BIOINFORMATION
Discovery at the interface of physical and biological sciences

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Volume 7(7)

Glycation of calmodulin binding domain of iNOS may increase the chance of occurrence of tuberculosis in chronic diabetic state

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Received November 18, 2011; Accepted November 18, 2011; Published December 10, 2011

Abstract:
Tuberculosis is known to occur more in cases of chronic diabetes mellitus. The exact cause of such an association is mostly unknown. Recently we have shown using tools of computational biology that glycation of the subunits of respiratory burst enzyme NADPH oxidase may impair intra-macrophage killing of *Mycobacterium tuberculosis*. Since glycation of proteins including subunits of NADPH oxidase will be significantly increased in long standing uncontrolled diabetes we have concluded that it may be an important factor for increased association of tuberculosis in diabetic state. Analogous to NADPH oxidase, role of NOS is proved beyond any doubt for killing of intracellular pathogen like *Mycobacterium tuberculosis*. Based on the above mentioned premises, in this work we have studied glycation of various domains of iNOS using tools of computational biology and observed that glycation of K531 of Calmodulin binding domain of iNOS may impair the enzyme activity. We have concluded that the above phenomenon can happen at chronic diabetic state which may render the host susceptible to tuberculosis.

Key Words: Diabetes mellitus; tuberculosis, NOS, reactive oxygen species, reactive nitrogen species, glycation.

Background:
Mycobacterium tuberculosis is an intracellular pathogen. After entering into the body it goes inside the macrophages by the process of phagocytosis. Unlike many pathogens it does not allow a very successful fusion of the phagosome with lysosome of the macrophage and stays inside the stable phagosome. It also inhibits the respiratory burst activity of the phagosome to a large extent reducing generation of reactive oxygen species (ROS) inside the phagosome. All these facts have made it a very competent pathogen infecting almost one third of the world population **[1].**

Analogous to ROS, nitric oxide synthase (NOS) derived reactive nitrogen species (RNS) plays important role in killing of intracellular bacteria. *Mycobacterium tuberculosis* is also not an exception in this regard. NOS has three isoforms namely inducible NOS, endothelial NOS and neuronal NOS popularly known as iNOS, eNOS and nNOS, respectively **[2].** Of these particularly iNOS is expressed in macrophages and plays important role for killing of intracellular pathogens including *Mycobacterium tuberculosis* **[3].**

Diabetes mellitus is an epidemic of the modern world. It is characterized by persistent hyperglycemia due to relative or absolute deficiency of insulin. Long standing diabetes with uncontrolled hyperglycemia is known to cause increased non-enzymatic glycation of proteins. It is also known to be associated with increased occurrence of tuberculosis. There is evidence to believe that increased association of tuberculosis in chronic uncontrolled diabetes is directly correlated with the extent of protein glycation. In this context the consequence of

glycation on NADPH oxidase activity is investigated earlier using tools of computational biology and it is observed that there is possibility of glycation induced inhibition of the enzyme activity. This *in silico* observation, if proved to be a fact experimentally, can serve as an explanation for increased association of tuberculosis in diabetic state **[4].**

In similar ways there may be glycation induced inhibition of NOS. In this work we have developed this hypothesis using tools of computational biology. Glycation induced inhibition of NOS may reduce generation of RNS and thus rendering a diabetic host more susceptible to tuberculosis.

Methodology:

Three isoforms of NOS proteins in human – iNOS, nNOS and eNOS are taken into consideration. The sequences are taken from UniProtKB database **[5].** The accession numbers for the proteins are as follows – iNOS: P35228; nNOS: P29475; eNOS: P29474. Sequence and domain annotations of all the isoforms are noted from corresponding uniprot id database **[6-8].** The complex structures of calmodulin and calmodulin binding region of NOS proteins are taken from Protein Data Bank (PDB) **[9]** having PDB code - iNOS: 3HR4 **[10]**, nNOS: 2O60 **[11]** and eNOS: 1NIW **[12],** respectively. The glycation of the ε amino group of lysine (Lys) residues are predicted using NetGlycate 1.0 server **[13].** Molecular diagrams are drawn using pymol **[14].**

