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Analysis of the effect of CFEt on insulin binding sites of erythrocytes in diabetic rats

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Abstract

In this paper, we investigated the insulin-receptor-binding effect of *Cassia auriculata* flower extract (CFEt) in streptozotocin induced male wistar rats, using circulating erythrocytes as a model system. We measured the levels of plasma glucose and plasma insulin and the binding of insulin to cell-membrane erythrocyte receptors (ER) in CFEt and glibenclamide treated diabetic rats. The mean specific binding of insulin to ER was significantly lower in diabetic control rats than in CFEt, glibenclamide treated diabetic rats, resulting in a significant decrease in plasma insulin. Scatchard plot analysis demonstrated that the decrease in insulin binding was accounted for by a lower number of insulin receptor sites per cell in diabetic control rats when compared with CFEt treated rats. High-affinity (K_{d1}), low-affinity (K_{d2}), and kinetic analysis revealed an increase in the average receptor affinity in ER from CFEt treated diabetic rats. The results suggest an acute alteration in the number of insulin receptors on ER membranes in streptozotocin-induced diabetic control rats. Treatment with CFEt (2.40 ± 0.15) improved specific insulin binding, with receptor number and affinity binding diabetic rats (0.95 ± 0.06). These biochemical observations were supplemented by histopathological examination of pancreas section. The data presented here showed that CFEt increase total ER membrane insulin binding sites with a concomitant (7.08 ± 0.51) increase in plasma insulin.

Keywords: Glucose, insulin binding sites, plasma insulin, *Cassia auriculata*, glibenclamide.

INTRODUCTION

Erythrocytes are known to respond to physiological stimuli via a diversity of receptor and effectors (Minetti and Low, 1997). Erythrocytes have specific surface receptors, which have binding characterization similar to those of the insulin receptor found in classical target tissues for insulin action (De pirro et al., 1980; McElduff, 1981). The metabolic effects of insulin are initiated by the binding of insulin to cell surface receptors (Bortoli et al., 1997; Clark and Harrison, 1983; Yamaguchi et al., 1983). Diabetes mellitus has been attributed to defects in insulin binding to its receptors and post-receptor defects (Bortoli et al., 1997). During severe uncontrolled hyperglycemia, there is decreased specific binding of insulin to erythrocytes receptors (Pari and Latha, 2004). A number of studies have shown that human erythrocytes can be used as a cellular model for assessing the status of insulin receptors in diabetes (Gambhir et al., 1978; DePirro et al., 1980). The erythrocyte insulin receptor serves as a model for studying insulin/receptor interaction in humans

Abbreviation: B_{m1}- Receptor numbers of high binding sites; B_{m2} - Receptor numbers of low binding sites; CFEt- *Cassia auriculata* Flowers extract; ER – Erythrocyte receptors; K_{d1} - Dissociation constant of high binding sites; K_{d2} - Dissociation constant of low binding sites; K_e - Dissociation constant of empty sites; K_f - Dissociation constant of filled sites.

(McElduff and Eastman, 1981; Ward and Harrison, 1986). Insulin resistance is a common pathological state in which target cells fail to respond to ordinary levels of circulating insulin (Kahn and Flier, 2000; Matthaei, 2000).

Cassia auriculata L. (Ceasalpiniaceae) is a shrub that has attractive yellow flowers, commonly used for the treatment of skin disorders and body odour. It is a native plant present in different parts of India. Indigenous people use various parts of the plant for diabetes mellitus. It is widely used in ayurvedic medicine as a "Kalpa drug" which contains five parts of the shrub (roots, leaves, flowers, bark and unripe fruits) which are taken in equal quantity, dried and then powdered to give "Avarai Panchaga Choornam", for the control of sugar levels and reduction of symptoms such as polyuria and thirst in diabetes (Brahmachari and Augusti, 1961) . A literature survey showed that a decoction of leaves, flowers, and seeds of the *C. auriculata* mediate an antidiabetic effect (Shrotri and Aiman, 1960). Thus, the available reports show that very little work has been done with respect to *C. auriculata* flowers, other than its hypoglycemic effects.

