EXPRESSION OF INSULIN RECEPTORS IN INSULIN TARGET ORGANS IN STREPTOZOTOCIN DIABETIC MODEL: AN IMMUNOHISTOCHEMICAL STUDY*

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Abstract: Insulin receptors are transmembrane proteins involved in insulin regulated glucose uptake signaling pathway, highly expressed in target tissues like liver, adipose tissue, skeletal muscle and also in several other types of mammalian cells. Several studies have documented a reduced expression of insulin receptors in insulin resistance and diabetes. In the present study, clinical diabetes was induced experimentally with streptozotocin in a rodent model, and maintained for 45 days along with non diabetic rats. Tissues like liver, heart, skeletal muscle and adipose tissues analyzed by immunohistochemical method using anti-insulin receptor α antibody, showed expression of insulin receptors indicated by an intense brown coloration. It was noted that immunopositivity was observed in two locations viz., the cytoplasm and the plasma membrane of the target tissue cells. In the present study, a reduced expression of insulin receptors among the diabetic rats, when compared to non diabetic rats has been demonstrated by immunohistochemistry in liver, heart, skeletal muscles and adipose tissue, potentiating the fact that there is indeed a reduction in expression of insulin receptors in diabetes mellitus.

Key words: Insulin receptors, Diabetes, Immunohistochemistry

INTRODUCTION

Diabetes, a global health problem of man and animals, is characterized by metabolic deregulation primarily of carbohydrate metabolism, manifested by hyperglycemia resulting from defects in insulin secretion, impaired insulin action, or both [1]. The worldwide prevalence of diabetes in human beings has been estimated to be 8.3 percent with 387 million people currently diabetic which is expected to rise to 592 million by 2035 and the world has recorded 4.9 million deaths due to diabetes in 2014. In South East Asia approximately 75 million people are diabetic and the disease is recognized as a serious problem affecting the world (International Diabetes Federation, 2014).

The current exponential increase in incidence of diabetes is mainly influenced by lifestyle situations or habits like lack of physical activity, sedentary lifestyle, increased body mass, coupled with stress

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which prevail as etiologies for obesity and metabolic syndromes that initiate the insulin resistance or pre diabetes which proceeds to full blown type 2 diabetes mellitus. In insulin resistance or prediabetes, there is reduced expression of insulin receptors on the plasma membranes resulting in hyperinsulinemia and subsequent hyperglycemia.

Insulin receptors are the transmembrane proteins, also called as receptor tyrosine kinases expressed by all mammalian cells [2] and play an important role in regulated glucose uptake pathway mediated by the hormone insulin in the cells [3]. The insulin receptor located on the plasma membrane has two pairs of subunits IRα and IRβ, the former resides on the outer membrane which participates in binding to insulin hormone, and the latter resides on the inner plasma membrane which participates in phosphorylation of substrates and effector molecules ultimately mobilizing the GLUT-4s from the GLUT-4 storage vesicles (GSVs), to translocate onto the plasma membrane which in turn transport glucose molecules from blood into the cells. Alterations in receptor properties significantly affect the qualitative and quantitative responses of cells to its corresponding extra cellular messenger or docking protein [2].

In diabetes and insulin resistance, several researchers have documented a reduced expression of insulin receptors by microscopy [4,5], radioautography, microradiography [6], fluorescent microscopy [7], and electron microscopy [8] in several animal models, cell cultures and intra vital imaging techniques. As there are scarce reports available on immunohistochemical demonstration of insulin receptors among controlled experiments in rodent models in induced diabetes by streptozotocin, the present study was designed to compare their expression in several tissues that are considered as target organs for glucose storage.

**MATERIALS AND METHODS**

The current investigation was carried out in the Department of Veterinary Pathology, Veterinary College, Bengaluru, with a prior approval from the Institutional Animal Ethics Committee (IAEC). A total of twenty healthy adult female Wistar albino rats were subjected to the experiment in the present study. The rats were maintained in appropriate ambient conditions with 12 hours light and dark cycles, with an adlibitum access to clean water and rodent feed (Nutrilab Rodent, Provimi, Bengaluru). They were acclimatized to the animal house conditions for two weeks prior to the actual experimentation. The rats were randomly divided into two groups of ten animals each, as non diabetic group and diabetic group.

**Induction of diabetes:** The rats of diabetic group were injected intra peritoneally with streptozotocin (Sigma) at 45 mg/kg body weight in cold citrate buffer and their serum glucose levels after 72 hours were estimated. Those rats showing more than 200 mg/dL of serum glucose only, were considered as diabetic and used for the study. The rats of non diabetic group were injected intra peritonially with only cold citrate buffer and the serum glucose levels were estimated after 72 hours.