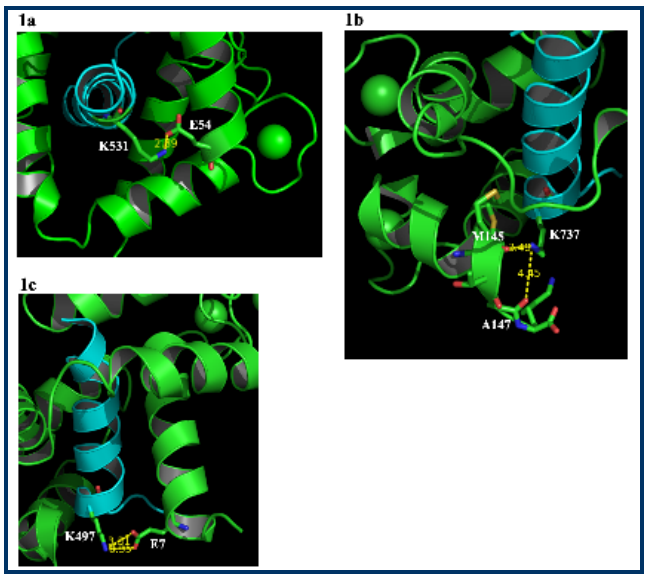

Figure 1: Interaction between calmodulin and calmodulin binding peptide region of NOS proteins (in 1a-iNOS, 1b-nNOS and 1c-eNOS, respectively) through predicted glycated lysine residue of calmodulin binding region of NOS proteins are depicted. Both calmodulin and calmodulin binding region of NOS proteins are shown in ribbon diagram with calmodulin in green and NOS proteins in blue colour. The Ca+2 ions are represented as green spheres. The lysine residues of calmodulin and interacting partner residues of NOS proteins are shown in stick mode with carbon, nitrogen and oxygen atoms in green, blue and red colour, respectively. The interaction between the atoms is shown by yellow dashed lines with distance in Å. All residues are labeled by one letter amino acid code with residue number obtained from corresponding PDB file.

Results and Discussion:

Domain classification of all the three isoforms of NOS proteins are given in **(Table 1, see supplementary material)**. It is observed that the total number of residues is varied but the domain architectures are similar in all the three isoforms. The lysine residues of each domain which are predicted to be glycated are also mentioned in **Table 1 (see supplementary material)** NO production is regulated by interdomain interaction between NOS and calmodulin **[10].** So we have analyzed all the complex structures of calmodulin and NOS proteins obtained from PDB. Calmodulin binding region of NOS proteins have lysine residue which is predicted to be glycated in all the three isoforms **(Table 1, see supplementary material).** Upon manual visualization of the complex structures, it is found that the predicted glycated lysine residue K531 is making salt bridge with acidic residue E54 (distance 2.89 Å) in iNOS-calmodulin complex **(Figure 1a).** Further, E54 is important residue for making the EF-hand loop that coordinates Ca^{+2} ions. So, if K531 becomes glycated, the ε amino group is not expected to form salt bridge with partner residue of calmodulin. In such a situation the coordination to Ca^{2+} ion will be blocked due to improper conformation of the EF-hand loop. Furthermore, if EF-hand loop is not properly formed, it will also weaken the interaction between iNOS and calmodulim. In case of nNOS-calmodulin complex the predicted glycated lysine residue K737 is interacting with polar group (peptide oxygen atom) of M145 and A147 **(Figure 1b).** However, the distances are higher (3.49 Å and 4.45 Å, respectively) than the distance in iNOS-calmodulin complex (2.89 Å). Further, neither of these two residues is coordinating to Ca^{2+} ion. In case of eNOS-calmodulin complex the predicted glycated lysine residue K497 is interacting with acidic residue E7 with two carboxylate oxygen atoms at distances 3.31 Å and 4.35 Å, respectively **(Figure 1c).** Here also, this E7 is not coordinating to Ca^{+2} ion. So if the lysine residues (K737 and K497) of nNOS and eNOS become glycated, the interaction between calmodulin and NOS may not be affected when compared to iNOS. In these cases the coordination to the Ca^{+2} ions is not expected be affected.