We have already reported the antiperoxidative effect of aqueous extract of *C. auriculata* flowers (CFEt) in diabetic rats (Pari and Latha, 2002). In our lab, we found that CFEt improves plasma insulin, decrease glucose levels, scavenging free radical and also antioxidant activity in diabetic rats (Latha and Pari, 2003). CFEt reverses the changes in the levels of antihyperlipidemic effect (Pari and Latha, 2002). The effects produced were compared with glibenclamide, a reference drug.

However studies on the effects of CFEt on insulin receptor in streptozotocin diabetic rats have not been studied to the best of our knowledge. To better understand how CFEt produces antihyperglycemic effect, we have analyzed the effect of CFEt on insulin binding sites of erythrocytes in diabetic rats.

MATERIALS AND METHODS

Animals

All the experiments were carried out with male Wistar rats aged seven to eight weeks (180 - 200 g), obtained from the Central Animal House, Rajah Muthiah Medical College, Annamalai University, India. The animals were housed in polypropylene cages and provided with water and standard pellet diet (Karnataka Agro Food Corporation Limited, Bangalore, India) *ad libitum*. The animals used in the present study were approved by the ethical committee, Annamalai University.

Chemicals

Streptozotocin was obtained from Himedia Laboratory Limited, Mumbai, India. All other reagents used were of analytical grade.

Plant material

C. auriculata flowers were collected freshly from Neyveli, Cuddalore District, Tamil Nadu, India. The plant was identified and authenticcated at the Herbarium of Botany Directorate in Annamalai University. A voucher specimen (No.231) was deposited in the Botany Department of Annamalai University.

Preparation of plant extract

Five hundred g of *C. auriculata* flowers were extracted with 1,500 ml of water by the method of continuous hot extraction at 60ëC for 6 h and evaporated (Jain, 1968). The residual extract was dissolved in water and used in the study.

Induction of experimental diabetes

A freshly prepared solution of streptozotocin (45 mg/kg intraperitoneally) in 0.1 M citrate buffer, pH 4.5 was injected intraperitoneally in a volume of 1 ml/kg body weight. After 48 h of streptozotocin administration, rats with moderate diabetes having glycosuira and hyperglycaemia (that is, with a blood glucose of 200 - 300 mg/dl) were taken for the experiment (Siddique, 1987).

Experimental procedure

In the experiment, a total of 36 rats (30 diabetic surviving rats, six normal rats) were used. The rats were divided into six groups of six rats each. Group 1-Normal untreated rats. Group 2- Diabetic control rats given 1 ml of aqueous solution daily using an intragastric tube for 45 days. Group 3 - Diabetic rats given CFEt (450 g/kg body weight) in 1 ml of aqueous solution daily using an intragastric tube for 45 days. Group 4 - Diabetic rats given glibenclamide (600 g/ kg body weight) in 1 ml of aqueous solution daily using an intragastric tube for 45days.

At the end of 45 days, all the rats were killed by decapitation after inducing anaesthesia (Pentobarbitone sodium, (60 mg/kg body weight). Blood was collected in tubes containing potassium oxalate and sodium fluoride solution for the estimation of blood glucose and plasma was separated for assay of insulin.

Measurement of blood glucose and plasma insulin

Blood glucose was estimated colorimetrically using commercial diagnostic kits (Sigma Diagnostics (I) Pvt Ltd, Baroda, India) (Lott and Turner, 1975). Plasma insulin was assayed by ELISA using a Boehringer-Mannheim kit with an ES300 Boehringer analyzer (Mannheim, Germany).

Preparation of purified erythrocytes

The erythrocytes were separated using a Percoll density gradient. Mononuclear leucocytes were separated from the erythrocytes by the use of Pasteur pipettes. The erythrocytes receptor assay was performed according to a modification of the method by Ghambir et al. (1978). The erythrocytes were washed three times by centrifugation (4°C, 400 x g) in 10 ml of buffer G containing (in mmol/L), tris (hydroxymethyl) methylamine, 50; 4-(2-hydroxyethyl)-1-peperazine ethanesulfonic acid, 50; $MgCl_2.6H_2O$, 10; $CaCl_2$, 10; ethylenedia-minetetraacetic acid (EDTA), 1% human serum albumin, (pH 7.8) for 10 min. On each occasion, the supernatant was removed and the cells re-suspended in buffer G, and respun. After the final wash of the cells, the supernatant was removed and the cells were left in 4 ml of buffer G containing 1% human serum albumin. This suspension contained 4 - 6 x 10^9 cells/ml.

