Chapter-4

Diminished synthesis of insulin and its impact on platelet aggregation in cerebrovascular accident

Introduction

Environmental stress factors are also the cause of strokes or cerebrovascular accident which is one of the prevalent causes of disability (neurologic disability) and death (Thapaetal.2013; Donnan et al.2008). Thrombus due to embolism or the atherosclerotic plaque from the different and far off a part of the body (Mattioli et al. 1994) or from the brain, all of these can produce transient ischemic attack (TIA) and from the clinical studies and experiment it has also been reported that the thrombosis is the cause of ischemic stroke (IS) or the development of atheromatous plaque in any one of the major arteries like carotid, cerebral or vertebral arteries of the brain accumulated with macrophage, lipids, debris, fibrous tissues and thrombosis owing to excessive aggregation of platelets where stenosis appeared in the lumen of artery and lead to ischemic stroke due to the impaired blood deliver in the brain even though stenosis is very uncommon case. Regarding hemorrhagic stroke, IS may transform into hemorrhagic however unadulterated hemorrhagic stroke is mostly not the consequence of crack of atherosclerotic plaque. Hypertension and hemorrhagic stroke are the well known reasons behind cerebral amyloid angiopathy (Lammie 2002; Vasilevko et al. 2010). So, it can be inferred that atherothrombus plaque in the brain arteries plays the critical role in stroke.

In the above context it may be mentioned here that although no mechanism of the development of atherosclerosis are well explained in different cases but the mechanisms underlying the proatherogenic function of platelets are increasingly well defined and involved specific adhesive interactions between platelets and endothelial cells at atherosclerotic prone sites (Kaplan and Jackson 2011), diabetes mellitus and hypertension are known to be the two major risk factors for atherosclerosis.

Results

The influence of insulin on the nitric oxide synthesis in normal PRP and in the PRP from the subjects affected with hemorrhagic and ischemic strokes

When PRP from the normal was incubated with different concentrations of insulin for different time intervals at 37°C, we observed that the maximal NO synthesis was reached when 15 μUnit/ml of insulin was added to the normal PRP and incubated for 2h. It was obtained that the utmost production of NO was 1.41 nmol/10^8 normal platelets/h (median), [range 0.63 to 1.98 nmol/10^8 platelets/h (n = 48)]. On the other hand, when the PRP from the ischemic stroke victims was treated with 15μUnits insulin/ml, the NO synthesis was 0.38 nmol/10^8 platelets/h (median), [range 0 to 0.68 nmol/10^8 platelets/h (n = 48)] under the similar conditions. In other word the insulin induced NO synthesis in ischemic volunteers and normal was analyzed from Wilcoxon signed rank test (where, z value = −4.1069, p value is 0, the result is significant at $p \leq 0.05$, two tailed). When the PRP from hemorrhagic stroke was used instead of ischemic stroke or normal, the NO production was 0.08 nmol/10^8 platelets/h (median), [range 0 nmol/10^8 platelets/h to 0.30 nmol/108 platelets/h]. Specifically, insulin induced synthesis of NO in ischemic platelets and hemorrhagic platelets was analyzed by Wilcoxon signed rank test (and z value = −4.0145, p value is 0, the result is significant at $p \leq 0.05$, two tailed) and the insulin induced NO synthesis in the platelets from hemorrhagic platelets and normal platelet was by Wilcoxon signed rank test (z value = −4.4573, p value is 0. The result is significant at $p \leq 0.05$, two tailed). HS and IS had different clinical characteristics like the different amount of NO production.

