Chapter-6

Aqueous extract of tobacco leaves (AETL) and hypoxia actuated hyperglycemia induced by dermcidin protein through the impediment of GLUT4 translocation and NO production

Introduction

Hyperglycemia or diabetes is one of the cardinal reasons of death around globe because it is the prime cause of the formation of atherosclerosis (American Diabetes Association; The National Heart, Lung, and Blood Institute; The Juvenile Diabetes Foundation International; The National Institute of Diabetes and Digestive and Kidney Diseases; and The American Heart Association, Circulation1999). Hyperglycemia/diabetes (type 1 & type 2) is due to the impairment of glucose induced insulin secretion as well as in the reduction of glucose uptake by the skeletal muscle cells even in the presence of insulin produced under normal circumstance. Long term exposure of hyperglycemia is recognized in recent research as a major pathogenesis in diabetic complication and as well as in atherosclerosis (Brownlee *et al*. 2005; Aronson *et al*., 2008). Hyperglycemia produces a large number of modifications at the cellular level that ultimately culminates to atherosclerosis (Paneni *et al*., 2013). Various kinds of stresses, including occupation (Ferrie *et al*., 2016; Norberg *et al*., 2007), hypoxaemia (Newhouse *et al*., 2017), emotional, all have been implicated in the development of hyperglycemia. Environmentally induced stresses, including hypoxia and tobacco leaf smoking have also been reported to produce hyperglycemia (A Report of the Surgeon General. Atlanta: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health, 2014). It has also been claimed that mitochondrial dysfunction plays a pivotal role in glucose toxicity in diabetes due to the stress factor where *O*-GlcNAcylation was found to elevate due to the hyperglycemia (Banerjee *et al*., 2015). Unfortunately, no mediator of the stress induced hyperglycemia has been so far identified. It has been also reported that the perturbed balance between the NO and $O_2^{\cdot -}$ plays the role in the genesis of hyperglycemia through endothelial cell dysfunction (Galougahi *et*

al., 2016) but how nitric oxide is involved in that mechanism is still remained discordant. The deposition of pancreatic amylin was previously thought to be involved in hyperglycemia (Kahn *et al*., 1999; Clark *et al*., 1996); the role amylin in the development of hyperglycemia still remains an unresolved issue.

In the above context, it should likewise be noted that although hyperglycemia (DM2) has been equated to atherosclerosis itself no mechanistic explanation is available for the pathogenesis of atherosclerosis even in context of dermcidin. We have herein reported that the synthesis of protein caused by unrelated environmentally induced stresses is determined to be dermcidin due to the expression of its gene. We have also analyzed about dermcidin induced inhibition of vesicle trafficking of GLUT4 to the plasma membrane triggered by insulin. Here we have also demonstrated that intravenous administration of dermcidin in the circulation of normal-non diabetic mice produced hyperglycemia within minutes mimicking diabetes in human. Furthermore, we also have explored that the protein possessed more anti-insulin property than glucagon.

Results

Expression of dermcidin isoform-2 protein in muscle cells of mice treated with aqueous extract of tobacco leaves (AETL)

Previously we reported the occurrence of stress induced DCN-2 protein in the state of low partial pressure of O_2 in case of high altitude ((Bank *et al*., 2014) and also in the circulation of acute myocardial infarction (AMI) patients (Bank *et al*., 2014). It is well known that cigarette can induce oxidative-stress in the human body (Lan *et al*., 2016) and also help in triggering diabetes (Borowitz JL & Isom GE, 2008; Bergman *et al*., 2012). To investigate the role of this stress inducer in the disease formation, cigarette extract was added to the homogenized muscle cells and incubated for 2h and production of dermcidin was analyzed by *in-vitro* translation by ELISA using by dermcidin antibody. It was found that the synthesis of dermcidin (350±7nM) was maximal at the amount of 10 (w/v%) of AETL (Fig-6.1). From the Pearson test for the correlation "r" between the increase amount of cigarette extract (w/v) and the increase production of dermcidin was determined to be r= + 0.9339 (two tailed p value, $p<0.05$), indicating that the increase concentration of nicotine was highly and directly correlated with dermcidin production.

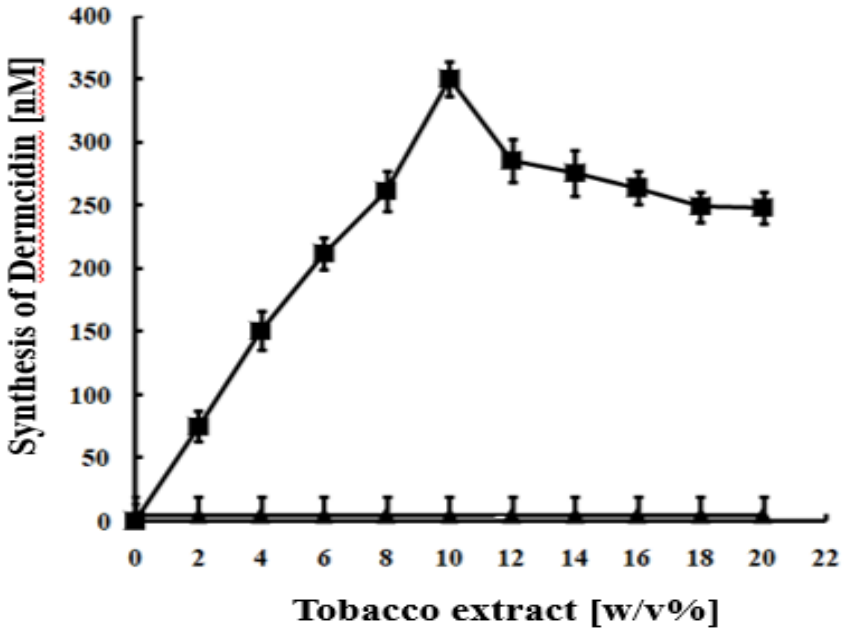


Fig-6.1: Synthesis of dermcidin in muscle cells of mice in presence of different (w/v) of AETL *Different amount of AETL 10 (w/v%) were incubated with homogenized muscle cells (skeletal muscle) of mice and synthesis of dermcidin was determined by in-vitro translation by ELISA by using dermcidin isoform-2 antibody. It was found that the synthesis of dermcidin isoform-2 was found to maximum in the presence of 10 (w/v%) amount of AETL. Here solid rhombuses (♦) indicated the synthesis of DCN-2 protein in the presence of different amount (w/v%) of AETL and (●) solid circles represented the DCN-2 synthesis in muscle cells without AETL.*

Expression of DCN-2 gene in neutrophil cells treated with AETL

So, stress can induce DCN-2 gene expression. In hypoxic condition when O_2 is depleted i.e. the partial pressure of O_2 was very low and in the presence of cigarette extract, DCN-2 protein was found to synthesize. Reverse transcription-polymerase chain reaction (RT-PCR) was performed to demonstrate the DCN-2 gene expression from the cDNA isolated from neutrophil cells treated with cigarette extract and to run the PCR, gene specific dermcidin primers (forward primer 5'- CAGCCAGCCTTTGTTGACTTAACAGGTGG-3' and reverse primer 5'- CGTAAAGCCTGCTGCTCCTGGG-3') were used (Klock *et al.*, 1976). In control experiment, amplification of the DCN-2 gene was done by PCR from cDNA of the sample where tobacco extract was not added. It was found that cigarette extract treated neutrophil cells over express DCN gene in lane-1.

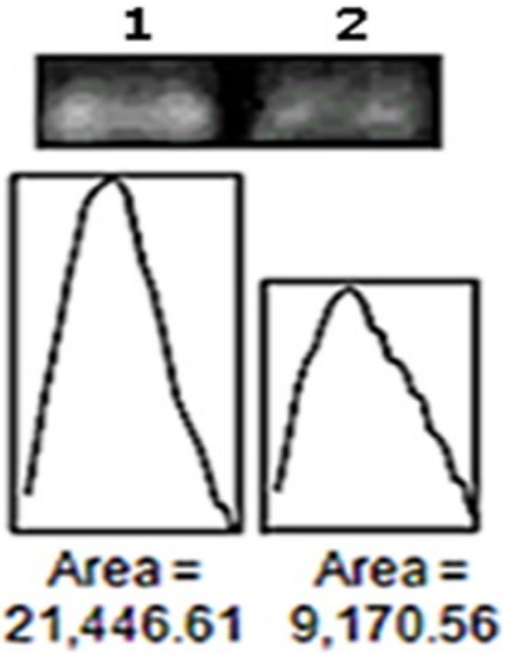


Fig-6.2: Analysis of the expression of dermcidin-isoform-2 gene by RT-PCR *mRNA was extracted from the AETL treated neutrophils. Expression of cDNA was determined by semi-quantitative RT-PCR and expression of dermcidin gene demonstrated in lane-1, and the control experiment in lane-2 demonstrated cells without AETL. From the Image-J analysis, it was found that the band intensity of cigarette induced dermcidin was 2.5.*

Expression of dermcidin gene in hypoxic condition

In the hill area where the partial pressure of oxygen is very low, dermcidin was synthesized there (Bank *et al*., 2014). So, to determine the protein in low oxygen, production of DCN protein was quantized by ELISA in hypoxic rat at different time intervals (6h, 12h, 24h and 7days), as described in the methodology. It was found that the expression of the protein was found to elevate in rat accordingly with the wing of time exposure in hypoxic condition and it was found that the release of dermcidin was maximal (92±4.7 nM) at 7day (168h).