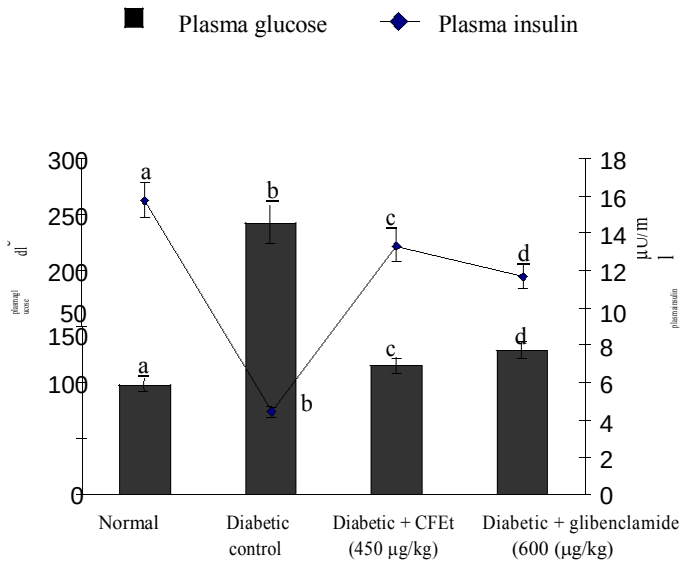

Figure 1. Effect of CFEt on the levels of plasma glucose and plasma insulin in normal and experimental rats. Values are given as mean ± S.D for 6 rats in each group.
Values not sharing a common superscript letter differ significantly at p<0.05 (DMRT).

Binding of ^{125}I to erythrocytes cells

Erythrocytes (4.5 x 10^9 cells/ml) were incubated at 15^{o}C with ^{125}I – insulin (40 pg in 25 l) with or without varying amounts of unlabelled insulin (0 to 0.5x10^5 ng) in a total volume of 0.5 ml. After 2.5 h of incubation, duplicate samples were placed in pre-chilled microfuge tubes along with the buffer and dibutylphthalate. Cell bound and free insulin were separated by centrifugation at 7000 g at 4^{o} C for 10 min. The radioactivity in the cell pellet and supernatant was determined in a gamma counter (ECIL, Hyderabad). The data was analyzed by Scatchard analysis (Scatchard, 1949). The recap-tor affinity and receptor numbers were derived for the physiological range of insulin i.e. between 0.1 and 100 ng/ml. Specific insulin binding (SB) was calculated as the percentage of radioactive insulin bound by 4 x 10^9 for erythrocytes. Non-specific binding is defined as the amount of radioactive insulin that remains bound in the presence of 10^5 ng/ml of unlabelled porcine insulin. All binding data were corrected for the non-specific binding to represent specific cell binding for purposes of comparison.

Competitive binding curves were obtained for each erythrocyte suspension. From these curves, the insulin receptor affinity and number of the receptor sites were determined by the Scatchard analysis.

Cell binding analysis

The results of the binding studies are presented in three ways: (1) the percentage binding of ^{125}I- insulin as a function of the total insulin concentration (competitive curve), (De pirro *et al* 1980) the bound-free insulin ratio plotted as a function of the bound insulin (Scatchard plot) and, (McElduff and Eastman, 1981) the average affinity profile. The total binding capacity or concentration of the binding sites was derived from the point where the linear extrapolation of the curve intercepts the horizontal axis and this was used to calculate the number of receptor sites per cell (Scatchard, 1949).

Experimental data suggest that the insulin receptor consists of two binding sites that undergo negatively co-operative site-site interactions such that the affinity of the receptors for insulin is inversely related to the receptor occupancy.