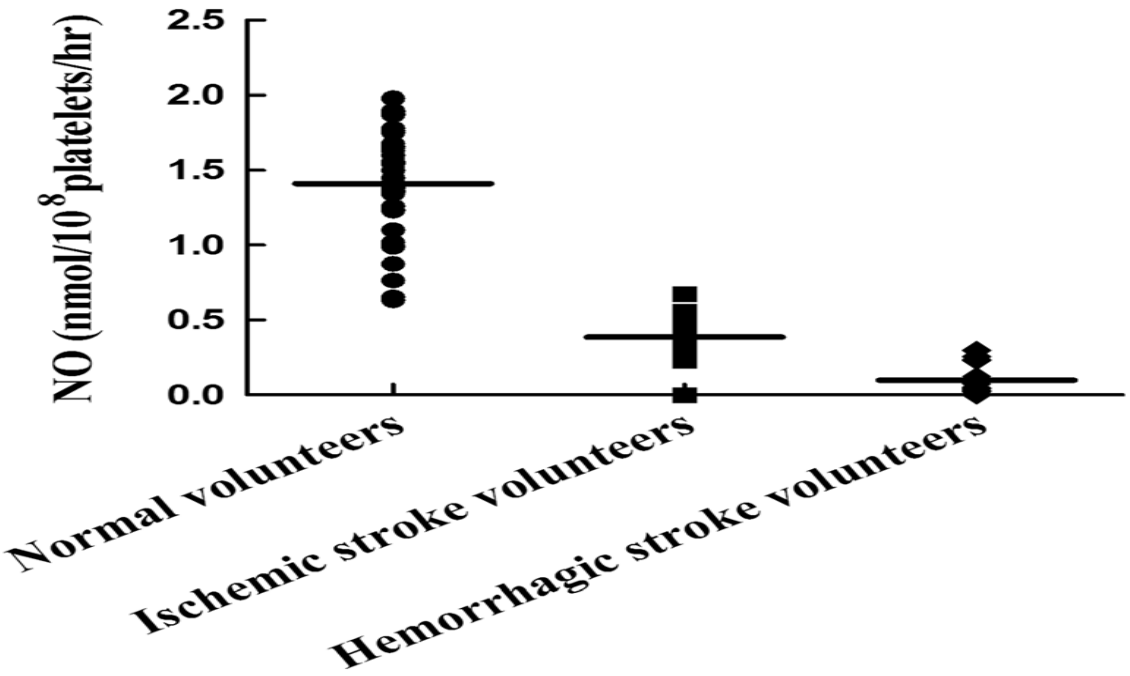

Fig-4.1: Nitric Oxide synthesis in the presence of insulin in platelets from ischemic stroke, hemorrhagic stroke and from normal*. PRP was prepared from normal volunteers and hemorrhagic stroke and ischemic stroke victims and that PRP was incubated with insulin (15μUnits/ml) for 2 h at 37 °C. After completion of incubation the NO synthesis was determined. This scattered graph demonstrated the median value of all 48 stroke victims and 48 normal volunteers. This figure explained the median of NO production in all volunteers and in each group.*

The plasma concentration of insulin in hemorrhagic stroke, ischemic stroke victims and normal volunteers

As described in Method section, during the presentation, the selected participants had no diabetes mellitus, however, when the plasma insulin levels were measured in the victims, it was found that while the insulin level in normal plasma was 16.85 µUnit/ml (median) ranging from 9 to 25 µUnit/ml (n = 48). The plasma insulin level in ischemic stroke was 1.3 µUnit/ml (median) ranging from 0 to 7 µUnit/ml (n = 22). The plasma insulin in hemorrhagic stroke victims was only 0.35 µUnit/ml (median), ranging from 0 to 7 µUnit/ml (n = 26). The significance "p" between the normal volunteers and ischemic stroke victims was ($p < 0.0001$). The "p" value between the hemorrhagic stroke victims and normal volunteers was ($p < 0.0001$).

