Chapter-7

Trypsin resistant oral insulin helps higher and sustained bioavailability of insulin in alloxan induced hyperglycemic mice

Introduction

The role of dermcidin in cardiovascular disorder and in the formation of diabetes was reported. We've got conjointly the neutralization of the effect of dermcidin, from our experiment, we have analyzed that dermcidin might be the cause of diabetes and aspirin/insulin neutralize the effect of dermcidin. Actually, we wanted to neutralize glucose level in diabetes patients and tried that through non-invasive method and we prepared oral insulin for the convenience of diabetic patients. Insulin, a hypoglycaemic macromolecule hormone discovered by Best and Banting, has a vital role in the carbohydrate metabolism in the transduction of energy for the survival of all forms of animal lives; there is no alternative to the effect of insulin. It is demonstrated in IDF DIABETES ATLAS, (Sixth edition), most of the 382 million people with hyperglycemia are aged between 40 and 59, and 80% of that people live in middle- and low-income nations. All types of diabetes are on the increase; particularly people with type-2 diabetes will increase by 55% by 2035. Insulin has the lifesaving property; sometimes it does not work that means insulin resistance in the system i.e. type-2 diabetes and sometime its concentration decreases in the body i.e. type- 1 diabetes. So, at the event of the diabetic condition, it is essential to inject insulin in the system as orally administration of insulin is disrupted by the digestive enzyme in the tract, and, so the essential hormone is not sufficient for its action in that way. Albeit different trials have been made to plan orally effective insulin for the control of hyperglycemia, the accessibility of oral insulin readiness has not yet been accomplished. We report in this an orally active trypsin safe insulin preparation by utilizing industrially accessible any sort of recombinant insulin by utilizing a straightforward strategy that can be executed anyplace without utilizing high innovation or costly instruments that requires prepared faculty with ability in protein science.

Result

Effect of oral ingestion of insulin preparation on the level of glucose and insulin in alloxan treated diabetic mice

When milk-insulin suspension (400 µL) (containing 0.08 U of insulin in the milk) as described in Methodology and followed by 3h 200 µL of the same preparation (0.04 U insulin) was fed to alloxan treated mice, that is reported to produce diabetic mice mimicking type I diabetes mellitus in human (Thurston et al, 1975) . It was found that the plasma glucose level of these mice decreased from 508±10 mg/dL (before the insulin preparation was fed) to 220±8 mg/dL at 3 h and to 130±10 mg/dL after 7 h ($p < 0.001$, $n = 20$). Thus, the feeding of milk-insulin preparation to the alloxan treated diabetic mice was found to control the hyperglycemic effect for 11 h.

In a separate experiment, using the same mice, when the plasma insulin concentration was determined at different hours after the feeding of the milk-insulin suspension preparation as described, it was found that the plasma insulin concentration in the alloxan treated mice, which was 3±1.1 µ Unit of insulin/ml before the feeding was found to increase to 13±1.4 µ Unit of insulin/ml at 3 h and to 18±1.5 µU of insulin/ml at 7 h after feeding.