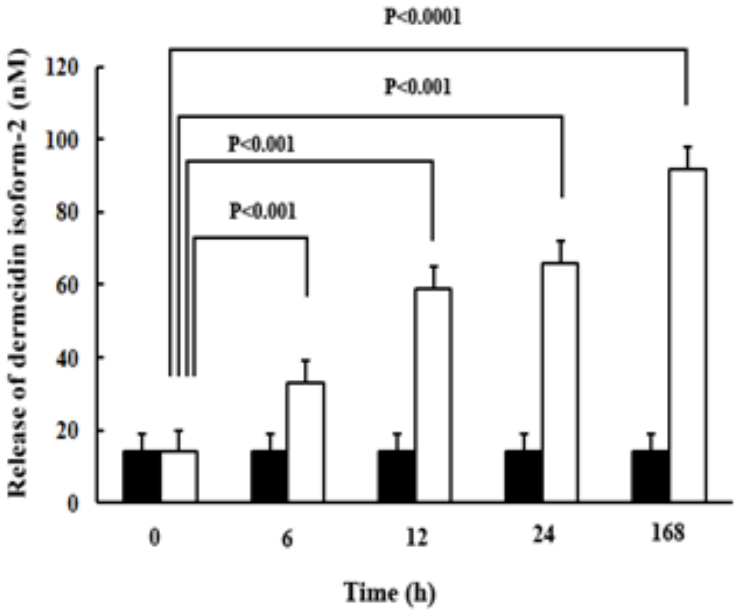


***Fig-6.3**: **Expression of dermcidin in hypoxia:** Synthesis of DCN was maximal (92±4.7nM) at 7days; it was found from the student-t test that 'p' value of DCN release between the control and 168h is p<0.0001; between control and 6h is p<0.001; between control and 12h is p<0.001; between control and 24h is p<0.001. Here solid bars ( ) represented the DCN level of rats which were not exposed to the hypoxic condition*

Effect of different altitude on dermcidin synthesis:

As with the increase of altitude partial pressure of oxygen is depleted, so there is a stressful condition in the hypoxic state. We wanted to investigate the synthesis of dermcidin in that hypoxic state if any.

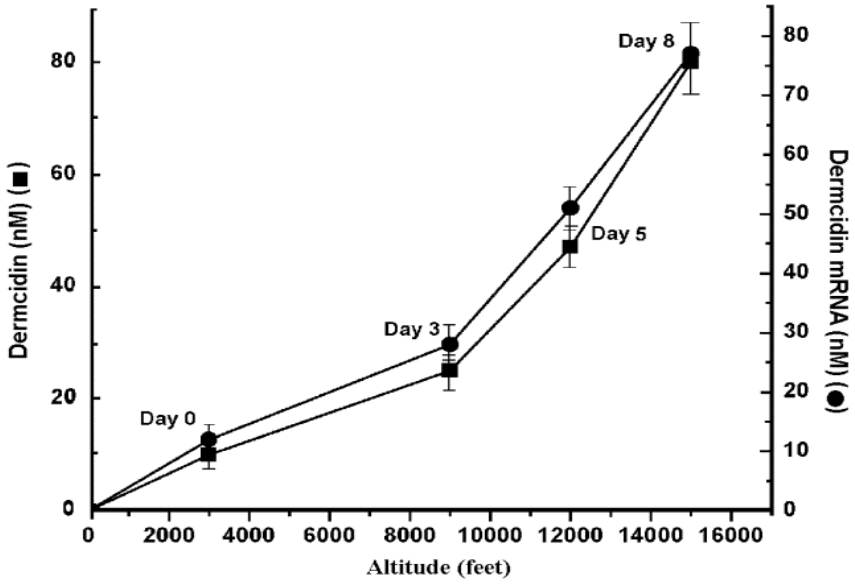


Fig-6.4: Dermcidin in high altitude*: In this figure solid squares (■) demonstrated the plasma dermcidin concentration and the solid circles (●) explained dermcidin synthesis (from dermcidin mRNA) in the leucocytes with increasing altitude. At 8th day and 15,000 feet height dermcidin was found to be maximum.*

Dermcidin isoform-2 injection can increase the glucose level in the circulation of mice

Previously it was reported from our laboratory, stress can induce dermcidin protein, which was likewise capable to activate platelet aggregation (Bank *et al*., 2014), and was a potent inhibitor of all forms of nitric oxide synthases (Ghosh *et al*., 2012), as it was known that inhibition of NOS might impair the glucose uptake by the muscle cells through the insulin signaling pathway (Sato *et al*., 2016) so it could produce hyperglycemia. To determine the effect of DCN-2 protein, it was injected into the overnight fasting mice. After injecting the DCN-2 protein (0.35µM), the plasma glucose level was found to increase in the mice and blood was drawn from the tail vein of mice at different time interval and production of Nitric Oxide (NO) in the withdrawal blood was

measured by methemoglobin method and NO level was found to decrease with the increase of glucose concentration in the circulation of mice.

When 15µM amount of aspirin or 30µUnits of insulin/mL was injected to the dermcidin induced hyperglycemic mice, the increased glucose level was found to normalize and concomitantly nitric oxide level was found to increase.

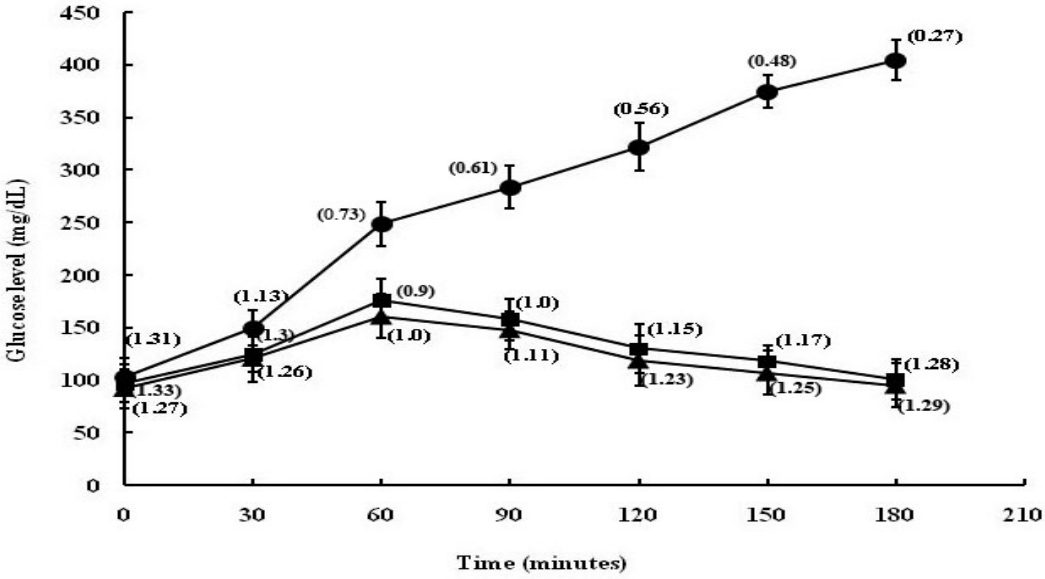


Fig-6.5: Increased level of glucose in the circulation after injecting dermcidin isoform-2 protein to the tail vein of mice *to determine the effect of DCN-2 protein, it (0.35µM) was injected to mice and they were found to be hyperglycemic. And when the blood was taken at different time intervals, the production of nitric oxide was found to decrease with the increase of glucose level. Injection of 15µM acetyl salicylic acid or 30µU insulin/ml was able to nullify the effect of DCN-2 induced hyperglycemia and helped in the increase of nitric oxide level (physiological concentration). Here, solid circles (●) represent the glucose level after injecting the DCN-2 protein; solid squares (■) represented the glucose level after the injection of acetyl salicylic acid and solid triangles (▲) represented the*