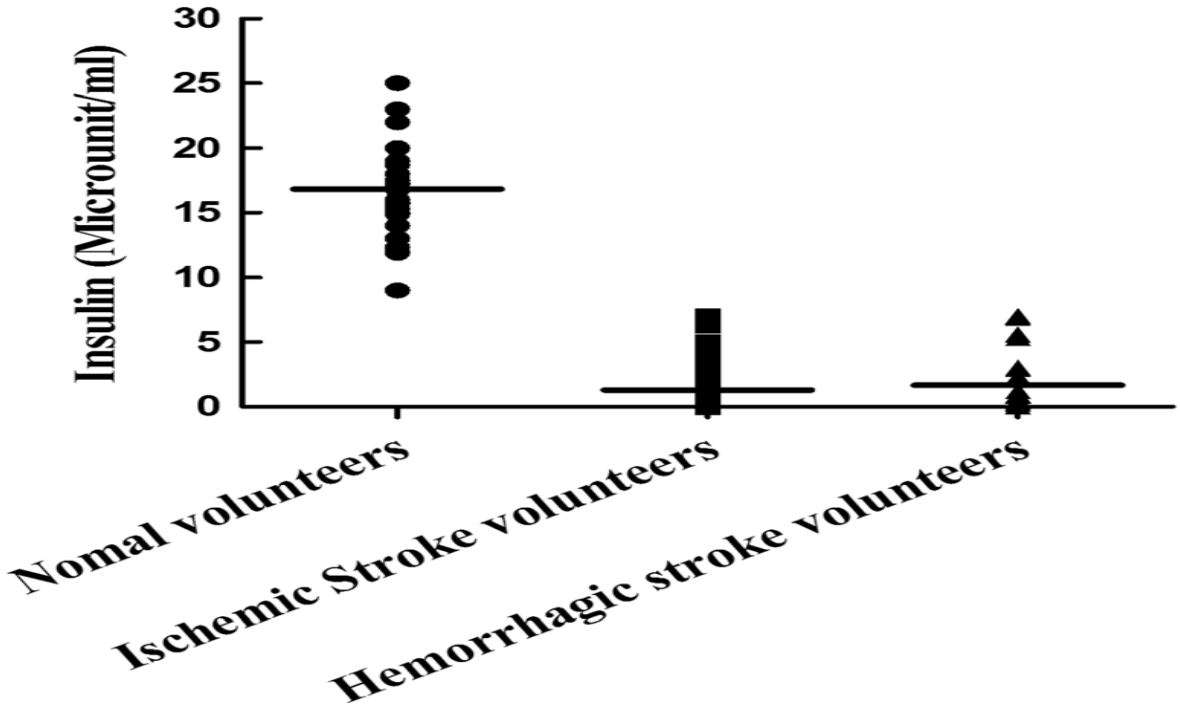

Fig-4.2: Plasma insulin level in ischemic, hemorrhagic stroke and normal volunteers*. Level of insulin in each volunteer was measured by ELISA as described in methods section.*

Each point indicates the amount of insulin (μUnits/ml) for the volunteers determined by at least 10 times in each case. Figures shown here are typical representatives of at least 48 sex and age matched normal volunteers and volunteers with stroke (n = 48, Ischemic stroke = 22, Hemorrhagic stroke = 26 and M = 20, F = 28).

Dermcidin isoform-2 protein concentration in the plasma of ischemic and hemorrhagic stroke victims

It has been recently demonstrated (Bank *et al.*, 2014; Ghosh *et al*., 2011) the function of dermcidin isoform-2 (DCN2), an environmental stress actuated protein (MW 11 kDa) in the circulation individuals affected with acute myocardial infarction (AMI) and as well as in acute coronary syndromes (ACS) and also in the efficient rise of arterial blood pressure through the impediment of insulin induced NO production by DCN2 (Ghosh *et al*., 2014). The protein was delineated to inhibit synthesis of insulin in animal model (Ghosh *et al*., 2014). To evaluate the role of DCN2 in the impaired NO synthesis in the stroke victims and on the inhibition of systemic insulin synthesis in the stroke victims by the stress induced protein as described above, the plasma DCN2 levels were determined in ischemic and hemorrhagic stroke victims (below table 4.1). As described in the Table, the plasma DCN2 levels in both ischemic stroke victims and in hemorrhagic stroke victims were significantly higher than that in normal counterpart.

Dermcidin concentration in Ischemic, Hemorrhagic stroke and in Normal volunteers		
Participants	**DCN2 level in plasma nM, median**	**"P" value***
A Normal (n = 48)	11.5 (ranging from 0 to 26)	Between **A** and **B** <0.0001
B Ischemic Stroke (n = 22)	142.5 (ranging from 77 to 172)	Between **A** and **C** <0.0001
C Hemorrhagic Stroke (n =26)	157 (ranging from 101 to 179)	Between **B** and **C** < 0.01

Table-4.1: *The cell free plasma (CFP) was made from the blood of normal volunteers, hemorrhagic stroke victims and from the ischemic stroke victims. The plasma DCN2 levels were measured by ELISA by DCN2 antibody which was ultra-purified by repeated gel electrophoresis as described in the Method and Materials. Polyclonal antibody against DCN2 was developed in the rabbit as demonstrated. Result had shown here the median DCN2 levels in the plasma ranges shown in the parentheses. *indicates two tailed paired "t" test.*

Acetyl salicylic acid resensitizes the platelets of hemorrhagic and ischemic stroke victims to the inhibitory effect of insulin in vitro in PRP

We have demonstrated before that acetyl salicylic acid (aspirin) was able to neutralize the effects of DCN2 (Bank *et al*., 2014), to determine whether the pre-treatment of aspirin with the PRP of ischemic or from the hemorrhagic stroke victims would be able to nullify the inhibitory effect of DCN2 on the insulin induced inhibition of platelet aggregation was determined. Aspirin (15μM) had no effect on the platelet aggregation inhibition (line A, hemorrhagic stroke and line B, ischemic stroke) when it was treated with PRP only. The pretreatment of 15 μM aspirin was able to negate the effect of DCN2 on the insulin induced platelet aggregation (line A1, hemorrhagic stroke and line B1, ischemic stroke).