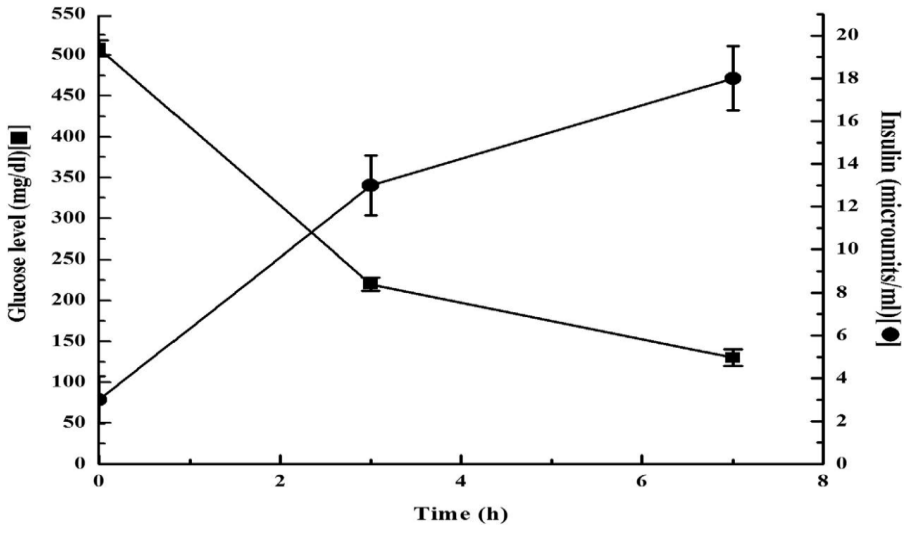

Fig-7.1: Level of glucose and insulin after oral ingestion of insulin preparation to the alloxan treated diabetic mice. *After ingestion of 0.4 mL insulin preparation (containing 0.08 unit insulin) and followed by after 3 h, ingestion of 0.2 mL of insulin preparation to the alloxan treated diabetic mice, the glucose level was decreased from 508±10 mg/dL to 220±8 mg/dL after 3 hr and glucose level was decreased to 130±10 mg/dL after 7 hr and insulin concentration were 3±1.1 µU of insulin/ml before the feeding of the insulin preparation was found to increase to 13 ±1.4 µU of insulin/ml at 3 h and to 18±1.5 µU of insulin/mL at 7 h respectively.*

The distribution of insulin at different parts of the body after feeding the oral insulin (milk-insulin) to the diabetic mice.

To determine whether the insulin was actually distributed in different parts of the body, the blood samples were taken from different parts of the body (as described in Table 7.1) at different time intervals and enzyme linked immunosorbent assay was performed by using insulin antibody. It was found that the insulin (µU/ml) was distributed in different parts of the body and increased with the time (Table 7.1).

Blood samples from artery and vein from different parts of the body	**Distribution of insulin (µUnits/ml) at different time intervals**		
	0 h	**3 h**	**7 h**
Tail vein	3 ± 1.1	13 ± 1.4	18 ± 1.5
Left & right superior vena cava	2.8 ± 1.2	14.2 ± 1.6	18 ± 2.2
Hepatic vein	4 ± 1.7	13.8 ± 1.9	17.1 ± 2.0
Femoral vein	3.5 ± 1.3	14 ± 1.1	17.3 ± 1.4

Table7.1: Distribution of insulin in different arteries of the diabetic mice after feeding the milk-insulin in the mice. *Milk-insulin preparation solution was prepared as described in Method and Material section. The solution was fed to the diabetic mice as described and blood was collected from different parts of the body at different time intervals. The amount of insulin in the blood samples were determined by ELISA using insulin antibody.*

The direction of the movement of milk-insulin preparation solution was calculated from Gibbs free energy (ΔG) equation

The direction of movement of the milk-insulin preparation was based on the thermodynamics of the system. The diffusion of a substance between two sides of a membrane: A(out) ⇋ A(in), thermodynamically resembles a chemical equilibrium. Here, A = Casein-Insulin complex.

A difference in the concentrations of substance on two sides of the membrane generates chemical potential difference:

$$\Delta G_A = G_A(in) - G_A(out) \qquad (1)$$

[ΔG_A is the chemical potential of A = Casein-Insulin complex, expressed in partial molar free energy] by putting the value of $\Delta G_A = RT \ln [A]$ in equation…. (1)

It was found that, $$\Delta G_A = -RT \ln \frac{[A]_{out}}{[A]in} \qquad (2)$$

By putting the value of A = Casein-insulin complex in equation…...(2)

$$\Delta G_A = -RT \qquad (3)$$

[as conc. of insulin in the external milieu $[A]_{out} = 0$, conc. of insulin in intestinal loop $[A]_{in} = 12 \times 10^4$ μ U/ml] By putting the value of Gas constant (R) and temperature (T)

in Kelvin scale in equation…(3) $\Delta G_A = -8.314$ J mol^{-1} $K^{-1} \times 310$ K [as, R = 8.314 J mol−1 K−1 and T = 37 °C = 310 K]

then, Δ GA = -2577 J. mol^{-1}

So, Δ GA < 0, i.e Δ G is negative which indicated that the emerge of combination of casein-insulin mixture to the milieu was energy independent and spontaneous process facilitated by diffusive transportation (natural entropy) (Bank *et al*. 2016).

