Chapter-5

The anti-anginal compounds regulate nitric oxide production in endothelial cells in acute coronary syndromes

Introduction

Cardiac pain is one of the distinctive symptoms in case of AMI (Wong *et al.* 2015). It is also known that the development of severe chest pain in AMI is more intense and severe compared to ACS (Goldberg *et al.* 2004). But the mechanism of the development of chest pain is remaining elusive (Gori *et al.* 2012; Setianto *et al* 2010; Sansen *et al.* 2007; Kono *et al*. 2014). We have reported that the decrease level of NO in normal plasma is the cause of cardiac pain (Ghosh *et al.* 2014). And it is known that the organic "nitro" compounds can reduce the pain through the NO (Lovich *et al.* 2015; Tanoue *et al.* 1999). It has been reported earlier that acetyl salicylic acid could be used as an anti-anginal compound through the systemic stimulation of nitric oxide syntheses (Karmohapatra *et al.* 2007). Nitro compound which functions through NO are used to reduce the chest pain, their mechanism of conversion of "nitro" compound to NO remains elusive. As NO is converted to NO_2 in the presence of O_2 in the circulation within 10^{-8} sec, various theories have been proposed. On the other hand, the major source of physiologic NO production has been reported to be occur in endothelial layers (Palmer *et al.* 1987). And the NO is called endothelial derived relaxing factor (EDRF) (Palmer *et al.* 1987). It is perhaps a misnomer and misleading in that endothelial cells, which do not produce NO by itself as there is no basal nitric oxide synthase (NOS) activity in these cells (Hisamoto *et al.* 2001; Förstermann *et al.* 1991). The presence of stimulator for the nitric oxide synthase is essential for the stimulation of NO in the endothelial cells (Hisamoto, *et al.* 2001; Förstermann *et al* 1991). Moreover, NO is not only produced in the endothelial cells, but it is also produced in different cells including erythrocytes, neutrophils and in hepatocytes, as such NO as the EDRF may not be acceptable (Guha *et al*. 2002; Sinha *et al*. 2002, Bhattacharya *et al*. 2013).

Results

The amino acid sequence of the purified pluri activator stimulated endothelial NOS (PLASENOS)

The PLASENOS was purified from the goat carotid arterial endothelial cells to homogeneity gel as demonstrated in the Materials and Methods. The purified protein was electrophoresed many times in the presence and absence of SDS (Fig-5.1a). After, many folds purification the specific enzymatic activity of the protein was found to be very high compared to that of its crude protein solution. This suggested that the degree of purification was acceptable. The end purified product was electrophoresed by PAGE (poly-acryl amide gel electrophoresis) and it was determined that its MW was 57 kDa, which was 69,444 folds purified over the starting homogenate of endothelial cells (Table-5.1). The amino acid sequence of this protein, was carried out at Harvard, Mass Spectrometry and Proteomics Resource Laboratory. The present result suggested that the protein comprised 264 amino acid residues as shown in the figure. The global alignment and multiple sequence alignment with peptide sequences in protein data bank demonstrated that the enzyme was a member of the protein disulfide isomerase gene family (Galligan *et al*., 2012). This enzyme catalyzes and regulates the formation-breakage of disulfide bonds between cysteine residues within proteins. This ensures that proteins would find its proper arrangement and conformation with the disulfide bonds in their fully folded state. In continuation, it will help the protein to perform its optimized functions at its exact folded state.

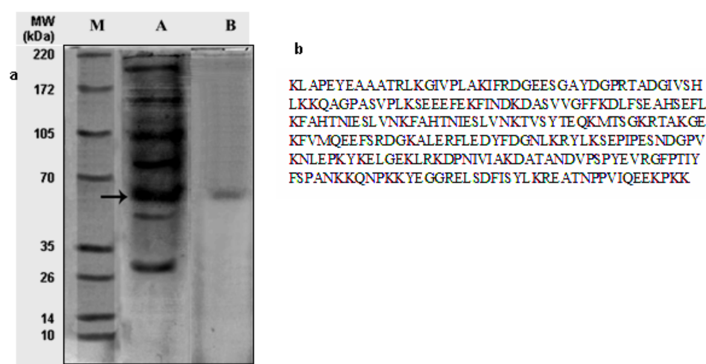

***Figure-5.1a**: Purification of PLASENOS*

PLASENOS was purified from membranous fraction of goat carotid artery endothelial cells at different stages of purification by SDS-PAGE as described in Methods. In the figure, PANEL-A demonstrated the electrophoresis of TritonX-100 treated membrane protein and PANEL-B demonstrated the sliced, triturated and electrophoresis of the 57 kDa band of PANEL-A in the absence of SDS. In each case, the target band was identified by Coomassie brilliant blue. The figure is a typical representative of 10 similar experiments using membrane protein 10 times. The arrow indicates the molecular wt. of PLASENOS protein. M denotes the molecular wt. marker protein.