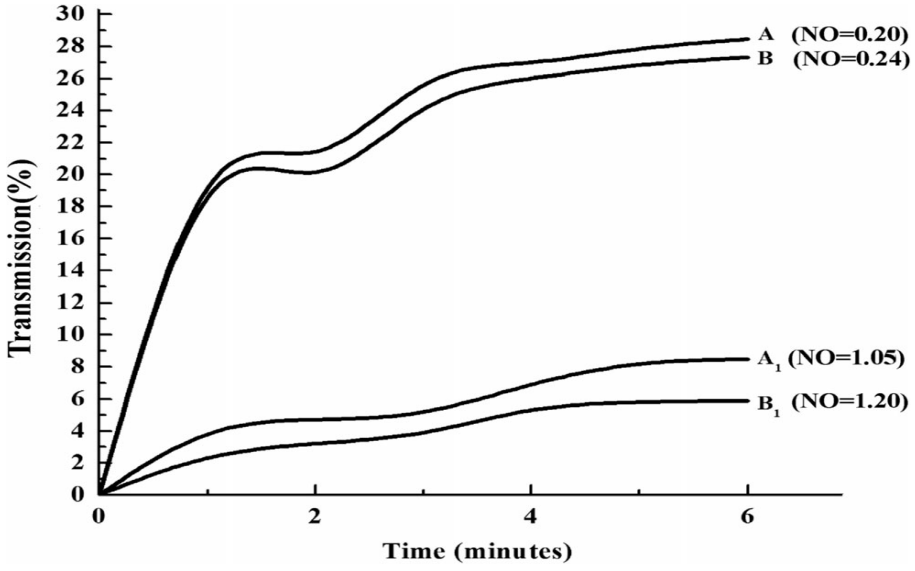

Fig-4.3: ***ADP induced aggregation of normal platelets, hemorrhagic stroke and ischemic stroke*** *(A1) represents the pre-incubated hemorrhagic stroke PRP with 15μUnits of insulin/ml for 2 h under identical condition like A and followed by incubation of 15 μM aspirin for 30 min at 37 °C. (B1) represents the pre-incubated ischemic stroke*

PRP with 15µUnits of insulin/ml for 2 h under identical condition like B and followed by incubation of 15µM aspirin for 30 minutes at 37ºC.

Dermcidin has a fundamental inhibitory effect on insulin synthesis

It was found that not only insulin stimulated NO production was impaired in the platelets from hemorrhagic or ischemic stroke victims, but the intrinsic synthesis of insulin itself was also severely impeded in the condition. We have delineated recently the role of a glucose activated nitric oxide synthase (GANOS) in the synthesis of insulin in liver cells (Bhattacharya *et al*., 2013). Interestingly, contrary to the assumption very little amount of GANOS was observed in the pancreas. We have also reported that for the synthesis of insulin in the liver, the synthesis of NO through the activation of GANOS by glucose were essential, and the amounts of the insulin synthesis was in the liver at least 10 times more than that produced in the pancreas at least in mice (Bhattacharya *et al*., 2013; Ghosh *et al*., 2010). As the inhibition of GANOS resulted in the inhibition of hepatic insulin synthesis (Bhattacharya *et al*., 2013), and as DCN2 was found to be a potent inhibitor of all forms of currently known nitric oxide syntheses (Bank *et al*., 2014), and as described above the amounts of DCN2 level in the plasma was found to be increased in the circulation of both ischemic and hemorrhagic strokes (Table-4.1), the role of DCN2 in the synthesis of insulin in the hepatic cells from the adult mice was investigated. It was found that addition of DCN2 in the hepatocyte suspension, in the amounts similar to the plasma DCN2 level in the stroke victims, totally obliterated the synthesis of the hormone stimulated by 0.02 M glucose. On the other hand the addition of 15 µM aspirin, which is reported to counteract the effect of dermcidin (Bank *et al*., 2014), could not only neutralize the inhibitory effect of DCN2, as described above, but also increased the systemic synthesis of the hormone at normal ranges when added to the reaction mixture in-vitro containing the mice liver homogenate.