As Δ GA < 0, from the experiment, it was found that the rate of efflux of the insulin (K1) from the intestinal loop to the external milieu (Tyrod's buffer) was 2.62 μ Units/ml/h whereas on the other hand entering of insulin from the milieu in the intestinal loop was undetectable (0 μ Units/ml/h) K2 = 0, because coming out of insulin from the intestinal loop was found to increase at different time and like a hyperbolic curve was constructed (Supplementary Fig. S1, indicates the process was not a simple diffusion but was facilitated diffusive transportation) i.e. no backward movement was found to occur in this case. It was inferred that casein-insulin complex that transported out from the intestinal wall was a spontaneous process. The possibility whether insulin-casein complex itself was capable of synthesizing insulin in the intestinal wall was carried. In order to purify the insulin mRNA from external milieu, the mRNA from milieu was applied to the column of oligo (dT)-cellulose and optical density measured at 260 nm. The fractions were eluted by the buffer containing 10 mM Tris (pH 7.5); 1 mM EDTA and 0.05% SDS. The fractions were translated in vitro by using plant ribosomes, mixture of all 20 amino acids (1 μ M each) and ATP (1 mM) as described (Chakrabarty *et al*. 2009). The fractions showing the highest activity for the in vitro translation of insulin were pooled. Synthesis of cDNA was performed by RT-PCR (Reverse transcriptase-polymerase chain reaction) of the isolated mRNA of external milieu for the amplification of the synthesized

product by using insulin gene specific primers, but no newly synthesized insulin was found in the intestines used in the experiment.

Extended presence of insulin in the trypsin treated oral insulin preparation solution.

From the above result it can be suggested that the recombinant insulin which was added in the milk solution was safe from the tryptic digestion in the digestive tract. To clarify that added insulin was actually resistant from the proteolytic effect of trypsin, the insulin incubated milk suspension solution was treated with pure trypsin (200 ng/ml) (physiologic level) at 37 °C for different time. And the survival time of insulin from the digestion of trypsin was analyzed by immunoblot analysis as demonstrated in the Method. It was found that 80.1% of the insulin survived from the trypsin treatment even after 3 h of incubation at 37 °C compared to the trypsin treatment on insulin only (0% insulin survived) (Fig. 7.2).