***Figure-5.1b**: Amino acid sequence of the electrophoretically purified PLASENOS* *After getting the single band from the gel electrophoresis, the protein band i.e. 57 kDa was sequenced and analyzed. Standard one letter abbreviation of the amino acids are: G glycine, P proline, A alanine, V valine, L leucine, I isoleucine, M methionine, C cysteine, F phenylalanine, Y tyrosine, W tryptophan, H histidine, K lysine, R arginine, Q glutamine, N asparagine, E glutamic acid, D aspartic acid, S serine, T threonine*

Table-5.1: Summary of the purification of PLASENOS:

Protein	Concentration of protein (mg/ml)	Specific activity (nmol/mg of protein/hr)	Folds of purification
Crude homogenized endothelial mass	187	0.009	1
Cytosolic fraction	62	0.0885	9.8
0.05% TritonX-100 treated membrane fraction	14	2.214	246
Purified protein after gel electrophoresis in the presence followed by in the absence of SDS	0.08	625	69,444

Table-5.1 *actually explained purification of our target enzyme in terms of nitric oxide synthesis activity [Specific activity (nmol/mg of protein/hr) i.e. Production of nitric oxide/mg of protein] and folds of purification was indicated here by comparing the specific activity of the protein in each stage [the specific activity of the protein in each stage/ground stage (crude homogenized endothelial mass)].*

Endothelial cells were prepared from goat carotid arteries as described in materials and methods section. Cytosolic fraction was isolated by centrifugation at 30,000g, next membrane bound proteins were extracted from Triton X-100 treated (TritonX-100 does not interrupt in the assay) membranous fraction and purified protein was prepared from the membranous fraction by electrophoresis in presence and followed by in the absence of

SDS as described in figure to see the activity of the 57kDa protein for the further studies. Specific activity (NOS activity) of both fractions was determined by synthesis of (nitric oxide) (mg protein)$^{-1}$ present in the fractions.

The properties of PLASENOS

The enzyme was found to be Ca^{2+} dependent for the stimulation of NO production. A certain isoform of NOS is known to be essentially dependent on the presence of calcium for its function. The arterial NOS was found to have no basal enzymatic activity, even in the presence of its substrate *l*-arginine alone (Lamas *et al*., 1992). The enzyme found to be activated in the presence of different stimulators as described. Some of these selected stimulators are clinically used, but their NOS related action was not obvious. As, for example, aspirin is extensively used. The endothelial cells of goat carotid arteries were treated with "nitro" compounds (10µM), aspirin (15µM), insulin (25µUnits/ml) and glucose (0.02M) at different time intervals. The maximum NOS activity (nmol/mg of protein/hr) was found at 30 min for each of the stimulator. Therefore, in each case, different stimulators were required to activate nitric oxide synthase in endothelial cells and it was endothelial derived constitutive nitric oxide synthase.