Histopathological study

The pancreas samples fixed for 48 h in 10% formal-saline were dehydrated by passing successfully in different mixture of ethyl alcohol – water, cleaned in xylene and embedded in paraffin. Sections of pancreas (4 - 5 m thick) were prepared and then stained with hematoxylin and eosin dye, which mounted in neutral deparaffinated xylene (DPX) medium for microscopic observations.

Statistical analysis

All data were expressed as mean ± S.D of number of experiments. The statistical significance was evaluated by one- way analysis of variance (ANOVA) using SPSS version 7.5 (SPSS, Cary, NC, USA) and the individual comparison were obtained by Duncans' Multiple Range Test (DMRT) (Duncan, 1957). A value of p<0.05 was considered to indicate a significant difference between groups. The data on insulin binding studies were analyzed by competition curve, scatchard plot and average affinity profiles. All values are expressed as Mean ± SD.

RESULTS

Effect of CFEt on plasma glucose and plasma insulin

Figure 1 shows the level of plasma glucose and plasma insulin of different experimental groups. The diabetic control rats showed a significant increase in the level of plasma glucose with significant decrease in the level of plasma insulin. Oral administration of CFEt and glibenclamide to diabetic rats significantly reversed the above biochemical changes.

Competitive binding curves on the binding of insulin to its receptor on erythrocytes

Figure 2 summarizes the ability of non-radioactive insulin to competitively inhibit the binding of ^{125}I-insulin to the insulin receptor on the cell membranes of erythrocytes in rats treated with diabetic, CFEt and glibenclamide.

Comparison of the plots showed that the insulin recap-tor on the cell membranes of erythrocytes from rats treat-ed with diabetic control bound significantly less ^{125}I- insulin than did cells from CFEt and glibenclamide treated diabe-tic rats at the same unlabelled insulin concentrations.

The percentage ^{125}I-insulin bound to the insulin recap-tor on the cell membrane of erythrocytes from diabetic rats was significantly lower than the percentage bound to those of CFEt and glibenclamide treated diabetic rats at very low unlabelled insulin concentrations (0 and 1 ng/ml). Comparison of the competitive curves of the percentage

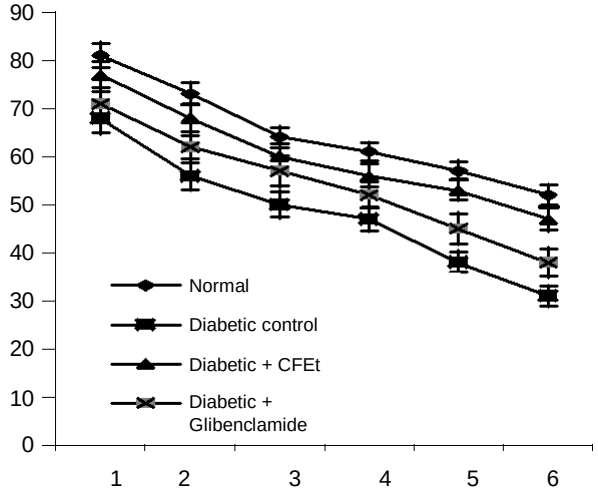

Figure 2. Competitive binding curves showing the effect of CFEt and glibenclamide in diabetic rats on the binding of insulin to its receptor on erythrocytes. Percentage of ^{125}I-insulin bound is plotted as a function of the unlabelled insulin concentration.

of ^{125}I-insulin bound to the insulin receptor on erythrocytes of diabetic, CFEt and glibenclamide treated diabetic rats showed slopes that decreased steadily at an unlabelled insulin concentration of 6 ng/ml.

Bound / free ratio

The bound/free (B/F) ratio of the labelled hormone is expressed as a function of the bound hormone, yielding a Scatchard Plot for erythrocytes (Figure 3) . Curvilinear plots were obtained for the diabetic, CFEt and glibenclamide rats. A greater B/F implies more bound hormone than free. Comparison of the plots showed that the insulin receptor on the cell membranes of erythrocytes from CFEt and glibenclamide treated diabetic rats had maximum B/F compared with diabetic rats.