*glucose level after the injection of insulin to the tail vein of mice; levels of Nitric Oxide (NO) (mean data) were demonstrated in brackets [**( )**] in nmol/mg/h.*

Dermcidin isofrom-2 induced inhibition of glucose uptake in skeletal muscle cells of mice

From the above result, it was found that DCN-2 is a hyperglycemic agent and this was due to the impairment of insulin receptors. As muscle cell is the major sites of insulin utilization, and then at the hyperglycemic state muscle cell would be unable to transport glucose, the rate limiting step of glucose utilization by muscle cells (Rodnick *et al*., 1992) and glycogen synthesis is stimulated by insulin, and defects in each of these step develops a peripheral insulin resistance. To test this possibility, chunks of muscle cell (0.025gm) were incubated in the presence of insulin (30μUnits/mL) and DCN-2 protein (0.35μM) in Kreb's buffer solution for 2h at 37°C; it was found that glucose uptake was inhibited in the presence of DCN-2 protein.

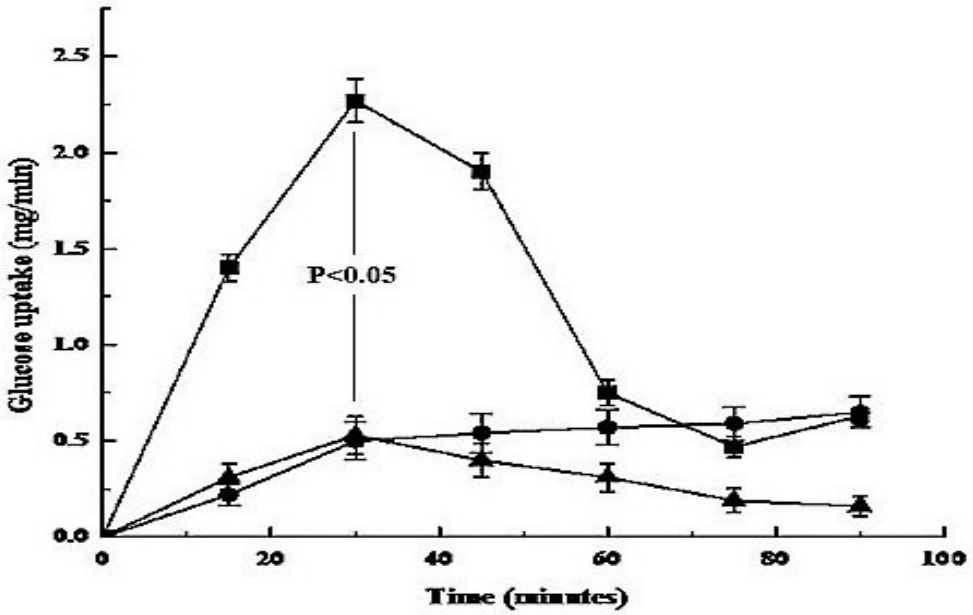


Fig-6.6: Impairment of glucose uptake in the muscle cells of mice by DCN-2 *This figure showed DCN-2 (0.35 μM) mediated inhibition of glucose uptake in the muscle cells of mice. From the figure, it was found that glucose uptake was hampered by muscle cells when DCN-2 was incubated even in the presence of insulin; here solid triangles (▲) represented the glucose uptake in the presence of DCN-2 and solid squares (■) represented the glucose uptake in presence of insulin where DCN-2 was not incubated; solid circles (●) represented the glucose uptake by muscles only.*

Determination of insulin binding on the muscle cells of mice by Scatchard plot

Equally it was found that dermcidin protein was a diabetogenic protein and it also impaired the glucose uptake in muscle cells, thus it was postulated that the protein might possess the critical role on insulin binding activity of the muscle membrane. To test the role of this dermcidin protein on the binding of insulin, pre-incubation of the protein in muscle cells was performed in the presence of insulin and in other separate experiment incubation of the protein was done with muscle cells both the presence of acetyl salicylic acid (15μM) and insulin (30μUnits/mL); and scatchard plot were performed; we included acetyl salicylic acid in our experiment because from the above it was found that aspirin was able to neutralize the effect of DCN-2 and normalized the glucose level. From the Scatchard plot analysis, a heterogeneous binding site population of insulin was revealed on muscle cells of mice in all instances. Previously it was also reported that acetyl salicylic acid (aspirin) was able to normalize the activity of DCN-2 by specific doses of aspirin in acute myocardial infarction (Bank *et al*., 2014). It was found that in the presence of DCN-2, binding of insulin was inhibited whereas acetyl salicylic acid (15μM) was able to sensitize again the muscle cells to the binding of insulin like normal. In each case the curvilinear binding sites of insulin were found like normal i.e. heterogeneous binding site population. In presence of dermcidin the binding of insulin low affinity (K_{d1})

= 1.28 and high capacity (n_1= 0.37X10^5 molecules/muscle cell). But in the presence of acetyl salicylic acid the binding of insulin; low affinity (K_{d1}'=1.99), high capacity (n_1'=1.12X10^5 molecules/muscle cell) binding sites of insulin were found close to the normal binding of insulin (control) where no acetylsalicylic acid or no dermcidin was added. So, DCN-2 induced binding of insulin was three times more inhibited in a hyperglycemic state of mice.

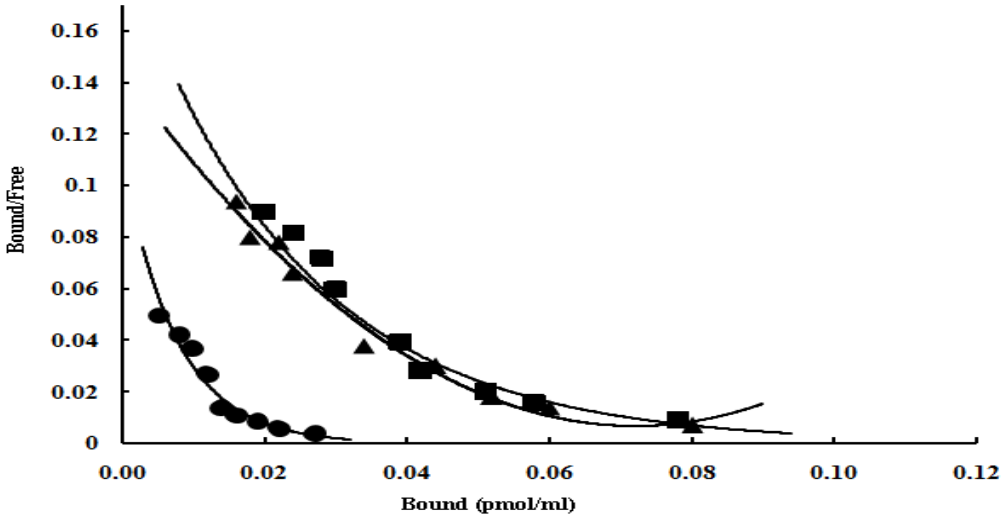
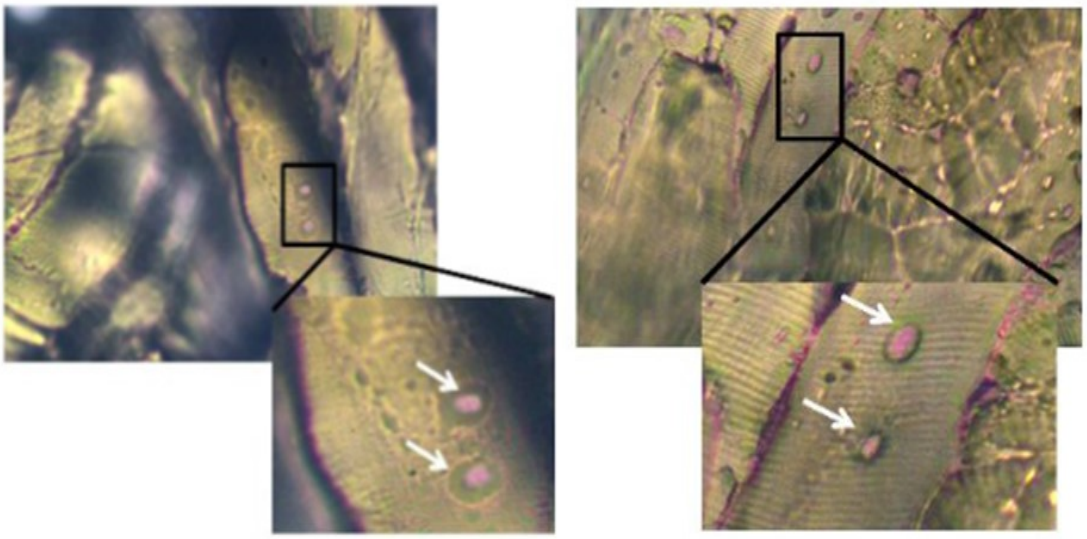