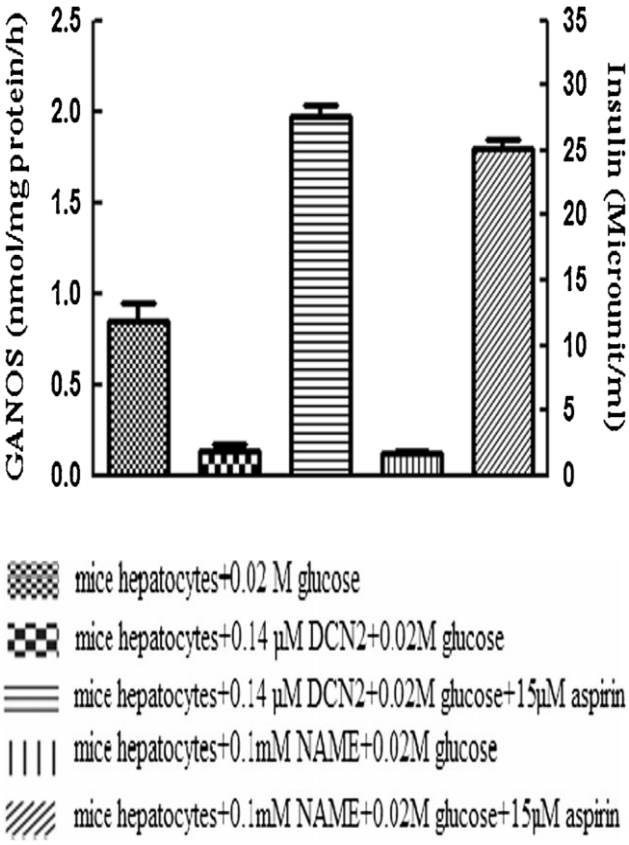

Fig-4.4: Dermcidin's role on insulin: *Liver hepatocytes are prepared by tyrodes buffer and incubation of 0.02M glucose in the presence and absence of DCN2 and NAME. In each cases GANOS activity (nmol NO/mg protein/h) and synthesis of insulin in the liver cells was measured. In some of the experiment it was found that in hepatocytes in presence of 15 μM aspirin can neutralize the effect of DCN2 and NAME. GANOS and insulin productions were measured by ELISA in the mixture of mice hepatocytes.*

The infection was not only the cause of body temperature in stroke patients

In stroke, fever is because of urinary tract infection, sepsis by catheter, pneumonia, upper respiratory tract contamination and gastritis where microorganism is the principle offender and contamination rely upon the age and the seriousness of stroke. But contagious stroke patients were excluded from the experiment as described in the method section because we wanted to avoid complicacy in the experiment which might have been interfering in the whole process. Be that as it may, many cerebral patients were found with high fever. C-responsive protein was discovered not all that raised in stroke patients in this analysis. Blood was withdrawn at the time of admission of stroke patients, but body temperature generally increases in stroke patients after two-three days of the stroke during the staying in hospital. Fever due to infection was emerging at later time points;

this suggested that if the preexisting infection was excluded, early fever in stroke patients could be an indication of neurological origin. However, fever control may be neuroprotective in patients with subarachnoid hemorrhage (Oddo *et al*., 2009) because high temperature causes the transformation of ischemic penumbra into infarction, increases the blood brain barrier breakdown (Reith *et al*., 1996.).

Discussion

From the above experiment, a type-I diabetes like condition was found to prevalent in ischemic and hemorrhagic stroke victims and an insulin resistance state was more acute in hemorrhagic stroke. So, there is a stressful condition in the cerebrovascular where hyperglycemia and plaques in the brain artery were found to develop like AMI.

So, we analyzed the stroke patients in case of thrombus formation and platelet aggregation, and we wanted to decipher the function of dermcidin effect if any. It was observed that the systemic impairment of insulin in stroke patients. From the previous reported results, it was found that GANOS (glucose activated nitric oxide synthase) is essential for the synthesis of insulin in hepatic cells (Bhattacharya *et al*., 2013). It was also found that due to the presence of dermcidin the insulin receptors were impaired as this protein inhibited the insulin production through the inhibition of nitric oxide, which is a stimulator of GANOS enzyme and it is crucial for the synthesis and the emanation of insulin. And a type I diabetes like condition was found in the stroke patients. It might also be argued that due to the lacking of insulin, NO signaling cascade is hampered in the hemorrhagic and ischemic stroke patients and that is may be one of the causes of severe platelet aggregation in the arteries of the brain. Our results also demonstrated that the use of insulin with 15 μM aspirin might surpass the dermcidin effect and restored the activity of GANOS, thus prognosis of stroke could be prevented. Insulin induced NO production is completely eliminated in hemorrhagic stroke patients.