Scatchard analysis of affinity profile and receptor binding sites in erythrocytes

To analyze the above changes in affinity more precisely, we calculated K_d, K_e and K_f. 'Dissociation constants of high-binding sites' (K_{d1}) were significantly decreased in diabetic rats and increased in diabetic rats treated with CFEt and glibenclamide. 'Receptor numbers' (B_{m1}) for K_{d1} was maximum in rats treated with CFEt and glibenclamide when compared with diabetic rats. Further 'Dissociation constants of low binding sites' (K_{d2}) analysis (Table 1) showed a significantly decreased affinity in erythrocytes from diabetic rats and significantly increased affinity in rats treated with CFEt and glibenclamide.

Similarly, 'Receptor numbers' (B_{m2}) (Table 1) were also

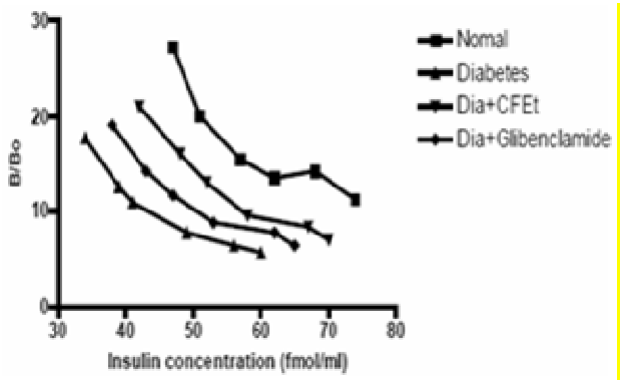

Figure 3. Scatchard plot showing the effect of CFEt on the binding of insulin to its receptor on erythrocytes of normal and experimental rats. Bound/Free ratio is plotted as a function of the insulin bound B/Bo. Di a- Diabetic control.

decreased in diabetic rats whereas CFEt and glibenclamide administration significantly increased the B_{m2}. Analysis of the data shows that erythrocytes from rats treated with CFEt and glibenclamide had an 'empty site' affinity (K_e), which began to decrease with increasing occupancy of the receptor sites by ^{125}I-insulin; the average receptor affinity progressively decreased to the 'filled site affinity' (K_f) (Table 1).

Histopathological changes

Pathological changes of pancreas (Figure 4) include parenchymal inflammation (Figure b) and atrophy of -cells and vascular degenerative changes in the islets (Figure c) in diabetic control rats. The pathological changes were reduced in rats treated with CFEt (Figure d) and glibenclamide (Figure e).

DISCUSSION

Insulin receptors have been demonstrated in cells of a large variety of tissues from different animal species. It has been shown that binding of insulin receptors in different tissues is heterogeneous, which may be due to a multiple class of receptors, to negatively co-operate bet-ween receptors or combination of both (Ward and Harri-son, 1986). Insulin binding to receptors is the first event in insulin action and this first step represents a major control point for insulin effects *in vivo*. Insulin bind-ing to receptors is not a fixed biologic process but rather is subjected to modulation by alterations in either recap-tor or affinity (DePirro et al., 1980). Many studies have shown decreased insulin binding in diabetes mellitus (Olefsky and Kolterman, 1981; Kolterman et al., 1981). In the present study, the number of insulin binding sites decreased in diabetic rats, whereas CFEt increased the number of bind

Table 1. Effect of CFEt and glibenclamide on K_{d1}, K_{d2}, K_e, K_f , B_{m1} and B_{m2} in erythrocytes of control and experimental rats.