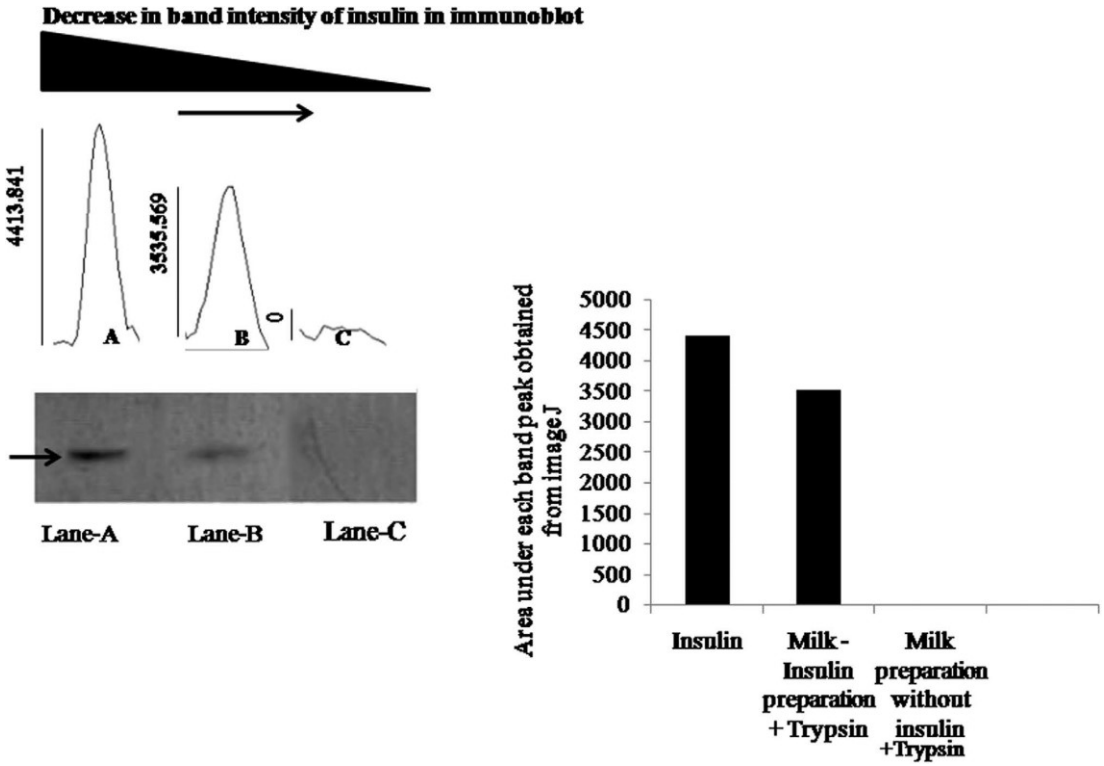

Figure7.2: Western blot analysis of trypsin treated oral insulin preparation solution. *The figure exhibited the augmentation of insulin in trypsin treated milk-insulin solution. It was seen that trypsin was not able to debase insulin in the solution (milk-insulin). In the figure, Lane-A indicated the band of insulin (only) by western blot analysis where trypsin was not added and an arrow line (→) specified that insulin band in Lane-A; Lane-B showed the band of insulin in trypsin treated insulin preparation after 180 min and Lane-C represented trypsin treated milk preparation without insulin by western blot analysis using insulin antibody. From the Image-J analysis, it was found that 80.1% of insulin remained unaltered in lane-B compared to insulin (alone) in lane-A after 180 min whereas no positive band was found in Lane-C.*

Discussion

Here, we found that the incubated mixture of insulin and milk at 23°C for two and half hours was an important step before the precipitation of protein by the treatment of 0.6M acetic acid and it was also observed that when acetic acid was treated with insulin-milk mixture without pre-incubation of milk and insulin no hypoglycemic effect on the insulin preparation as described above could be found in alloxan treated diabetic mice. It has been suggested that milk could have a role on the inhibition of trypsin digestion in our digestive tract (Shehadeh *et al*., 2001; Whitmore *et al*. 2012). It has also been argued that milk can protect insulin in new born babies from the tryptic effect (Kinouchi *et al*., 2000; Shulman, R. J. 1990) our results interpreted that casein might be involved in the protection of insulin from the trypsin digestion in the digestive tract of alloxan induced hyperglycemic mice where casein bound insulin co-precipitated by acetic acid play the role. One of the most crucial steps in this experiment was the incubation of milk with insulin and precipitation by acetic acid and as a result serine protease could not show its effect on insulin molecule. Intestinal loop experiment explained that the milk-insulin came out from the intestinal tract to the circulation by the thermodynamically favored system which depended on Gibbs free energy (ΔG) of the reaction so the process was a facilitated diffusion which does not require energy and the process is mediated by transporter protein which is membrane embedded. From the result, it can also be demonstrated that the orally fed milk-insulin was disseminated uniformly in different arteries of the studied mice. Despite the modern painless injection, unpleasantness and anxiety of insulin injection are still prevailing, especially for the baby and under aged persons. As the diabetics who need repeated insulin injections in a lifetime, so, insulin injection continuously might make other problems specially soreness, bruising, infection, irritation commonly seen at the injected area. Muscle cramp some time occur.

Lipohypertrophy at the site of injection is often found where lump can be developed. Hypertrophy can numb the area of injection, so sometime we are unable to feel the pain and the most problematic is that uneven growth of cell can inhibit absorption of insulin. In that sense this oral insulin might be the alternative and safer for the insulin injection. From the above experiment it was found that our oral insulin is basal insulin which demonstrated from its duration of action. So, our preparation of oral insulin contained only recombinant insulin and common food preparation which are available commercially. Therefore, this oral insulin might be helpful for the young and old diabetes patients to normalize the hyperglycemia especially for the economically disadvantaged people all over the world.