

Activity of Indoleamine 2, 3 Dioxygenase (IDO) in Type 2 Diabetes Mellitus Patients in Pakistan

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Abstract

Background: To assess the activity of Indoleamine 2, 3 Dioxygenase (IDO) in Type 2 Diabetes Mellitus Patients in Pakistan

Methods: In this prospective study, activity and expression of IDO, was assessed in sera of diabetics and healthy controls (n=28). Colorimetric Assay was performed to analyze IDO activity in samples.

Results: A significant difference was observed between the means of control and diabetic patients with a p-value of 0.0001.

Conclusion: IDO concentrations were significantly higher in the serum of samples of diabetes mellitus patients as compared to control.

Key words: Diabetes Mellitus, Indoleamine 2, 3 dioxygenase, kynurenine pathway

Introduction

Indoleamine 2, 3 dioxygenase, an intracellular enzyme, regulates the degradation of L-tryptophan through kynurenine pathway. Upon induction of IFN- γ , dendritic cells (DCs), macrophages, fibroblasts and endothelial cells specify IDO protein. Specific immune systems are modulated by IDO in the course of various inflammatory and autoimmune diseases. Diabetics with over expression of IDO are susceptible to various bacterial, viral and fungal infections. Diabetes mellitus is a worldwide issue, responsible for effecting approximately 381 million individuals. According to WHO, 7 million people are affected by diabetes in Pakistan in 2012. Diabetes characterized by hyperglycemia, is a group of metabolic diseases resulting from irregularity in secretion or action of insulin or both. Long-term damage, irregular function and organ failure specifically the kidneys, nerves, eyes, heart and blood vessels are involved in chronic hyperglycemia of diabetes.¹ Diabetics are found to be more susceptible to certain bacterial, viral and fungal infections and have enhanced prevalence of cardiovascular, atherosclerotic, cerebrovascular disease and peripheral arterial.² There are two classes of Diabetes Mellitus i.e. Type 1 and Type 2 of which Type 2 constitutes 90% of the

cases. According to the World Health Organization (WHO), in 2015, approximately 422 million individuals were affected with diabetes. Prevalence is rapidly increasing and this number is determined to almost double by 2030. According to World Health Organization (WHO), in Pakistan, Seven million people are affected with diabetes mellitus, both types. Currently, Diabetes Mellitus is incurable, but depending on the type of diabetes suffered by the individual it can be managed. The goal for the treatment of diabetes is controlling blood glucose levels, in turn to prevent disease complications.

Indoleamine 2, 3 dioxygenase (IDO), heme containing enzyme, regulate catalytic degradation of tryptophan.³ IDO is involved in the modulation and downregulation of immune system by tryptophan degradation.⁴ Tryptophan, an essential amino acid (AA) and is vital for all living organisms and required in various metabolic pathways. Degradation of tryptophan results in the production of kynurenines leading to the suppression of cell proliferation. Immunomodulatory effects are produced in the biological systems⁵. IDO is involved in progression of several inflammatory reactions occurring in major diseases ranging from cancer, autoimmunity, infection and allergic reactions.⁴

Immunology of IDO is complex in nature. According to current knowledge, activity of IDO directly affects T-cells. Apoptosis is induced by IDO either through depletion of tryptophan or through tryptophan metabolites. The first mechanism discovered for IDO activity was tryptophan depletion. Induction of IDO is predominantly triggered by macrophages and dendritic cells. During inflammation, IDO is significantly upregulated by Interferon gamma (IFN- γ), which in turn is extremely fundamental for the development of T-cell and initiation of inflammatory response against infections and diseases. Enzymatic activity of IDO is indicated by the rate of tryptophan degradation and the ratio of kynurenine to tryptophan (Kyn/Trp) can be used as an indicator for

degradation of tryptophan as well as in the activation of immune system.⁶

Many inhibitors of IDO are being studied but 1-methyl tryptophan (1MT) has been considered as a potent inhibitor of enzymatic activity of IDO.³ 1MT has favorable pharmacokinetic characteristics such as oral availability, low protein binding and low clearance.⁷ IDO is involved in the progression of numerous pathological conditions ranging from various cancers, infections, allergies and autoimmune diseases. IDO and its differential response have been studied in several systems.

Current study focuses on the assessment of activity of indoleamine 2, 3 dioxygenase in diabetes mellitus type 2. According to a WHO survey in 2014, global prevalence of prevalence of diabetes was estimated to be 9% among adults aged 18 and above years. In Pakistan, approximately 7 million individuals are affected by diabetes. IDO has never been studied previously in relation to diabetes mellitus type 2.