Groups	Normal	Diabetic control	Diabetic + CFEt (450 μg/kg)	Diabetic Glibenclamide (600 (μg/kg)
Dissociation constant of high affinity binding sites (K_{d1}) [$x10^{-10}$ M^{-1}]	3.28±0.19 [a]	0.95 ± 0.06 [b]	2.40 ±0.15 [c]	2.15 ± 0.11 [d]
Dissociation constant of low affinity binding sites (K_{d2}) [$x10^{-8}$ M^{-1}]	25.42±1.51 [a]	6.49±0.45 [b]	12.65±0.78 [c]	15.64± 0.84 [d]
Number of high affinity binding sites (B_{m1}) [f mol/mg]	54.98± 3.27[a]	12.48± 0.86[b]	34.49± 2.21[c]	30.50±1.66[d]
Number of low affinity binding sites(B_{m2}) [f mol/mg]	119.04±7.07[a]	65.39± 4.49[b]	98.83±6.28[c]	92.01±5.01[c]
Affinity constant for empty receptors (K_e) [x10 $^{-8}$ M^{-1}]	16.54±0.98[a]	5.37±0.37[b]	7.91±0.50[c]	7.68±0.42[c]
Affinity constant for filled receptors (K_f) [$x10^{-7}$ M^{-1}]	3.70± 0.18 [a]	0.84± 0.04 [b]	1.82± 0.11 [c]	2.20± 0.11 [d]

Values are means ± SD for 6 rats in each group. [abcd] With in columns, means with different superscript letters differ significantly at $p < 0.05$ (DMRT).

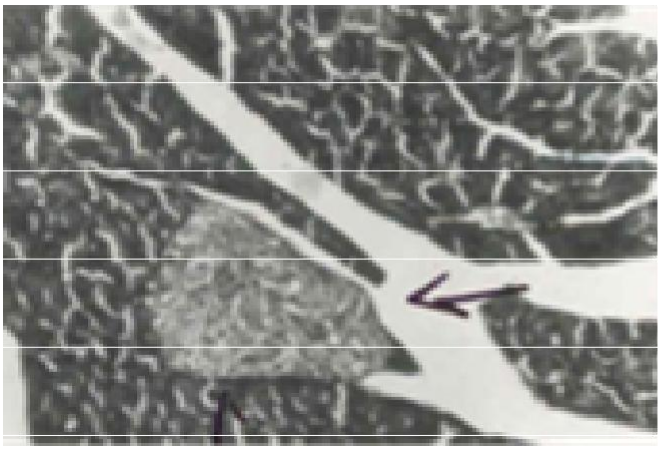

Figure 4a. Normal rat pancreas: H&E x 20. Pancreas showing in islets.

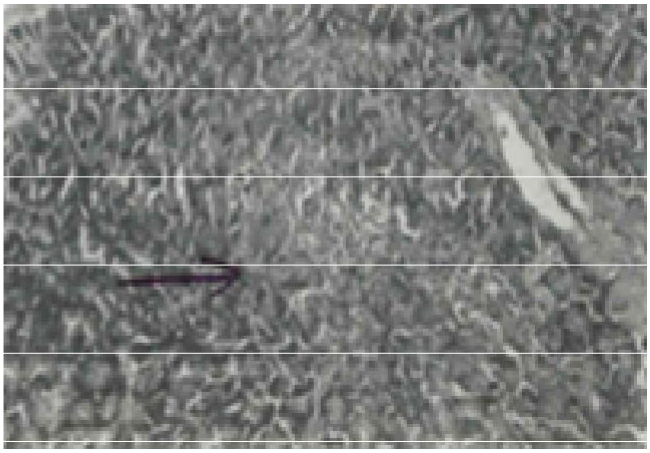

Figure 4b. Diabetic control rat pancreas: H & E x 20. Parenchymal inflammation

binding sites. This treatment significantly decreases plasma glucose and increases plasma insulin level. In the present study, the number of insulin binding sites decreased in diabetic rats, whereas CFEt increased the number of binding sites. Hyperglycemia caused a significant reduction in the receptors of erythrocytes and insulin target tissues. In the present investigation, treatment with CFEt and glibenclamide showed significant antihyperglycemic activity. This is probably indicative of efficacy of the drugs. In addition, despite that the CFEt stimulate insulin secretion and glibenclamide sensitizes the receptors, we have also shown that diabetic rats treated with CFEt increased the number of insulin binding sites (Latha and Pari, 2003). Using erythrocytes as the study tool, we have shown a decreased specific binding of insulin to erythrocyte insulin receptors in the diabetic rats. This phenomenon appears to be not only due to a significant decrease in the receptor concentration per cell but also to a marginal decrease in the affinity of the receptor. As insulin levels decreases in diabetic rats, insulin binding to to its erythrocyte receptors also decreases (Olefsky and and Reaven, 1976; Sukhinder and Clandinin, 2001).

