Results:

Table 1: Showed the clinical and routine laboratory characteristics of CAD patients and controls.

Table 2: Showed inducible nitric oxide synthase levels and mRNA expression in the studied groups. The level of iNOS was found to be significantly higher in the patients group when compared with control group (P<0.0001). Also, iNOS expression was found to be positive in about 54.8% of the patients group.

Moreover, the level of iNOS was found to be significantly higher in diabetic coronary artery disease patients than non-diabetic coronary artery disease group (P=0.0001). Also, iNOS expression was found to be positive in about 71.4% of diabetic patients versus 21.4% of non-diabetic patients **(Table 3 & Figure 2)**.

Table 4: Showed that, the level of iNOS and its mRNA expression increase significantly within the patients group with increase age of the patients (<0.05). Also, patients with dyslipidemia (serum LDL-C >3.37 mmol/l and serum HDL-C <0.90 mmol/l) had a significantly higher serum level of iNOS and more iNOS expression than patients without dyslipidemia **(Table 5)**. Moreover, a statistically significant positive correlation has been detected between serum iNOS levels and serum total cholesterol, LDLc and triglycerides. While, significant negative correlation has been detected between serum iNOS levels and ejection fraction **(Table 6).**

Table 7: Showed that, ejection fraction was significantly lower in patients with iNOS mRNA expression than patients without gene expression (P<0.001).

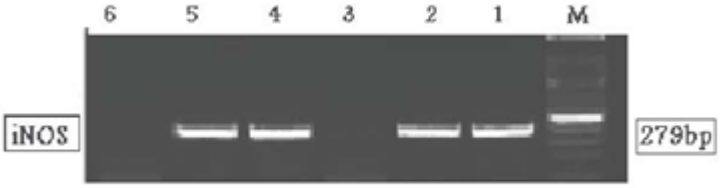

Figure 1: PCR for induced nitric oxide synthase (iNOS) – cDNA:
iNOS specific amplification of cDNA yielded a PCR product of 279 bp in lane 1,2,4,5

Table 1: Characteristics of CAD patients and controls.

Parameter	***CAD-non diabetic N=14***	***CAD- diabetic N=28***	***Control N=20***
Age (years)	58.0 ± 6.52	57 ± 4.98	56.4 ± 5.09
Waist/hip ratio	0.981 ± 0.162	0.961 ± 0.182	0.992 ± 0.231
Body mass index (kg/m²)	28.12 ± 5.87	27.73 ± 4.71	28.93 ± 3.86
Total cholesterol (mmol/l)	5.32 ± 0.44	5.49 ± 0.58	3.98 ± 0.56
Triglycerides (mmol/l)	2.03 ± 0.15	2.31 ± 0.35	0.98 ± 0.12
HDL cholesterol (mmol/l)	0.96 ± 0.03	0.87 ± 0.12	1.3 ± 0.06
LDL cholesterol (mmol/l)	3.40 ± 0.41	3.54 ± 0.17	2.13 ± 0.021
Glucose (mmol/l)	5.33 ± 0.34	6.92 ± 1.32	5.01 ± 0.78

N= Number of cases Data: Mean ± SD

Table 2: Inducible nitric oxide Synthase mRNA expression and serum levels in the studied groups.

	Patients group (n=42)	**Control group** (n=20)	***X²***	***p***
iNOS level (IU/ml)	25.18±5.6	7.61±1.55		0.0001
Gene expression:				
Positive	54.8% (no=23)	0%	15.14	0.0001
Negative	45.2% (no=19)	100% (n=20)		

X²:Chi-square with Yates correction Significant P: <0.05

Table 3: Inducible nitric oxide synthase expression and levels in diabetic versus non-diabetic patients.

	Diabetic patients (n=28)	**Non-diabetic Patients** (n=14)	***X²***	***p***
iNOS level (IU/ml)	28.48±5.6	16.45±5.62		0.0001
Gene expression:				
Positive	71.4% (no=20)	21.4% (no=3)	7.51	0.006
Negative	28.6% (no=8)	78.6% (n=11)		