Fig-6.7: Inhibition of the binding of insulin on muscle cells of mice due to the presence of dermcidin isoform-2 *This figure demonstrated the binding of insulin on skeletal muscle cells of mice in presence of a) only insulin (30µUnits/mL) b) insulin (30µUnits/mL)+dermcidin isoform-2 (0.35µM), c) insulin (30µUnits/mL)+acetyl salicylic acid (15µM)+dermcidin isoform-2 (0.35µM). In the above figure, solid squares (■) demonstrated the binding of insulin in the presence of insulin only; solid triangles (▲) represented binding of insulin in the presence of insulin and DCN-2; solid circles (●) represented binding of insulin in the presence of DCN-2 only on muscle cells. From the figure it was found that binding of insulin was inhibited in presence of dermcidin and the dermcidin induced inhibition was corrected by acetyl salicylic acid and as a result, insulin again was able to show its binding effect on muscle cells of mice.*

Dermcidin induced impairment of GLUT-4 translocation in muscle cells of mice

From the previous study, it was found that a GLUT-4 protein leads a role in glucose uptake in muscle cells (Derave *et al*., 1999; Karlsson *et al*., 2009, Lauritzen *et al*. 2008) and maintains the glucose homeostasis in cells. Skeletal muscle is insulin sensitive and insulin helps in glucose transportation, so in diabetic condition, impairment of insulin blocks the signaling pathway of glucose transporters.

To find out whether the binding of insulin was inhibited by dermcidin was actually mediated through impairment of GLUT-4 protein; the immunofluorescence (IF) analysis was performed in grated muscle cells in the presence and absence of dermcidin. In set-I experiment, pre-incubated (2h) muscle cell with dermcidin was incubated with insulin in tyroid's buffer for 30 minutes at 37°C and direct IF was performed. Simultaneously, the set-II experiment was performed in the same way except the condition of preincubation of muscle cell with DCN-2. From the IF technique under a fluorescent microscope with the help of fluorescent tagged GLUT-4 antibody, it was found that the GLUT-4 translocation was hampered in presence of dermcidin isoform-2 (PANEL-B).



PANEL-A **PANEL-B**

Fig-6.8: Impairment of GLUT-4 translocation due to the presence of dermcidin isoform-2 *This figure demonstrated the GLUT-4 translocation on the muscle cells of mice in presence and absence of dermcidin isoform-2; here* ***Panel-A*** *represents the IF study of GLUT-4 translocation on muscle cells of mice in the presence of insulin where DCN-2 was not incubated and Panel-B represents the IF study of GLUT-4 translocation on muscle cells of mice in presence of DCN-2. From the figure it was found that GLUT-4 translocation was inhibited in* ***Panel-B*** *where DCN-2 was added to the reaction mixture.*

Synthesis of GLUT-4 in muscle cells of mice

GLUT-4 was a well-known protein for the transportation of glucose through its translocation. We reported that glucose plays the role of synthesis of insulin in the liver and also demonstrated that GLUT-4 was synthesized in liver cells by glucose activated nitric oxide synthase (Bhattacharya S *et al*. 2013). From this study, it was found that DCN-2 can inhibit the GLUT-4 translocation. It was found in fig-6.5 that acetyl salicylic acid was able to abolish the effect of DCN-2 and helped in the decrease of sugar level *in-vivo* mice; it was also observed in fig-2C that binding of insulin on muscle cells of mice was resuscitated in the presence of acetyl salicylic acid. From our laboratory it was claimed that dermcidin isoform-2 is a potent inhibitor of all forms of nitric oxide synthase.

Thus, the relation between nitric oxide production and GLUT-4 expression in the presence of DCN-2 and AETL was determined in the study. For this purpose four separate experiments were demonstrated - a) homogenized muscle cells were incubated with insulin (30µUnits/mL) for 2h; b) homogenized muscle cells were incubated with insulin (30µUnits/mL) and dermcidin isoform-2 (0.35µM) for 2h; c) homogenized muscle cells were incubated with insulin (30µUnits/mL) and AETL 100 (w/v%) for 2h

and d) incubation of muscle cells with insulin (30μUnits/mL) and 1mM NAME (N^G-methyl-*l*-arginine acetate ester). In all cases mRNA was isolated in respective incubated mixture and *in-vitro* translation was performed. Synthesis of GLUT-4 was quantized by ELISA by using GLUT-4 antibody from the translation products and simultaneously the production of nitric oxide of the above-mentioned mixtures was measured by methemoglobin method. From this experiment it was found that the synthesis of GLUT-4 was inhibited in the experimental mixture where b) dermcidin isoform-2, c) nicotine and d) NAME were respectively present. From the Student's t-test, it was found that the GLUT-4 production of the above-mentioned mixture was significantly different ($p<0.05$) from the GLUT-4 production in muscle cells in the presence of insulin only. The production of nitric oxide was also inhibited in these cases compared to the production of nitric oxide in insulin incubated muscle cells only. NAME was added in the experiment to check the production of Nitric Oxide.

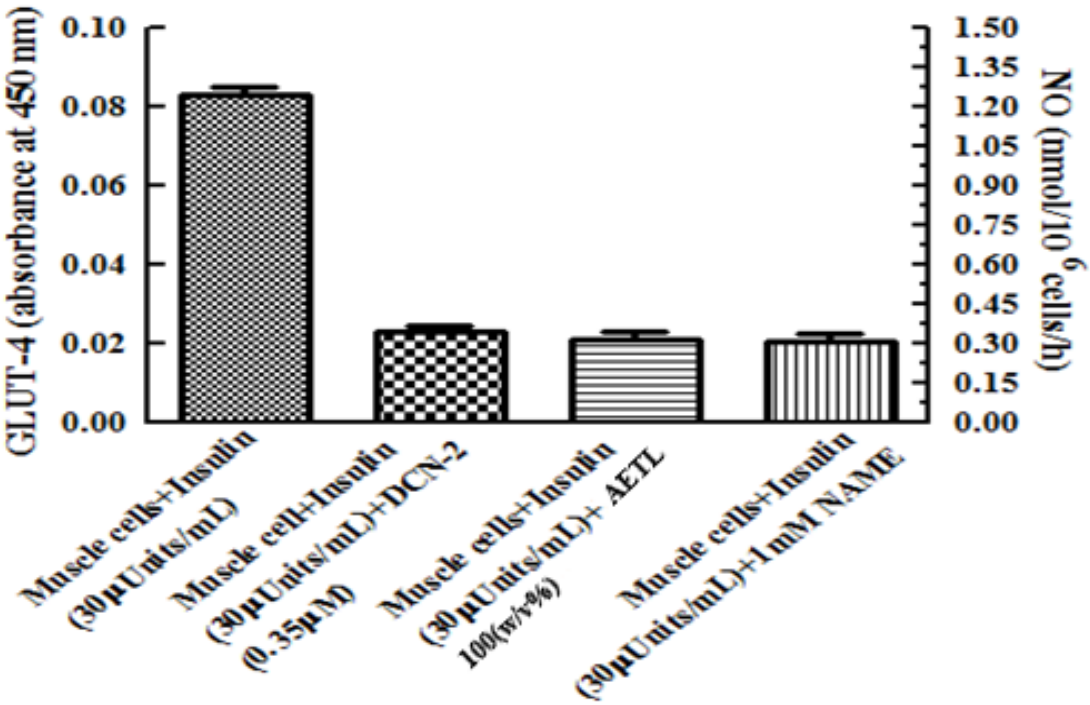


Fig-6.9: GLUT-4 synthesis in muscle cells *This figure demonstrated that the synthesis of GLUT-4 relates to the production of Nitric Oxide (NO). Here, muscle cells synthesized maximum GLUT-4 with concomitant increased production of NO in the presence of insulin only; on the other hand, in the presence of DCN-2, nicotine and NAME synthesis of GLUT-4 was diminished with the decreased production of NO. Synthesis of GLUT-4 in muscle cells in presence of insulin was significantly different ($p<0.05$) from the synthesis of GLUT-4 in presence of DCN-2, AETL and NAME.*

Glucagon and dermcidin isoform-2 induced emancipation of stored glucose

From the above figure-6.5, it was found that dermcidin isoform-2 played the critical part in the development of hyperglycemia within minutes. Glucagon is also a well-known physiologic hyperglycemic agent. Though secretion of glucagon is necessary in hypoglycemic state, but it was found that glucagon played one of the major roles in the formation of diabetic state. In the consequence of impairment of insulin, stored glucose was being released from liver and muscle cells by glucagon signaling pathway. The least amount of glucose was also found to store as glycogen in kidney cells.