Calmodulin binding to NOS is universally required for its functional activity and in case of iNOS the Calmodulin binding may be an effective stimulator of the enzyme irrespective of the availability of the calcium ion **[10, 15].** It is in this context our *in-silico* observation is important. We have found that glycation of Lys531 in the Calmodulin binding domain of iNOS is causing a structural change in the molecular microenvironment that is not suitable for Ca^{+2} and Calmodulin binding. Although Lys737 and Lys497 are glycated in the other two isoforms of NOS, the possibility of glycation induced inhibition of Calmodulin (or Ca^{+2}) binding is less apparent in the other two isoforms. Therefore possibility of Lys737 and Lys497 glycation induced inhibition of Calmodulin binding is comparitively less in nNOS and eNOS, respectively when compared to iNOS. Glycation is practically more possible in macrophages compared to other cells or its precursors since glucose transporters are expressed more on the mature macrophage membrane that is expected to transport more glucose inside the macrophage from extra-cellular environment particularly at hyperglycemic state **[16].** At the present moment there are experimental evidences to support this idea **[17].** Therefore in persistent hyperglycemia proteins like iNOS that reside inside the macrophage are expected to be glycated more which has the potential to inhibit

Calmodulin-iNOS interaction. A vast literature is in the favor of absolute requirement of Calmodulin binding for functioning of iNOS [**18].**

Therefore, if such *in silico* observation proves to be experimentally true there is enough chance of glycation induced inhibition of iNOS activity. It is needless to explain that glycation induced inhibition of iNOS will make the chronic diabetic host more susceptible to tuberculosis. It is also known that reactive nitrogen species (RNS) play an essential role in host defense against *Mycobacterium tuberculosis* in the mouse model of tuberculosis as evidenced by the increased susceptibility of mice deficient in the inducible isoform of nitric oxide synthase (iNOS) **[19].** This fact makes it almost evident that if inhibition of iNOS happens due to any reason, it will make the host more susceptible to tuberculosis. Since glycation is the hallmark of chronic uncontrolled diabetes, glycation induced inhibition of iNOS can be considered as a cause of increased association of tuberculosis in diabetic state. In this connection it is worth mentioning that in diabetic state endogenous inhibitors of NOS is described in the literature **[20]** and that is also thought as a link between increased incidences of tuberculosis in diabetic state **[4].**

Conclusion:

We show structural models to support that there is a high possibility of glycation induced inhibition of iNOS which may serve as a causative factor for more tuberculosis infection in diabetic state. It should be noted that this hypothesis should be validated using experimental inference.

Acknowledgement:

RB has done the computational analysis and DB developed the basic hypothesis. RB and DB acknowledge DST, New Delhi for financial assistance.

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Edited by P Kangueane

Citation: Bhattacharyya & Banerjee. Bioinformation 7(7): 324-327 (2011)

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Supplementary material:

Table 1: Domain classification and predicted glycated lysine residues in iNOS, nNOS and eNOS. Sequence annotation is given according to the position of amino acid in protein sequence.

iNOS (total residues 1153)			nNOS (total residues 1434)			eNOS (total residues 1203)		
Domain classification		Predicted glycated residues	Domain classification		Predicted glycated residues	Domain classification		Predicted glycated residues
Range	Description		Range	Description		Range	Description	
509-529	Calmodulin binding	531K	1-205	Interaction with NOSIP	24K,38K,131K,143K	98-486	Interaction with NOSIP	108K, 395K, 397K, 429K
539-677	Flavodoxin-like	549K	17-99	PDZ	24K,38K	491-510	Calmodulin binding	497K
623-654	FMN binding		163-245	PIN binding	207K	520-703	Flavodoxin-like	544K, 610K, 631K
730-970	FAD binding FR-type	730K,738K,872K	730-750	Calmodulin binding	737K	649-680	FMN binding	
767-778	FAD binding		755-774	Tetrahydrobiopterin binding	759K	756-1002	FAD binding FR-type	773K
903-913	FAD binding		760-940	Flavodoxin-like	776K, 847K, 861K	793-804	FAD binding	
978-996	NADP binding		886-917	FMN binding		935-945	FAD binding	
1076-1091	NADP binding		995-1242	FAD binding FR-type	1012K, 1160K, 1127K	1010-1028	NADP binding	
			1032-1043	FAD binding		1108-1123	NADP binding	
			1175-1185	FAD binding				
			1250-1268	NADP binding				
			1348-1363	NADP binding				