**Tissue collection and processing:** The rats from diabetic and non diabetic groups were sacrificed sequentially two each during 15th and 30th day, followed by the remaining six rats on 45th day of the experimental study. Tissues like heart, skeletal muscle (gastrocnemius), liver and adipose tissue, with a size not more than 0.5 - 1 cm thick were collected immediately after sacrificing the animals and fixed in 10% neutral buffered formalin and processed by routine paraffin embedding technique [9]. Sections of four to five micron thickness were cut using Microm semi-automatic microtome (Thermo Fisher) with disposable blades.

**Immunohistochemical procedure:** Formalin fixed paraffin embedded sections mounted onto 3-aminopropyltriethoxy-silane (APES) adhesive coated slides were subjected to the immunohistochemical procedure for the demonstration of insulin receptor α (IR α) as detailed below.

Lyophilized rabbit polyclonal anti-insulin receptor α (Boster Immunoleader, USA., Code No. PA1205, supplied by Genxbio, India) was the primary antibody used. The antibody was reconstituted with 0.2 ml distilled water and stored at 4 °C and working dilution was prepared just before use at 1: 500 ratio with distilled water. Secondary antibody was peroxidase conjugated goat anti-rabbit IgG (gamma chain specific from Boster Immunoleader, USA., Code No. BA1054 - 0.5, supplied by Genxbio, India). The working concentration was prepared at 1: 500 ratio in phosphate buffer saline as the diluent just before
the use. Tissue sections were dried at 37 °C for three hours. The paraffin tissue sections were deparaffinized using xylene and rehydrated using descending grades of ethanol. The sections were covered with neat formic acid and incubated at room temperature for 10 minutes as a pretreatment for antigen retrieval and washed with wash buffer thrice. The sections were incubated at room temperature for fifteen minutes covered with 3 per cent H₂O₂ in methanol to block endogenous peroxidase followed by washing in three changes of wash buffer. Heat induced epitope retrieval (HIER) was carried out by immersing tissue sections in boiling citrate buffer (pH 6.0) and were cooked for 25 minutes at 98 °C. Sections were allowed to cool down to room temperature for approximately 30 minutes and later washed in three changes of wash buffer. Protein blocking was carried out with 0.2 percent BSA (Bovine serum albumin) in PBS (phosphate buffer saline) at room temperature for 30 minutes and washed in three changes of wash buffer. The primary antibody was applied to the sections at a dilution of 1 μg/ml concentration (1:500 dilution) in distilled water, and incubated overnight at 4 °C for approximately 12-16 hours. After washing with three changes of wash buffer, the sections were incubated with 1:500 concentration of secondary antibody for 90 minutes and washed thrice with wash buffer. DAB (Diaminobenzidine) enhanced liquid substrate was applied for 5-10 minutes and washed with distilled water. The sections were stained for nucleus with Harris haematoxylin for 30 seconds and then dehydrated with ascending grades of ethanol, cleared with xylene, mounted with an aqueous mounting medium and observed for reaction under the microscope.

RESULTS

Localization of insulin receptors was demonstrated by immunohistochemistry in various tissues like liver, heart, skeletal muscle and adipose at different time intervals comprising 15th, 30th and 45th day of the experimental study. The brown colored granular chromogenicity in the cell was considered as immunopositive reaction for insulin receptors. The comparison between the groups was made based on the number of positive cells, granularity, staining characteristics like color intensity, localization like cytoplasmic or membranous, focal, multifocal or diffuse distribution, histologic site specific distribution and any other cell or tissue specific characteristic features. In general, the insulin receptor molecules were expressed in the cytoplasm as well as plasma membranes of several types of cell populations in liver, heart, skeletal muscle and adipose tissue.

Expression of insulin receptors in non diabetic group: The insulin receptors in the non diabetic rats was both cytoplasmic and membranous in expression and the positive immune reaction was brownish in colouration with granularity in the cytoplasm which was diffuse in distribution:

1. The insulin receptor immune reaction in liver was diffuse involving all the cells of hepatic lobules with a prominent peri-portal distribution. The hepatocytes revealed both cytoplasmic and cell membranous distribution. The immune reaction was diffuse, brownish and granular in the cytoplasm and, linear, brown and well defined on the cellular membrane. Variation in the expression of insulin receptors was observed with several hepatic cords intensely stained compared to others. The insulin receptor positivity was also observed in lining endothelial cells of veins, arteries and sinusoids, red blood cells and the lymphocytes. These observations were consistent during all the three time intervals for insulin receptor expression in hepatic tissue (Fig 1).