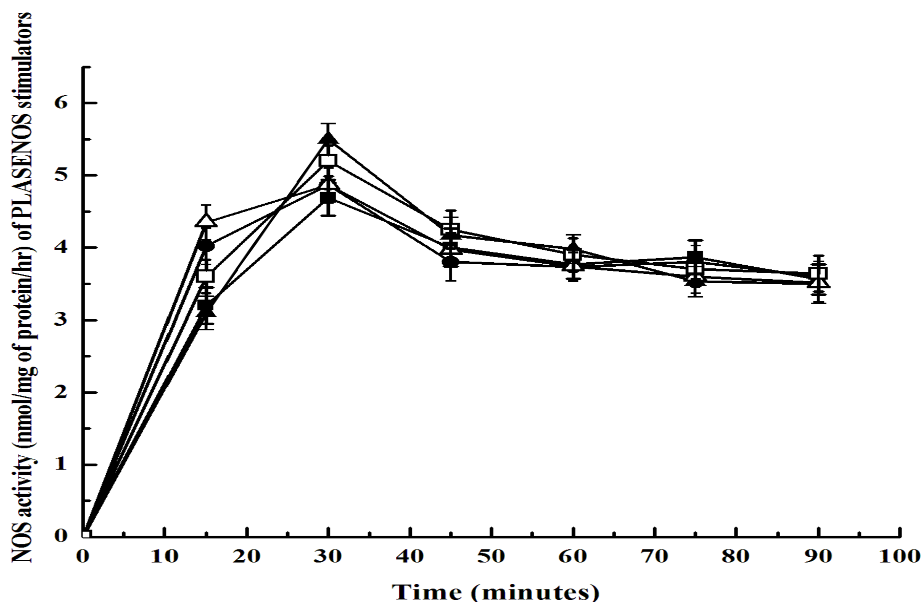

***Figure-5.2**: **NOS activity of different PLASENOS stimulators** This figure demonstrated the NOS activity of purified PLASENOS in presence of different stimulators [nitroglycerine (▲), isosorbide nitrate (□), insulin (Δ), aspirin (●) and glucose (■)]. Endothelial cells from goat carotid arteries were incubated with these stimulators at different time intervals. After incubation NOS activity i.e. the synthesis of NO was performed by methemoglobin method. The result shown here are mean ± SD of 10 different experiments using separately purified PLASENOS for each of the stimulators.*

Activation of PLASENOS by different stimulators of the enzyme

Although the enzyme had no elementary enzyme activity for the NO synthesis even in the presence of *l*-arginine, the arterial cells were procured to be activated by unrelated and different compounds which were demonstrated in the above experiment. This suggests that therapeutic responsiveness at emergent situation might be beneficial and at the normal physiological condition, this NOS activation might not be a prime requirement. This provision of NOS like activation may be regarded as an adaptive response during the

severe pathological conditions. The electrophoretically purified enzyme from each sample (different "nitro" compounds, aspirin or insulin or glucose) was added to the different concentration of *l*-arginine and synthesis of nitric oxide were determined. It was found from the Lineweaver-Burk plot that the purified and activated PLASENOS protein showed V_{max} of 5 nmol NO formed/mg protein/h in all the cases where this protein was activated by different stimulators ((A) Nitroglycerine, B) Isosorbide nitrate, C) Insulin, D) Aspirin, E) Glucose)). It was also noted that the NO synthesis activity of PLASENOS was completely inhibited in the presence of 0.1 mM NAME (N^G-Nitro-L-arginine methyl ester hydrochloride) in the reaction mixture.

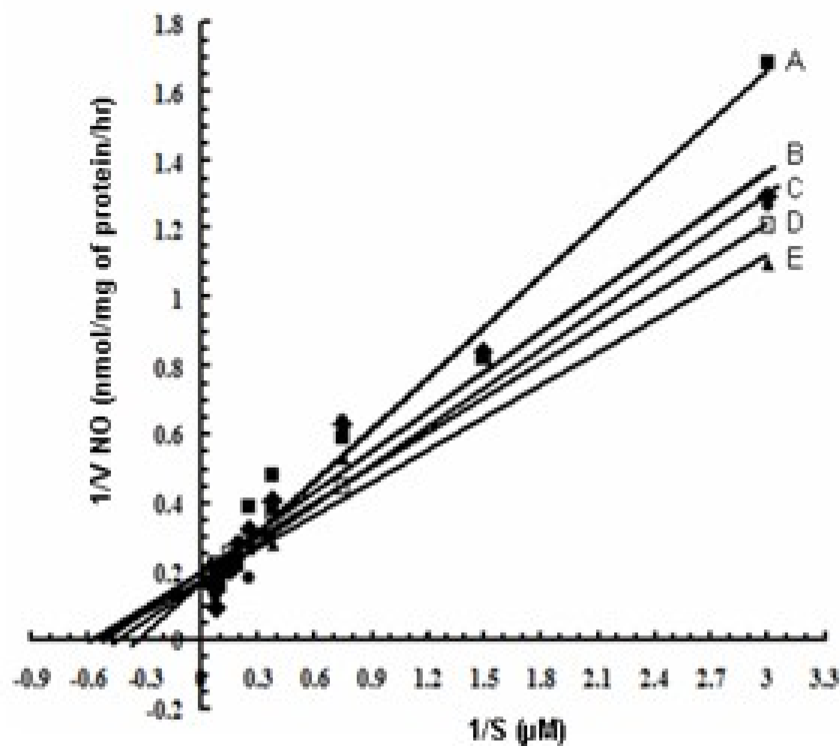

Fig-5.3: K_m and V_{max} analysis of PLASENOS in presence of stimulators

This is a Lineweaver-Burk plot of purified PLASENOS protein in presence of A) Nitroglycerine, B) Isosorbide dinitrate, C) Insulin, D) Aspirin, E) Glucose. The L-B plot