To study the efficacy of the dermcidin isoform-2 and glucagon on the emancipation of glucose from liver, kidney and muscles, chunks of these cells were separately incubated with (a) glucose+insulin+dermcidin isoform-2 (b) glucose+insulin+glucagon (c) glucose+insulin. It was found that the release of glucose from the cells was higher in each case where dermcidin was added rather than glucagon. From the experiment, it was found that the effect of dermcidin and glucagon on the release of glucose was maximum in liver cells (Fig-6.10: A). It was also found from the experiment that glucagon had little role in

muscle and kidney cells in the release of glucose (Fig-6.10: B, C) where as DCN-2 imparted the major role.

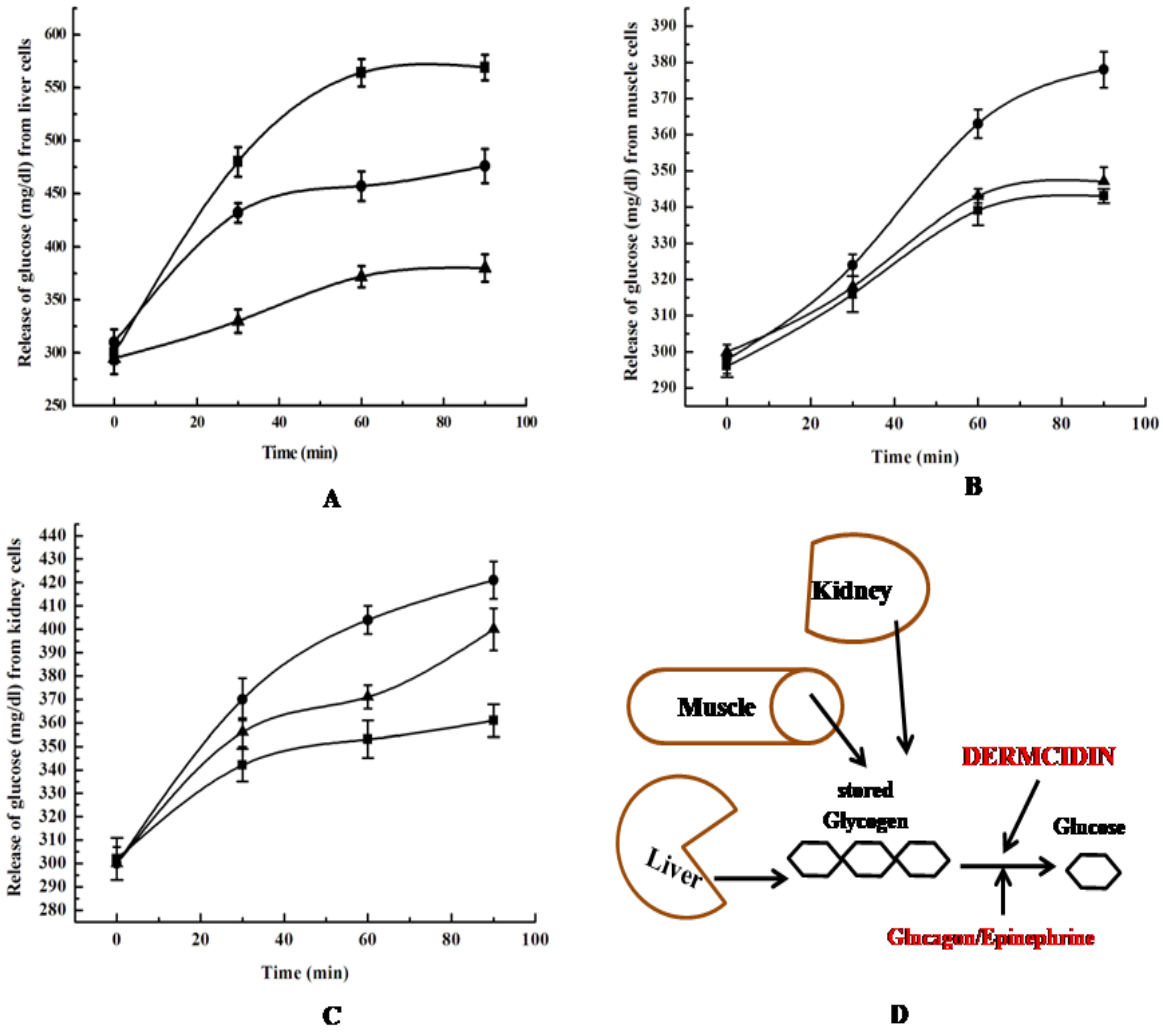


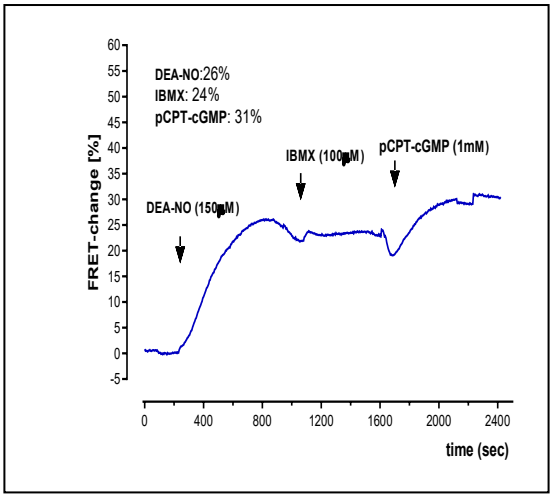
Fig-6.10: Release of stored glucose by dermcidin isoform-2 from liver cells, muscle cells and kidney cells; ***A*** *demonstrated that DCN-2 was able to release stored glucose from liver cells in the presence of glucose and insulin showed in solid squares (■); solid circles (●) represented the release of glucose from the liver in presence of glucagon, insulin and glucose; solid triangles (▲) represented the release of glucose the in presence of insulin and glucose from the liver.* ***B*** *demonstrated release of glucose from muscle cells in presence of DCN-2, insulin and glucose represented in solid circles (●); Release of glucose in the presence of glucagon, insulin and glucose represented in solid*

*triangles (▲); release of glucose in presence of insulin and glucose only represented in solid squares (■). **C** demonstrated the release of glucose from kidney cells in the presence of DCN-2, insulin and glucose presented in solid circles (●), solid triangles (▲) represented the release of glucose in the presence of glucagon, glucose and insulin; solid squares (■) represented the release of glucose in the presence of glucose and insulin. **D**, this diagram demonstrated that glucose was released from stored glycogen (glycogen of liver, muscle and kidney cells) in the presence of DCN-2 and glucagon.*

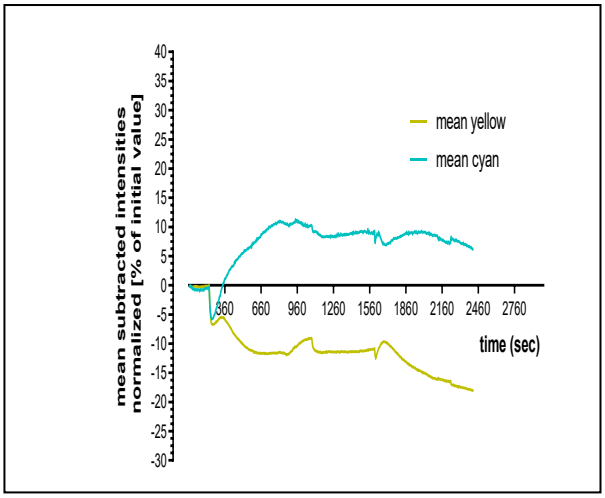
DEA-NONOate induced cGMP regulation

Nitric oxide, a messenger molecule of cGMP, can regulate the cGMP level. It has also been reported that dermcidin can inhibit the nitric oxide (Ghosh R *et al.*2011). To decipher the role of dermcidin in the regulation of cGMP, genetically encoded cGi-500 i.e. a cGMP sensor tagged CHO cells was used and dermcidin (1µM and 2µM) was added to the cGi-500 tagged CHO cells and followed by DEA-NONOate (DEA-NONOate is the combination of diethylamonium and nitric oxide, when this DEA-NONOate was added to the CHO cells, it released nitric oxide slowly in the solution) was added to the same cell, it was found that DEA-NONOate induced cGMP regulation was inhibited in the presence of dermcidin and it was analyzed by the % of FRET changes [Figure-6.11 (G,H,I) (J,K,L & M,N,O)]. Whereas, when dermcidin was not incubated but all other conditions were same, the increase level of cGMP was observed by FRET measurement and analysis (Figure-4 (A, B, C) (D, E, F). So, dermcidin might impart a major role in the inhibition of cGMP. To evaluate above FRET study further, nitric oxide was measured in kreb's buffer solution in the presence of DEA-NONOate and dermcidin. It was also found from the methemoglobin experiment that the released NO from DEA-NONOate was inhibited in the presence of dermcidin.