During all the time intervals, the cardiomyocytes showed a diffuse, granular, intense brown staining reaction in the sarcoplasm as well as on the sarcolemma. The cross striations were well appreciable and specifically prominently stained in longitudinal sections of cardiomyocytes. Brown colored cytoplasmic granularity was also appreciable in the cross sections of cardiomyocytes. In addition other cells such as connective tissue mast cells and the macrophages also showed very intense insulin receptor positivity in the interstitial spaces. The insulin receptor expression was also observable on pericardium, pericardial fat, capillaries and red blood cells (Fig 3).

The insulin receptor immunopositivity in rhabdomyocytes was similar to that of cardiomyocytes which also showed brownish granular diffuse distribution, prominently on the cross striations and also on sarcolemma (Fig 5). In adipocytes the insulin receptor immune reactivity was observed on cell membrane and in the vertices which was uniform, well defined and brownish, observed during all the three time intervals (Fig 7).
Expression of insulin receptors in diabetic group: In diabetic rats, the expression of insulin receptors was observed to be reduced in general which was characterized by absence or negligible presence of granular brownish colored immune reaction in the cytoplasm as well as the cell membrane in all the organs examined. The staining intensity was very much reduced during 15th day interval, which persisted during 30th day, but showed mild improvement during 45th day of the experiment.

During 15th day, the liver showed extremely reduced immunopositivity with only occasional hepatocytes showing light brown staining reaction. The reduced expression was persistent during 30th day, however a few small clusters of hepatocytes at the centrilobular or periportal areas showed slight immunopositivity. During 45th day, there was an improvement in the number of immunopositive cells with multifocal areas of liver showing mild expression of insulin receptors, although the staining intensity was mild (Fig 2).

The cardiac musculature showed lack of expression of insulin receptor positivity during 15th day, mild expression during 30th day with few cardiomyocytes showing discontinuous staining reactions visible on the cross striations and on the sarcolemma, and a mild improvement with several isolated cardiomyocytes showing immunopositivity during 45th day of observation (Fig 4). There was a reduction in cytoplasmic granularity and intensity of brown colouration in the cross section of heart tissue.

Majority of the rhabdomyocytes were immunonegative during 15th day with diffuse loss of cross striations. Similar reaction persisted during 30th day, although mild granularity was observed in the cytoplasm and on cross striations in a few scattered rhabdomyocytes. However, during 45th day, there was a mild improvement in insulin receptor positive reaction in several rhabdomyocytes that were patchy and focal in distribution (Fig 6).

In adipose tissue during 15th day, there were large adipocytes interspersed with small variable sized adipocytes, however, a mixture of both large and small adipocytes were observed during 30th day, with visible reduction and discontinuity in staining intensity on the plasma membrane. During 45th day, there was moderate improvement in insulin receptor expression with intense staining reaction observable only in a few large and small adipocytes (Fig 8).

DISCUSSION

The insulin receptors play an important role in insulin regulated uptake of blood glucose into the cells. They are the heterotetrameric complex molecules found on the plasma membrane of all mammalian cells [2]. Interaction of insulin with its receptor tyrosine kinase triggers intracellular cascade of kinases that leads to cellular uptake of glucose in insulin target cells. The mechanism involves binding of insulin to IRζ unit of insulin receptor on the outer cellular membrane, which activates the IRβ located on the inner side of the plasma membrane that participates in phosphorylation of tyrosine kinases, several substrates and effector molecules, ultimately stimulating the translocation of glucose transporters onto the plasma membrane, which in turn channelize the movement of glucose molecules across the membrane from blood into the cell, in an energy independent mechanism [10].

In the present study, the expression of IR was demonstrated apart from hepatocytes, adipocytes, rhabdomyocytes and cardiomyocytes also in

Legend for Figures

Fig 1: Liver from non diabetic group showing intense immunopositivity for IR in hepatocytes (Liver, IHC, X 100). Fig 2: Liver from diabetic group showing multifocal small clusters of IR immunopositive hepatocytes distributed sporadically in the hepatic parenchyma (Liver, IHC, X 100). Fig 3: Heart from non diabetic group showing diffuse expression of insulin receptors in majority of the cardiomyocytes (Heart, IHC, X 200). Fig 4: Heart from diabetic group showing reduced expression of insulin receptors in the cardiomyocytes. (Heart, IHC, X 200). Fig 5: Skeletal muscle from non diabetic group showing a good expression of insulin receptors on the sarcolemma and the cross striations of rhabdomyocytes (Skeletal muscle, IHC, X 200). Fig 6: Skeletal muscle from diabetic group showing reduced expression of IR immunopositivity in rhabdomyocytes with discontinuous staining in sarcolemma and cross striations, and reduced cytoplasmic granularity (Skeletal muscle, IHC, X 200). Fig 7: Adipose tissue from non diabetic group showing well defined, regular and continuous plasma membranes with intense brown coloration in adipocytes (Adipose tissue, IHC, X 200). Fig 8: Adipose tissue from diabetic group showing irregular cells with ill defined plasma membranes showing discontinuity in staining reaction. (Adipose tissue, IHC, X 200).
pericardium, endocardium, hepatic sinusoidal and cardiac vascular endothelial cells, erythrocytes, lymphocytes, mast cells and macrophages of non diabetic rats which indicated that low grade expression of IR occurs in many different types of cells apart from insulin target organs such as skeletal muscle, adipocytes and hepatocytes [2,11].