X²:Chi-square with Yates correction Significant P: <0.05

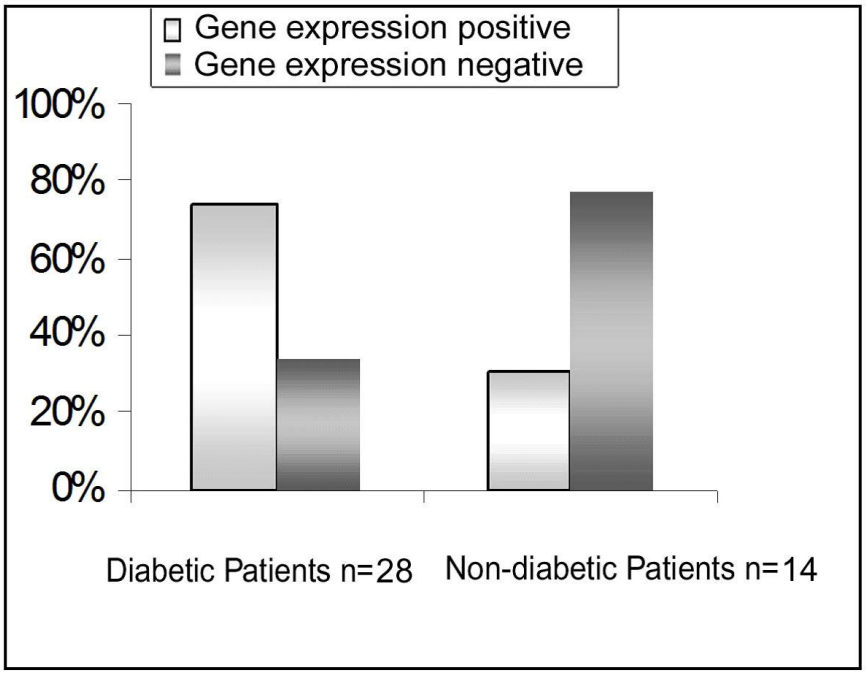

Figure 2: **Inducible nitric oxide synthase *mRNA* expression in the diabetic versus non-diabetic patients.**

Table 4: Effect of age on iNOS levels and iNOS mRNA expression in the patients group.

	Group A (n=12)	**Group B** (n=30)	X^2	*p*
iNOS level (IU/ml)	17.3±8.15	23.42±8.53		0.01
Gene expression:				
Positive	25% (no=3)	66.7%(no=20)	4.44	0.035
Negative	75%(no=9)	33.3% (no=10)		

Group A= Patients < 60 years. Group B= patients ≥ 60 years.

Table 5: Effect of dyslipidemia on iNOS levels and its mRNA expression in the patients group.

	Patients with dyslipidemia (n=28)	**Patients without dyslipidemia** (n=14)	X^2	*p*
iNOS level (IU/ml)	26.17±5.89	19.39±4.22		0.0001
Gene expression:				
Positive	67.8%(no=19)	28.6%(no=4)	4.33	0.037
Negative	32.2% (no=9)	71.4%(no=10)		

X^2:Chi-square with Yates correction Significant P: <0.05

Table 6: Correlation between serum iNOS levels and other parameters for all studied patients.

	r	p
Age	0.28	0.066
EF	-0.39	0.032*
Bl glucose	0.26	0.086
SGOT	0.071	0.64
SGPT	0.091	0.55
Cholesterol	0.58	<0.001***
TG	0.61	<0.001***
HDL-c	-0.091	0.55
LDL-c	0.69	<0.001***
SBP	0.71	<0.001***
DBP	0.42	0.004**
MBP	0.69	<0.001***
HR	0.27	0.075

*= Significant correlation.

Table 7: Study of ejection fraction in patients with and without inducible NOS mRNA expression

	Patients without gene expression (n=19)	**Patients with gene expression** (n=23)	***p***
Ejection fraction	0.58±0.1	0.52±0.13	0.001

Discussion:

The development of atherosclerosis is a dynamic process that results from excessive inflammatory and fibroproliferative responses. In human atherosclerosis, the three isoforms of NOS are present and although strong evidence suggest that eNOS plays an important role in protection of the vessel walls from atherosclerosis [16], the role of iNOS in modulating the development of atherosclerosis remains unclear. So, this study aimed at evaluation of the expression of iNOS in patients with coronary heart disease and diabetes mellitus and detection of its serum levels.

In the present study, the level of iNOS was found to be significantly higher in the patients group when compared with control group. Also, iNOS mRNA expression was found to be positive in about 54.8% of the patients group. These results were in agrement with the results of Valance and Chan (2001) [5]; they reported that iNOS expression is seen mainly in conditions of infection or inflammation and that this inducible isoforms of NOS can be rapidly induced by microbial endotoxins or cytokines stimulation.

Also, expression of iNOS in active atherosclerotic plaques has been detected. It is possible that iNOS contributes to tissue damage or other features of plaque development or stability [1,17].