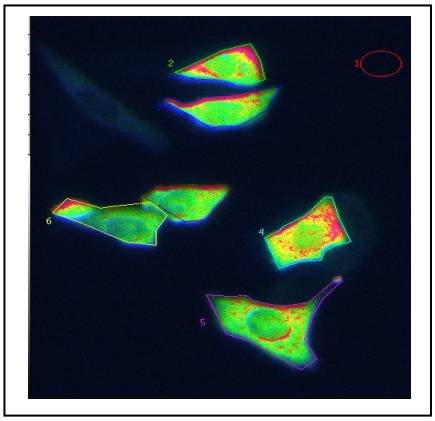
A


DEA-NO:26%
IBMX: 24%
pCPT-cGMP: 31%
DEA-NO (150µM)
IBMX (100µM)
pCPT-cGMP (1mM)
FRET-change [%]
time (sec)

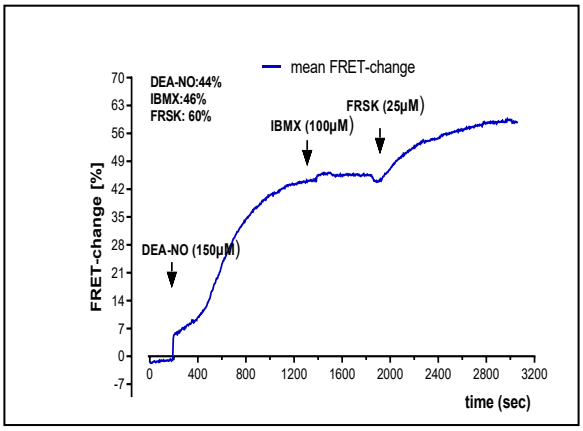
B


mean yellow
mean cyan
mean subtracted intensities normalized [% of initial value]
time (sec)

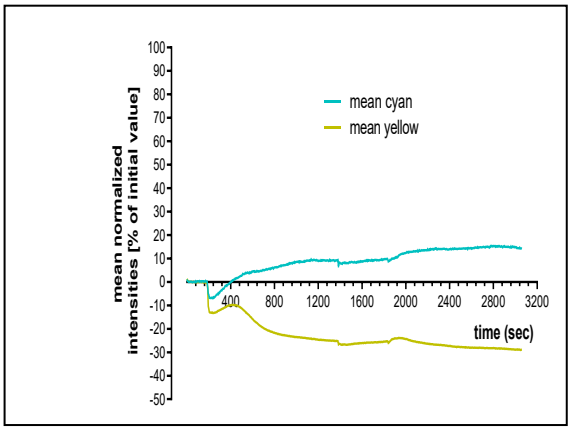
C



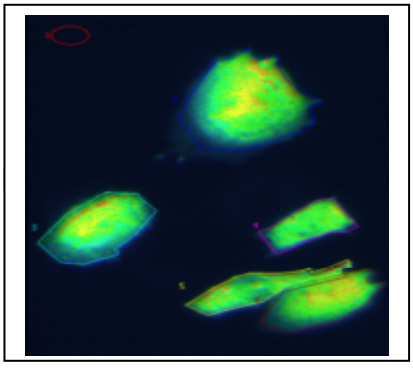
D


mean FRET-change
DEA-NO:44%
IBMX:46%
FRSK: 60%
DEA-NO (150µM)
IBMX (100µM)
FRSK (25µM)
FRET-change [%]
time (sec)

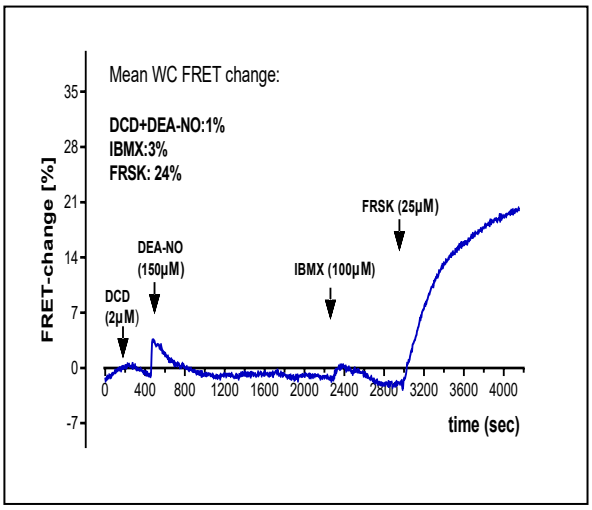
E


mean cyan
mean yellow
mean normalized intensities [% of initial value]
time (sec)

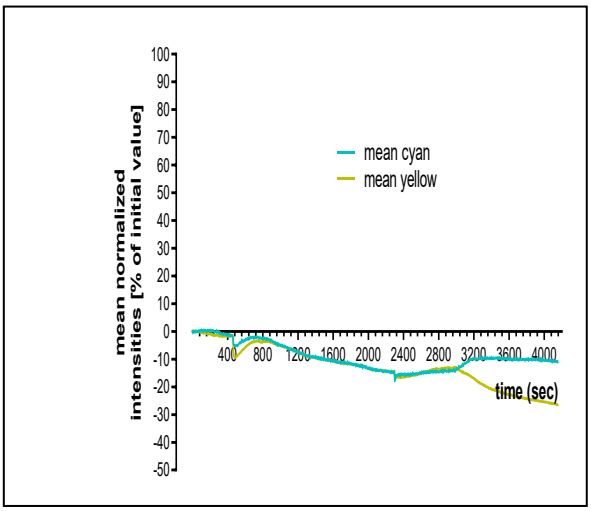
F



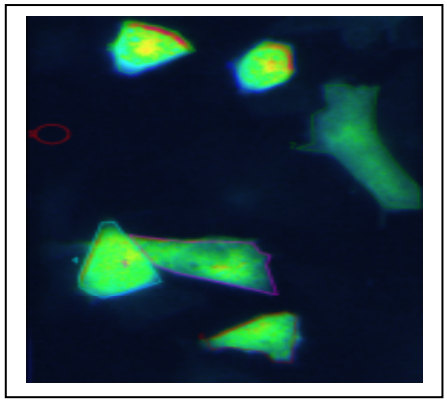
G


Mean WC FRET change:
DCD+DEA-NO:1%
IBMX:3%
FRSK: 24%
FRET-change [%]
DCD (2µM)
DEA-NO (150µM)
IBMX (100µM)
FRSK (25µM)
time (sec)

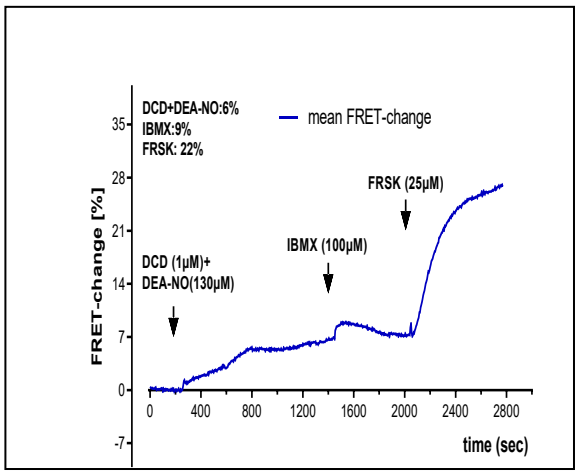
H


mean normalized intensities [% of initial value]
mean cyan
mean yellow
time (sec)