was constructed by adding the different concentration of l-arginine and velocity was the production of nitric oxide at different arginine concentration with the stimulators. From the figure it was found that in all cases V_{max} *was almost same i.e. 5 (nmol/mg of protein/hr). The* K_m *of nitroglycerine, isosorbide dinitrate, insulin, aspirin and glucose were 0.4 μM, 0.47μM, 0.45μM, 0.4μM and 0.5μM respectively (*$K_m \approx 0.5$ *μM).*

Discussion

It was reported that NO is involved in cause of pain and we wanted to identify if any NOS enzyme might be involved in case of chest pain in AMI or ACS. It has also been demonstrated the presence of a distinctive nitric oxide synthase (NOS) protein like activity in carotid-artery endothelial cells of goat, this NOS was actuated by 5 different inducers of the enzyme, including nitroglycerin, isosorbide dinitrate, insulin, aspirin and glucose. These all compounds have been demonstrated to control anginal pain through the synthesis and activation of NO. It is reported that acetyl salicylic acid (aspirin) can control all ailments in ACS (Pollack *et al*., 1995), we have reported before that insulin was capable of controlling angina pain through the synthesis of NO (Sinha *et al.*, 1999). We have also delineated that the aspirin could be able to control pain in ACS and that might be due to the turn on NOS by the aspirin in lieu of its widely-known effect, as a cyclooxygenase inhibitor (Karmohapatra *et al*., 2007). Glucose has also the ability to stimulate NO synthesis, which might control pain, but the mechanism needs to be translated. Thrombus in the heart arterial wall does not let the plasma glucose to reach at the site of ache in the heart. It has been reported that the enlargement of thrombus not only occlude the supply of O_2 in the muscle cells of the heart, but also that thrombus could block the availability of nutrients (including glucose), water and minerals that are very crucial for the cardiac function and inevitably may steer to ACS. It might be considered that the glucose supply is blocked due to the thrombus formation in the arterial wall and as such NO production through the PLASENOS enzyme activity was impaired and that can be the cause of anginal pain. Insulin might have a very crucial role in NO synthesis (Sinha *et al*., 1999) and boost the conveyance of glucose which can also trigger NO synthesis. So, the transformation of the nitro compounds directly to NO to function as an anti-angina-agent which may be not sufficient. Our results explained that if arginine is

available in sufficient level, then the presence of PLASENOS would provide a continuous supply of NO for the anti-anginal outcome.

In this context PLASENOS has an important role to provide NO which is supposed to control chest pain (cardiac). It was found from this experiment that different stimulators induce nitric oxide synthase and can modulate pain through PLASENOS. We also revealed that aspirin and glucose were capable of producing NO (Bank *et al*., 2014; Karmohapatra *et al*., 2007; Bhattacharya *et al*., 2013]. It has been reported before that insulin can also directly activate the purified protein through its binding (Bhattacharya *et al*., 2001). Insulin was found to induce the activity of NOS in other tissues also and NO possessed the ability to execute carbohydrate metabolism like insulin, and nitric oxide can also trigger the PI-3 kinase and tyrosine kinase activity; so, it was demonstrated that nitric oxide might be called as a second messenger molecule of insulin (Kahn *et al*., 2000). Therefore, it could be postulated that aspirin, nitro compounds, insulin, and glucose all were able to regulate NO through the protein disulfide isomerase (PDI) expression. From the Lineweaver-Burk plot it was found that all compounds have nearly the same maximal velocity (Vmax) of enzymatic reaction of PDI. From the amino acid sequence of PLASENOS it is explained that this nitric oxide synthase protein was a protein disulfide isomerase family member which have a role in disease (Galligan *et al*., 2012).

We have revealed that nitric oxide can modulate pain in AIHD (acute ischemic heart diseases). From this experiment it has been found that insulin, nitro compounds and glucose all are able to diminish ischemic heart pain and they could do so by the PDI expression through NO production in the arterial endothelial cell membrane *in-vitro*. Thus, it might be inferred that PDI expression is somehow associated with pain modulation through the nitric oxide pathway.