The insulin receptors, though documented to be transmembranous [2], the immunopositivity was observed both on the cell membrane and in the cytoplasm of cells of various organs examined in the present study. Several studies have shown that the insulin–receptor complex, although originates as a membrane ligand, is gradually internalized after effecting its signal transduction and recycled back to express again on the plasma membrane [3,12] which substantiates the demonstration of cytoplasmic immunoreactivity in the present study. It has been postulated that IR signaling is dependent on its localization wherein the internalized IRs regulate mitogenic activity and membranous IR for the metabolic balance [13]. Studies have also indicated that IR \( \alpha \) is internalized more than IR \( \beta \) [13], which supports the cytoplasmic detection of IR in several cells, as in the present study.

Thus the “present study documents the immunohistochemical demonstration of intracytoplasmic as well as membranous localization of insulin receptors in liver, heart, skeletal muscles and adipose tissue in normal and STZ induced diabetic rats”.

The expression of IR \( \alpha \) in muscle, adipose tissue and liver in diabetic group was lesser in comparison with that of control group in the present investigation. Decreased IR expression similar to the present study in tissues such as liver [14], kidneys [5], heart [6], skeletal muscles [8] and adipose tissues [15] in diabetes have also been recorded by many researchers. Several authors have demonstrated down regulation of IR accompanied by diminished insulin signaling causing insulin resistance [16-18] and attributed to several inductive factors such as endoplasmic reticulum stress which abrogates IR expression, free fatty acids, deficiency of architectural transcription factor (HMGA1) of IR gene, defects in IR gene expression and others. Studies have shown concentration dependent down regulation of IR in insulin target cells with palmitate [19] and with transfection of lipid to skeletal muscle cells and adipocytes [20]. Dasgupta et al. [18] have observed about 85–90% of IR \( \beta \) degraded in skeletal muscle with almost equal amount of reduction in its gene expression in obese type 2 diabetic db/db mice. These observations specify that FFA is one of the important causes of IR reduction and insulin resistance in diabetes. Fatty acids cause phosphorylation of protein kinase \( \epsilon \) (PK\( \epsilon \)) which move to nucleus and phosphorylates HMGA1 and down regulates its expression by deactivating its transcription factor Sp1. HMGA1 (High mobility group protein) is an architectural transcription factor and induces transcriptional activation of IR gene by recruiting Sp1 and c/EBP \( \beta \) to the IR promoter. Downregulation of HMGA1 causes attenuation of IR gene and its protein expression which seriously compromise insulin sensitivity. In the present study, the decreased IR expression could be attributed to the elevation in serum cholesterol, triglycerides and fatty acid levels induced by significant damage to organs that express GLUT-2 like liver and kidney along with pancreas by STZ in rats.

In addition, STZ also causes structural damage especially due to increased membranous peroxidation by free radicals leading to loss of cholesterol that compromise integrity of cell membrane with reduced number of caveolar proteins where the receptors are localized in more numbers [21], could be contributory for loss of membranous insulin receptors and also for induction of ER stress [22]. In diabetes mellitus, the decreased uptake of glucose compromises the carbohydrate, lipid and protein metabolism in cells. Studies have shown that ER stress plays pivotal role in the onset of insulin resistance [23]. The endoplasmic reticulum stress activates the Jun-NH\(_2\) Kinase (JNK), which in turn induces serine phosphorylation of IRS-1, which further suppresses the intracellular insulin signaling [24]. ER Stress in diabetes is probably due to altered mitochondrial function and disruption of mitochondria associated membrane (MAM) which induces a reduction in the protein synthesis, are also associated with hepatic insulin resistance [25].

In diabetes mellitus, hyperglycemia is a prominent factor that is present in insulin resistance and might have a role in IR protein regulation [5]. In support of this hypothesis, Tiwari et al. [5] found a significant decrease in both IR \( \alpha \) and IR \( \beta \) subunits in the kidneys of rats made diabetic with hyperglycaemia by administration of STZ.
From the present study it could be concluded that, insulin resistance in STZ induced diabetes model is associated with the reduction in the expression of insulin receptors on the membranes of cells of insulin target organs.

REFERENCES