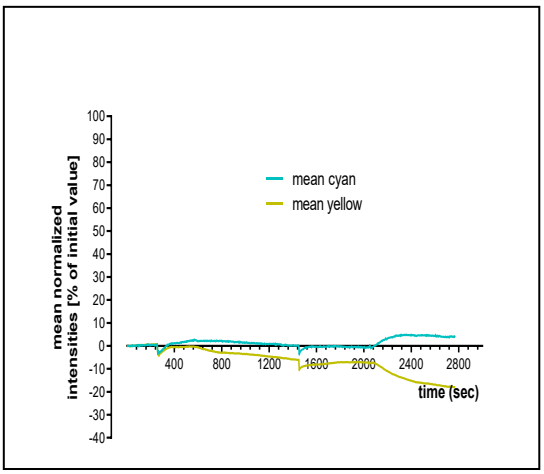
I



J


DCD+DEA-NO:6%
IBMX:9%
FRSK: 22%
mean FRET-change
FRET-change [%]
DCD (1µM)+ DEA-NO(130µM)
IBMX (100µM)
FRSK (25µM)
time (sec)

K


mean normalized intensities [% of initial value]
mean cyan
mean yellow
time (sec)

L

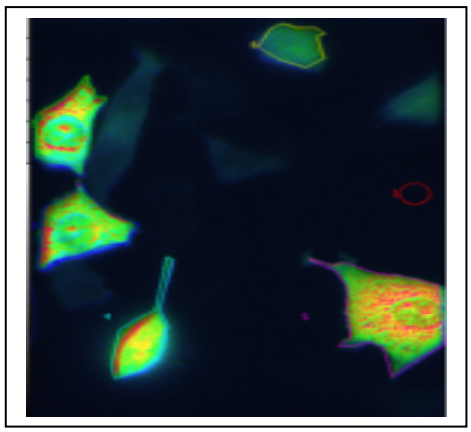


Figure-6.11: DEA-NONOate induced cGMP regulation in presence of DCN (unpublished data). *Here A, B, C & D, E, F indicates the increase level of cGMP in the presence of DEA-NONOate and the data were analyzed by % FRET changes in the CHO cells tagged with cGi-500 sensor and graph B & E show the normal emission of fluorophore antiparallelly; where as G, H, I & J, K, L indicated the inhibition of cGMP in the presence of dermcidin (2μM)+DEA-NONOate & (1μM)+DEA-NONOate respectively and H, K graph show the fluorophores are not emitting properly (lost antiparallel emission). In each case 1st square diagram demonstrated the % FRET changes, 2nd square demonstrated % of intensities of cyan and yellow fluorescence and the 3rd square diagram indicated the corresponding cell (under fluorescence microscope).*

Discussion

We found that the increase level of dermcidin protein in normal volunteers was due to the low partial pressure of O_2 in high altitude area. So, when O_2 is decreased in the atmosphere i.e. in hypoxic condition, dermcidin might be synthesized. From the found result, it can be determined that the dermcidin level is correlated (calculated coefficient of correlation "r" was +0.9405) with the increase of altitude. The level of dermcidin was measured by ELISA in the plasma or in the mRNA isolated from the neutrophil cells of the volunteers. Increased dermcidin level in the circulation might precipitate AIHD in case of high-altitude illness though it is known that atherosclerotic plaque rupture and massive platelet aggregation occur in AIHD, but it is very difficult to determine the plaque rupture if it occur. However, we also found the raised concentration of dermcidin in the circulation of AIHD patients from our laboratory (Ghosh *et al*., 2011).

As it was claimed before that prothrombotic conditions is formed by the slowly ongoing enlargement of atherosclerosis (Vilahur *et al*., 2011), and both type 1 and type 2 diabetes are responsible for the genesis of atherosclerosis (Laing *et al*., 2003; Aronson *et al*., 2002), and the systemic insulin synthesis was found to reduce in presence of upraised dermcidin concentration. So, it can be assumed that increased dermcidin level might be the causative factor for AIHD due to prothrombotic conditions in case of HAI. We have also found the role of a dermcidin protein in the development of hyperglycemia. Hyperglycemia is a major risk factor for stroke, acute myocardial infarction and chronic kidney disease (Kannel & McGee, 1979; Hanefeld *et al*., 2004; Chen *et al*., 2016; Gheith *et al*., 2015). In stroke and AMI, thrombus formation played the main role in disease progression and prognosis. Dermcidin gene expression led the critical role in the pathophysiology of the disease through blood thrombogenicity which influenced thrombus formation on disrupting atherosclerotic plaques. It can be assumed from

different experiments that hyperglycemia and atherosclerosis are the equivalents, but the mechanistic correlation remains elusive and discordant. From our results, it was found that the stress induced protein DCN was synthesized in the presence of AETL. Synthesis of dermcidin was not a metabolic process, but the gene of DCN was expressed due to the presence of nicotine. At low oxygen concentration (partial pressure of oxygen was decreased) i.e. in hypoxic condition, increased level of DCN protein was found in the plasma of rat. Therefore, DCN was synthesized in various stressful conditions and it could also be assumed that the pathophysiology of smoking induced diabetes might be involved with DCN protein synthesis. Administration of that stress induced protein to the tail vain of fasting mice and development of hyperglycemia demonstrated the effect of that protein on the genesis of diabetes and impairment of GLUT-4 translocation. It was reported that nitric oxide was obviously involved in diabetes and was found that resistance of insulin impaired the synthesis of nitric oxide in type II diabetes; in diabetic condition, due to the decreased insulin production, the basal endothelial nitric oxide synthesis is decreased possibly due to the reduced insulin mediated vasodilation in skeletal muscle cells (Petrie *et al.* 1996). As nitric oxide imparts in the inhibition of plaque formation in the wall of the arteries, impaired nitric oxide might play the important role in making of atherosclerosis. So, there is a loop among hyperglycemia, insulin resistance, impaired nitric oxide, hypertension, atherosclerosis and cardiovascular disease. So, here we demonstrated in our experiment that the production of nitric oxide by the very low dose of acetyl salicylic acid was able to reduce dermcidin induced hyperglycemia in mouse models.

Endothelial dysfunction is associated with decreased NO bioavailability and impaired activation of the NO receptor soluble guanylate cyclase (sGC) in the vasculature and in platelets (Cortese-Krott *et al*. 2018). Recent data demonstrate that an intact sGC/PDE5/PKG-dependent signaling pathway exists in RBCs, which remains fully

responsive to NO and sGC stimulators/activators in patients with endothelial dysfunction (Cortese-Krott *et al.,* 2018). Similar to RBC, neutrophil has been shown to influence the NO regulation in some our previous work. The report reveals that NO provides contractile support of heart muscle via a cGMP/ protein kinase G (PKG)-dependent mechanism in contractile dysfunction associated with hyperosmotic stress (Morell *et al.,* 2017). Here, FRET results also uncovered that the released nitric oxide from DEA-NONOate compound elevated the cGMP level, which was calculated by CFP/YFP ratio and when dermcidin was added in the reaction mixture, it was found to affect the cGMP level. Simultaneously in a separate experiment when dermcidin was added in to the DEA-NONOate solution only, it was revealed from the nitric oxide assay (methemoglobin method) experiment that dermcidin might scavenge NO.

Other stress like high salt-induced hypertensive rat model or restraint stress model sGC has been shown to have involvement (Nomura *et al*., 2017).

Here, nitric oxide played the role to the elicit of insulin and we found that the binding of insulin in the muscle cells of mice was restored in the presence of aspirin and the binding restored insulin signaling pathway which inturn was able to restore the GLUT-4 protein translocation of glucose in the membrane of the muscle cells.