Patients and Methods

Total 89 blood samples were collected from which 61 were diabetic samples while 28 were control samples. Serum was extracted from blood samples through centrifugation at 14000 rpm for 5-7 minutes. Extracted samples were stored at -80 degree C. For colorimetric assay of Kynurenine two solutions namely Solution A and Solution B were prepared. Solution A was 30% trichloro-acetic acid (TCA) solution in glacial acetic acid while Solution B was 20% Ehrlich reagent (p-dimethylbenzaldehyde) in glacial acetic acid. The function of TCA is to precipitate proteins in sample and Ehrlich reagent imparts color which binds to IDO. 200µL of serum sample was added from diabetic patient as well as from healthy control in an Eppendorf tube. They were labelled on the basis of their well number. 100µL of solution A was added in the samples. This process was repeated for all the samples separately. The mixture was then centrifuged at 14000 rpm for 3 minutes. After centrifugation two separate layers are established. 125µL of supernatant was added from each 96 Eppendorf tube to a separate well of 96-well plate. After all the wells were filled and 125µL of solution B was added which resulted in an immediate change of colour. This change in colour was assessed through Dynex Technologies microplate reader by measuring the absorbance of light at 490nm. IDO activity corroborated in diabetic samples as well as control samples through kynurenine concentration absorbance procured from microplate reader. Different concentrations of kynurenine were checked for

absorbance at 490nm and a standard curve was established in 96-well plate by conducting colorimetric assay (Figure 1).

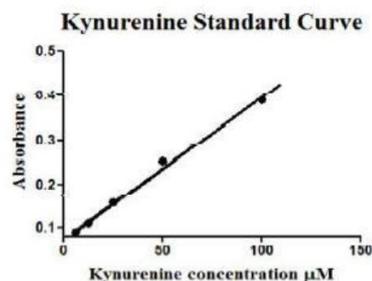


Figure 1: Kynurenine standard curve

Results

Enzymatic activity of IDO, due to the incongruity between IDO expression and activity. Six diabetics had hypertension (Table 1). A significant difference was observed between the means of control and diabetic patients with a p-value of 0.0001 (Table 2). Unpaired T-test was applied and it revealed significant up regulation in IDO expression with a p-value of 0.0095.

Table 1: Diabetics and controls- Demographic and clinical profile

Characteristics	Diabetic Samples (n=61)	Control Samples (n=28)
Age	58 (43-84)	33 (21-40)
Sex (M/F)	25/36	14/14
Hypertension (Y/N)	6/55	0/28
Cardiac Complications	6/55	0/28
Viral Infections (Y/N)	0/61	0/28

Table 2: IDO activity in diabetics and controls

IDO activity in samples			
Patients	Diabetic samples (n=61)	Control samples (n=28)	p-value
Kynurenine Conc. Absorbance Average	0.2521	0.0545	0.0001

Discussion

According to the World Health Organization (WHO), in 2015, approximately 422 million individuals were affected with diabetes. Prevalence is rapidly increasing and this number is determined to almost double by 2030. According to World Health Organization (WHO), in Pakistan, Seven million people are affected

with diabetes mellitus, both types. Being an underdeveloped country, Pakistan allots minimal quota for the health budget, 24 dollars per person cost of diabetes in Pakistan. IDO is involved in the up regulation of IFN- γ in diabetic patients and up regulates production through human islets cells.⁸

In this study, we established a correlation between up regulation of IDO and Diabetes Mellitus. We evaluated the enzymatic activity of IDO in serum of diabetes mellitus patients. A marked higher expression and activity of IDO was observed in diabetes mellitus patients as compared to healthy controls.

Indoleamine 2, 3 dioxygenase (IDO), heme containing enzyme, regulate catalytic degradation of tryptophan, an essential amino acid³. It has vital role in metabolism of tryptophan and performs cleavage in indole ring of a double bond at 2, 3 positions, consequently is also the rate limiting step of this catabolic pathway³. IDO is expressed in varying amounts by numerous tissues along with antigen presenting cells (APCs). It is intracellular in nature, extracellular form has not been reported yet. Activity and expression of IDO has been evident in trophoblast cells at fetal-maternal interface.⁹

Lately, a new enzyme has been reported having similar activity to IDO¹⁰. The enzyme is cited as indoleamine 2, 3 dioxygenase-2 (also proto indoleamine 2, 3-dioxygenase or indoleamine 2, 3-dioxygenase like protein) because of its structural and functional analogy with IDO. IDO is a monogenic protein and has 15 kb gene, constituted of 10 exons, present in chromosome 8 at the syntenic region in mice as well as humans. IDO is reported to be well conserved.¹¹ IDO gene transcription is strictly regulated and is prevalent in a limited number of cells. Only specific set inflammatory and genetic promoters can upregulate IDO transcription. Various pro-inflammatory promoters are strong inducers of IDO like type II interferons (IFN- γ) in turn type I interferons (IFN- α and IFN- β) are less potent inducers.¹² IDO protein is specifically expressed by macrophages, endothelial cells, fibroblasts and dendritic cells (DCs) upon the induction by IFN- γ .⁹ STAT-1 and IRF1 along with IFN- γ are involved in the IDO induction¹³. IFN- γ is strongly associated with inflammation and IDO induction; consequently various others potent inducers are also present to induce IDO expression along with IFN- γ . Tumor Necrosis Factors (TNF) and lipopolysaccharide (LPS) are potent inducers.¹⁴ Absence of IFN- γ is unaffected in the induction of IDO through LPS, although LPS requires TNF to support its responsiveness¹⁵ which