The data shown in Table 1 clearly demonstrate that insulin binding decreased at lower non-radioactive insulin

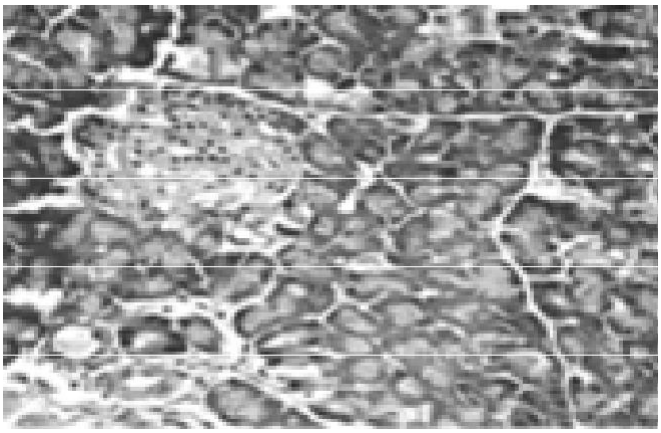

Figure 4c. Diabetic rats pancreas: H & E x 20. Atrophy of β-cells and vascular degenerative changes in the islets

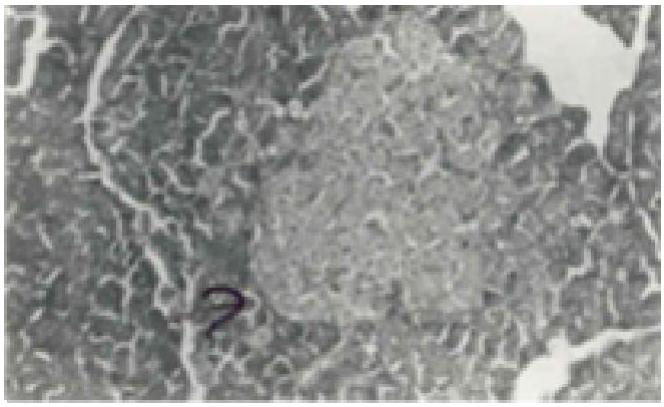

Figure 4d. Diabetic + CFEt rat pancreas: H&E x 20. Near normal appearance of islets

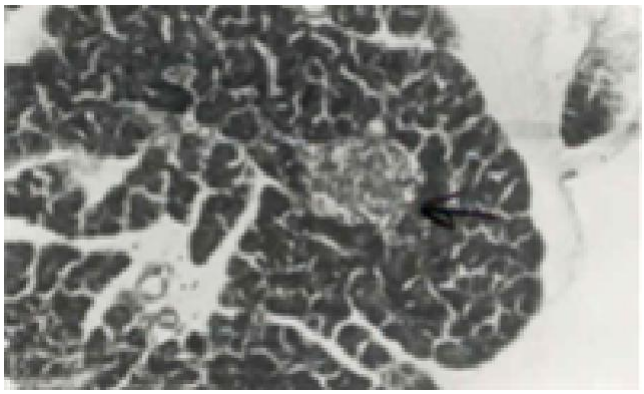

Figure 4e. Diabetic + Glibenclamide rat pancreas H&E x 20. Fatty infiltration in islet and shrinkage

concentrations, but the curves converge at the higher non-radioactive insulin levels, this pattern suggests a change in receptor affinity. The decrease in insulin binding attributed to a decrease in receptor affinity was confirmed by the decrease in the average affinity (K) for erythrocytes.

To investigate whether the decrease in insulin binding could be attributed to a decrease in the number of recaptor sites per cell, we used a Scatchard plot to analyze the data. Using this method of analysis, the x-intercept represents the number of insulin receptor sites per cell. The calculations revealed that a decrease in the number of receptor sites per erythrocyte in diabetic control rats compared with CFEt and glibenclamide treated rats. Therefore, in type 2 diabetic conditions the decrease in insulin binding due to defect in insulin secretion and its receptor could result in a decrease in the number of receptor sites per cell.