Excessive or inappropriate NO production by iNOS reacts with superoxide anions in the plaque to yield reactive oxidant species such as peroxynitrite contributing to cytotoxicity and tissue injury. Another possible outcome for NO coming from iNOS could be the activation of matrix metalloproteinase and induction of apoptosis in smooth muscle cells and macrophages [17, 18, 19].

In the present study, the level of iNOS was found to be significantly higher in diabetic coronary artery disease patients than non diabetic coronary artery disease patients. Also, iNOS mRNA expression was found to be positive in about 71.4% of coronary

diabetic patients versus 21.4% of coronary non-diabetic patients. This abnormal finding in diabetes mellitus may be related or modulated by several factors such as the degree of acute hyperglycemia, chronicity of hyperglycemia (disease duration), accumulation of advanced glycosylated end products, insulin concentrations and diabetic complications as autonomic neuropathy and microalbuminuria. Also, abnormality in iNOS, may be related to accelerated atherosclerosis (atherosclerosis) associated with type II diabetes mellitus [20,21]. Regarding gene study, it is possible that gene polymorphism may interact with other environmental conditions with the development of insulin resistance or metabolic syndrome which could progress to prediabetes or overt type II diabetes mellitus [22,23,24].

Hayden and Tyagi (2003) [8] have postulated that type II diabetes mellitus is a vascular disease with dysfunctional NOS enzyme reaction, resulting in endothelial cell dysfunction, atherosclerosis as an early manifestation and hyperglycemia as a later manifestation.

Song et al. (2000) [25] have reported that aging may elevate iNOS protein expression in rat skeletal muscle and that exercise training attenuates this elevation. Also, the present study has revealed that the level of iNOS and iNOS gene expression increase significantly within the coronary artery disease patients with increase age.The same results also reported by Santhanam et al. (2007) [26], they found that the iNOS has a role in the pathophysiology of vascular aging. This increase may be related to increase of nuclear factor kappa B (NF-B) which occurs by increase of cytokines that act directly or by phosphorylation of NF-B, inflammation, injury or immune response [27]. Aging is characterized by increase in susceptibility to injury and inflammation [28].

The present study showed that, patients with dyslipidemia have a higher serum level of iNOS and more iNOS gene expression than patients without dyslipidemia. Moreover, a statistically significant positive correlation has been detected between serum iNOS levels and serum total cholesterol, LDLc and triglycerides.

Peter et al. (2001) have concluded that, genetic deficiency of iNOS decreases diet-induced atherosclerosis and lowers plasma levels of lipoperoxides, a marker for oxidative stress, in Apo E-knockout rates [29]. Thus the presence of iNOS may be a factor in presence and progression of dyslipidemia and atherosclerosis [30]. Also, dyslipidemia has been reported to be associated with endothelial dysfunction and enhanced expression of iNOS with enhanced production of superoxide anion that shares in the formation of powerful oxidant, peroxynitrite that initiates lipid peroxidation in LDLc [31].

In the present study, ejection fraction was found to be significantly lower in patients with iNOS mRNA expression than patients without gene expression. Also, a significant negative correlation has been detected between serum iNOS levels and ejection fraction. Ferreiro et al. (2004) reported up regulation of iNOS expression in patients with heart failure with both ischemic and non ischemic causes, thus increase iNOS is an important element of heart failure syndrome [1].

Also, our study revealed a positive correlation between serum iNOS levels and systolic, diastolic and mean blood pressure. Several studies have demonstrated high iNOS level in patients with hypertension. Impaired endothelial dependant vasodilatation associated with abnormal iNOS may be an important factor in the development and progression of atherosclerosis and hypertension [19]. In addition endothelial dysfunction, in hypertensive patients may initiate vascular inflammation that leads to cytokine-induced activation of inducible NOS which favors the formation of peroxynitrite contributing to cytotoxicity and tissue injury [5,32].

Conclusion:

In conclusion, inducible nitric oxide synthase, the important signaling molecule in conditions of inflammation, atherosclerosis and tissue injury, was found to be higher with more gene expression in patients with coronary heart disease, diabetes, and with increasing age. Also, with the presence of dyslipidemia but its presence was associated with a lower ejection fraction, the marker of left ventricular systolic function of the heart. Recently with the availability of efficient transduction systems for in vivo gene transfer, as well as other methods of gene manipulation, the time is ripe to consider NOS gene therapy.

However, the present study has several limitations. First; we did not confirm the iNOS protein expression with iNOS RNA transcription, second we did not confirm that iNOS protein is an active configurations, capable of catalyzing the L-argenine to NO + L-citrullin. So, further extended study on large number of patients with quantification of iNOS protein and iNOS activity well be done.

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