reiterates metabolic pathways of IDO induction independent of IFN- γ present. Complex transcription and expression is enhanced by certain cell specific cytokines.

Tryptophan, an essential amino acid (AA) and is vital for all living organisms and required in various metabolic pathways. Animals are unable to produce tryptophan by themselves and hence are dependent on primary producers for the flow of tryptophan.

From the digestive system, tryptophan is taken to the liver where it is utilized in various ways: Biosynthesis of Serotonin, Protein Synthesis and Kynurenine degradation pathway. Various biological mechanisms are affected; kynurenine pathway metabolizes most of the tryptophan. It is imperative in the production of serotonin. Degradation of tryptophan results in the production of kynurenines leading to the suppression of cell proliferation.⁵ In tryptophan catabolism, two important enzymes are involved, e.g., Indoleamine 2, 3-dioxygenase (IDO) and Tryptophan 2, 3-dioxygenase (TDO). Tryptophan and certain metabolic steroids activate the enzyme.¹⁶

IFN- γ is the potent inducer of IDO and is expressed in several tissues.¹⁷ Activated T-cells secrete IFN- γ as well as other leukocytes having the ability for the induction of reactive nitrogen species (RNS) and reactive oxygen species (ROS) in macrophages and neutrophils.^{18,19} Inexplicable role of IFN- γ during an immune response is well documented during vigorous and sustained catabolism of tryptophan.²⁰ Although the biological significance of IFN- γ mediated degradation of tryptophan is not completely comprehended, researchers believe that it is linked in prevention of tryptophan supply to intracellular parasites, cancer cells and pathogens.²¹ In recent years, these roles have gained attention.

Immunology of IDO is complex in nature. In specific cases, IDO can proliferate the pathogen growth or be beneficial to any disease inflicting factor thus in turn damaging the host. In tissues, generation of immunosuppressive microenvironment is a matter of extensive research. According to current knowledge, activity of IDO directly affects T-cells. Apoptosis is induced by IDO either through depletion of tryptophan or through tryptophan metabolites. The first mechanism discovered for IDO activity was tryptophan depletion. On the other hand, antimicrobial effects of IDO are reversed by the addition of tryptophan. Inhibition of T-cells was observed to be reversed through numerous studies conducted by different researchers.²²

T-cells are detrimentally effected by metabolites

produced by the degradation of tryptophan through IDO i.e. 3-hydroxyanthranilic acid and quinolinic acid even if externally added. It is hypothesized that the metabolites bind to the receptors and either block or be directly toxic to the cells. T-cell affinity determined by molecular pathways to metabolites of IDO is yet to be studied. However, specific pathways such as activation of GCN2 kinase pathway by amino acid withdrawal and inhibition of mTOR (mammalian target of rapamycin) pathway are known.

Through advancement in knowledge of biological effects of IDO and computer mediated drug design strategies, study and synthesis of inhibitors of IDO has got attention from numerous researchers. Competitive and Non-competitive IDO inhibitors have been studied along with competitive inhibitors constituted from tryptophan derivatives and non-competitive inhibitors derived from β -barboline.²³

1-methyl tryptophan (1MT) is the most widely studied inhibitor of IDO. 1MT has favourable pharmacokinetic characteristics such as oral availability, low protein binding and low clearance²⁴. Methyl-thiohydantoin-L-tryptophan (MTH-Trp) was found to be much more potent inhibitor than 1MT during screening for IDO inhibitors of competitive nature.²⁵

In different pathological conditions, IDO has numerous roles in human beings. Enhanced activity of IDO has been reported in various tumors in their microenvironment²⁶ resulting in T-cell suppression. Poor prognosis of cancer is associated with IDO and elevated levels of kynurenine have been reported in patient samples.^{27,28}

Conclusion

1. Enzymatic activity of IDO measured through colorimetric assay indicates that IDO might be involved in progressing the symptoms and susceptibility to infections in diabetes mellitus patients.

2. Blocking IDO or inhibiting its production can provide new strategies as an advent intervention therapy for diabetes mellitus

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