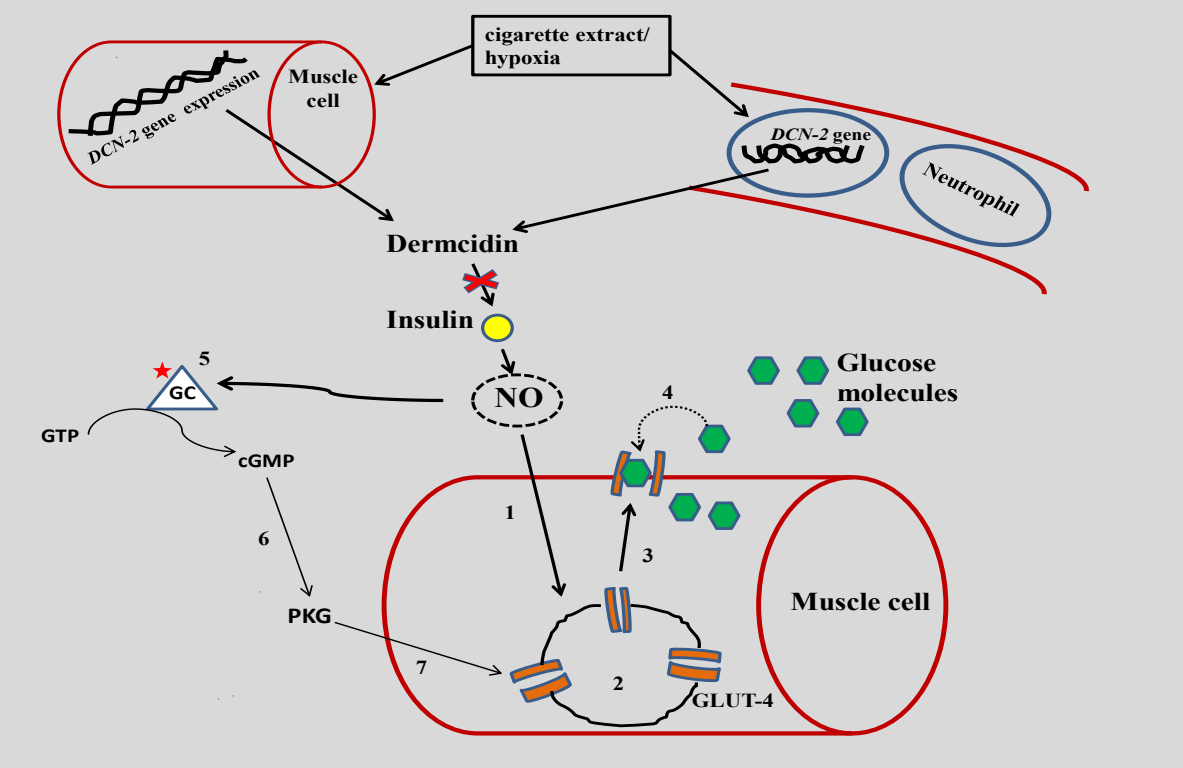


Fig-6.12 Schematic diagram of the mechanism of the inhibition of GLUT-4 translocation. *This diagram demonstrated that Aqueous extract of tobacco leaves was able to express DCN gene in muscle cells and neutrophils. As a result, dermcidin protein inhibited the synthesis of insulin (indicated in yellow circle) and as such the production of nitric oxide (NO) was impaired. This impaired NO was unable to produce signal transduction (1) in muscle cell of mice for the exocytosis of the GLUT-4 protein from the intracellular vesicles (2) to the plasma membrane i.e. GLUT-4 translocation (3) for the uptake of glucose molecule (4) from the circulation to the muscle cells.*

DCN-2 inhibited the insulin induced GLUT-4 translocation to the plasma membrane. GLUT-4 translocation in skeletal muscle cells of mice is a well-known phenomenon for maintaining glucose homeostasis (Derave W et al. 1999; Karlsson HK et al. 2009, Lauritzen HP et al. 2008). In the presence of insulin signal transduction, the latent pool of glucose transporters came out from the internal vesicles to the plasma membrane by

exocytosis. We herein demonstrated that DCN-2 protein counter effect the activity of insulin in comparison to glucagon and it was also observed that the rate of release of glucose (mg/dl min^{-1}) from liver cells was maximized.

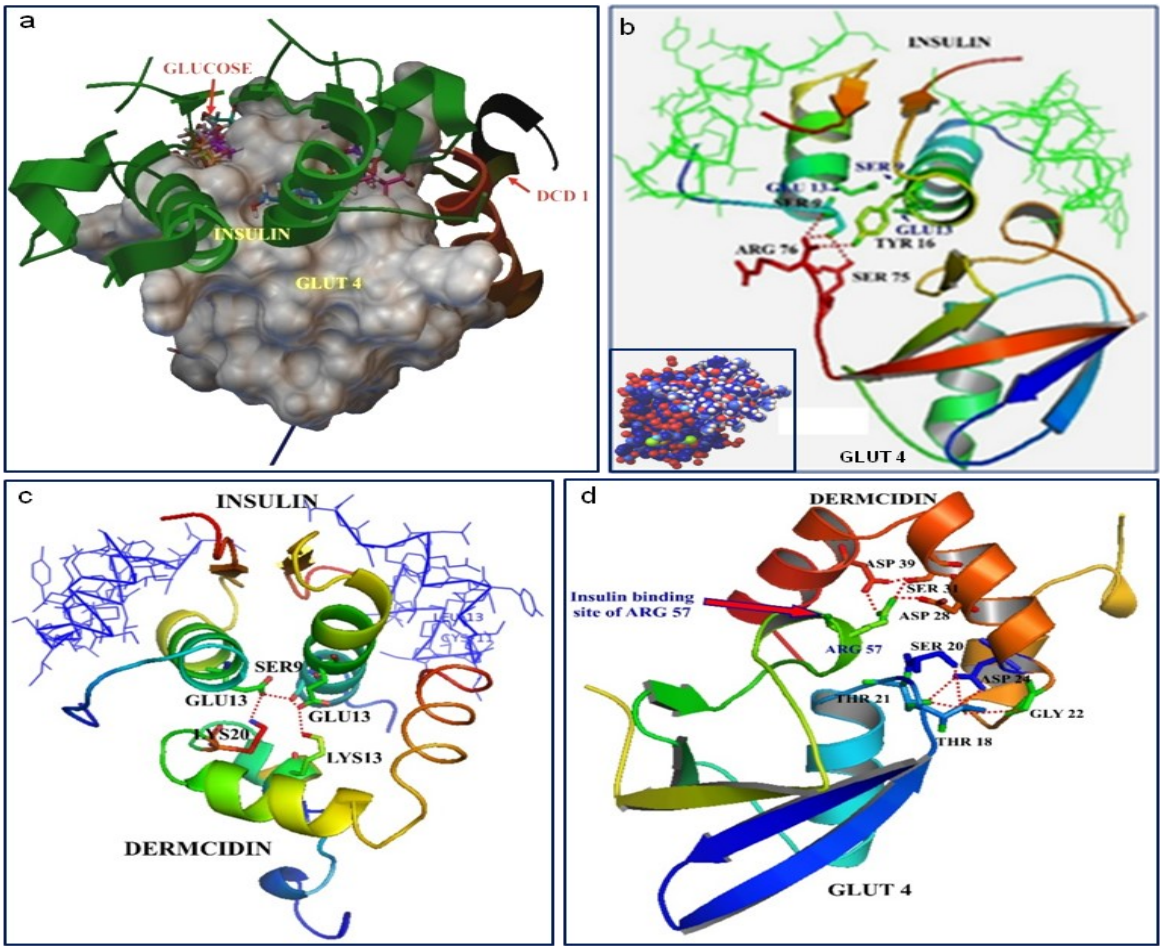


Fig-6.13: Dermcidin, insulin, GLUT4 protein interaction analysis (unpublished)

Therefore, a third party, i.e. dermcidin isoform-2 might play a pivotal role in the genesis of hyperglycemia. So, it can be concluded that the 11kDa protein DCN-2, which was found in the circulation of ACS and AMI (Ghosh *et al*. 2011; Bank *et al*. 2014), might play the role through its DNA synthesis (gene expression) in stressful condition (in presence of nicotine) in neutrophil and also in muscle cells as such insulin signaling is inhibited and consequently nitric oxide production is hampered and produce hyperglycemia through the inhibition of insulin induced GLUT-4 translocation and muscle cells were unable to uptake circulatory glucose. From the results, it can be

hypothesized that different types of stress induced dermcidin protein inhibited the nitric oxide syntheses and vis-à-vis occurrence of insulin resistance was found and it was also reported before that nitric oxide was able to mimic the effect of insulin as such nitric oxide is the "second messenger" of insulin (Kahn *et al*., 2000), so glucose uptake by skeletal muscle cells through the GLUT4 translocation was inhibited by impaired insulin induced signaling of NO or directly by impaired NO production and as a result dermcidin started to create the event of endothelial dysfunction due to the impairment of insulin in the circulation and as such predisposing to acute ischemic heart diseases slowly with formation of plaques in the arteries.