The co-operative interactions among receptor sites can be explained in terms of a “negative co-operativity” model. Negative co-operativity is a frequent occurrence in hormone-receptor systems with site-site interactions, resulting in a decrease in the apparent affinity of receptor for insulin when fractional saturation of the receptor increases. According to this model and a calculation of the number of receptor sites using Scatchard analysis, the decreased insulin binding observed is primarily due to a decrease in the number of receptor sites. The results suggest that the lower number of receptor sites per cell in diabetic rats could be the result of primary alteration in the receptor or might be secondary to some other alterations in the integrity of the membrane (Slater, 1984). Such changes in membrane integrity could be responsible for the decreased number of receptor site per cell. The resulting membrane dysfunction can impair transport of glucose across the cell membrane, which leads to the observed hyperglycemia.

The contributing effect of any alteration in receptor affinity was evaluated by average affinity. The significant decrease in the empty site affinity for erythrocyte sup-ports the concept of alterations in both receptor affinity and receptor sites, each contributing to decreased insulin binding. Some of the insulin receptors on the cell membranes of erythrocytes from diabetic rats can be affected by the cytotoxic effect of streptozotocin and become desensitized as the consequence of increased glucose concentration. Desensitization is associated with a total lack of insulin effect despite remaining insulin receptors. Several possibilities exist to explain the mechanism of changing receptor affinity and desensitization. Primarily, fluidity may be an important factor in the modulation of insulin binding and action. Further, the insulin receptor may be covalently associated with another protein that modulates receptor affinity. It is therefore, a possibility that over production of free radicals due to lipid peroxidetion may alter the interaction of insulin with its receptors, thus affecting the ability of insulin to differentially regulate its receptor and this regulator protein (Harmon et al.,

1980). Free radicals especially increased generation of NO in diabetic state may also affect the formation of the insulin receptor complex. Another possibility is that the receptor undergoes post-translational modification that alters binding and signal transmission properties (Kasuga et al., 1982). The post-translational modification could involve a change in the redox state of the receptor. The insulin receptor is composed of major subunits linked by disulfide bonds to various oligomeric forms. Reduction of the oxidized forms of the receptors could modify the affinity of insulin (Massague and Czech, 1982).

The advantage of using circulating erythrocytes for investigating the receptor status in human and animals is that they are more easily accessible than cells of primary insulin target organs, such as adipocytes and muscles (Okada, 1981; Tomasevic et al., 2003). The present study suggests that CFEt stimulates insulin secretion and glibenclamide sensitizes the receptors, due to increase in the binding of insulin to its receptors. Oral hypoglycemic agents have been reported to improve the insulin recaptor status (Ragoobirsingh et al., 1990; Proks et al., 2002; Patane et al., 2000). In our study, the administration of CFEt and glibenclamide increased the insulin binding to isolated erythrocyte insulin receptors.

In our study, histopathological observation in diabetic control parenchymal inflammation and atrophy of -cells and vascular degenerative changes in the islets in pancreas. The reaction is provoked by the increased production of highly reactive intermediates of streptozotocin, which are normally detoxified by endogenous GSH but when present in excess, can deplete GSH stores, allowing the reactive intermediate to react with and destroy pancreatic cells (Blum and Fridovich, 1985).

The above pathological changes were reduced in diabetic rats treated with CFEt and glibenclamide.

The histological evidence of diabetic control rats suggest that structural alte-rations at the end of 45 days are due to streptozotocin-induced free radical generation quite early in diabetes.

Thus in addition to blood glucose lowering effect, histopathoogical observations also supports the notion that CFEt at 450 μg/kg-body weight produced significant antihyperglycemic activity by protecting the erythrocyte against streptozotocin action.

In conclusion, the present study showed that oral treatment with the CFEt and glibenclamide improved the erythrocyte membrane insulin binding sites with concomitant increase of plasma insulin.

The molecular basis for each event that occurs after the binding of insulin to its receptor remains to be examined.

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