The Role of Dermcidin Isoform 2 in Different Conditions Predisposing to Acute Coronary Syndrome

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1 Introduction

Environmentally induced stresses have been recognized to be involved in the pathogenesis of different life-threatening conditions that includes cancer (Gaudet et al., 2013), diabetes mellitus both type 1 and type 2 (Maritim et al., 2003), hypertension (Briones & Touyz, 2010) and acute coronary syndromes (ACS) (Maxwell & Lip, 1997). However, it is only recently, an environmentally induced oxidative stress protein has been identified to be dermcidin isoform 2 (DCN-2) (Ghosh et al., 2010). This protein of molecular weight 11kDa has been reported to be synthesized due to various stresses which includes hypoxia, tobacco smoke and alcohol consumption. These stresses have been related to the development of acute myocardial infarction (AMI), one of the most deadly thrombotic disorder and a major killer of the human race (Page et al., 1971).

Diabetes mellitus (DM), an abnormal increase of blood glucose level in the circulation, is a major world-wide public health problem. The condition is classified as:

1. Type 1 diabetes mellitus (T1DM) where the systemic synthesis of insulin, an essential hormone for carbohydrate metabolism for energy transduction, was severely impaired (Pfeifer et al., 1981) and,

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2. Type 2 diabetes mellitus (T2DM) where the system became resistant to the hypoglycemic effect of the hormone (Bogardus, 1993).

Although the impaired insulin synthesis in the pancreatic β cells was originally believed to be solely responsible for production and secretion of insulin when stimulated by glucose (Hughes et al., 1992), more recently it has been reported that the extra pancreatic insulin synthesis (Kojima et al., 2004) particularly in the hepatic cells in the liver was a major source of glucose induced insulin synthesis and secretion. Indeed the amounts of insulin synthesized in the liver could be more than 10 fold than that in the pancreas (Ghosh et al., 2010). In this context it should be mentioned that the occurrence of DM either T1DM or T2DM almost always leads to atherosclerosis that is responsible to cause a pro-thrombotic condition leading to the development of acute coronary syndrome (ACS) due to atherosclerotic plaque rupture on the coronary artery where the platelets formed micro-aggregates embedded in fibrin mass (thrombus) which in consequence obstructs normal blood circulation in the musculature of the heart and thus initiating ACS (Colman & Walsh, 1987; Fuster et al., 1996). The environmentally induced stress protein DCN-2 was found to be involved in the genesis of both type 1 and type 2 DM leading to atherosclerosis. Thus DCN-2 may not only pre-dispose system to atherosclerosis but may actually be involved in ACS (Ghosh et al., 2011).

The environmentally induced stress protein might be involved in the pathogenesis of AMI. AMI is developed due to thrombosis in the pericardium (Carvalho et al., 1988), and that might extend to both left and right ventricle, usually cause cardiac cell death that appear as patchy dark spots on the heart surface. It has been reported by many investigators that the use of aspirin, through its ability to inhibit platelet aggregation not only reduce the occurrence of death in ACS, but the compound has been reported to improve all acute syndromes associated with the condition (Pollack et al., 1995). Unfortunately, however, aspirin has been reported to fail to inhibit platelet aggregation in AMI. Neither the mechanism of the resistance of the platelets to the inhibitory effect of aspirin in AMI nor the way to restore the sensitivity of the platelets to the aspirin effect that could be beneficial in AMI was until recently available. We have recently reported that the appearance of DCN-2 in the circulation in AMI, imparted the resistance of the AMI platelets to the inhibitory effect of aspirin (Bank et al., 2014, Scientific Reports).

It is generally believed that high altitude illness (HAI) is a cluster of syndromes leading to life threatening condition of pro-thrombotic disease due to the development of ACS where the decent to sea level from high altitude condition usually produced little or no effect on the ensuing ACS. As such, persons stationed at high altitude are particularly vulnerable to an attack of ACS. Unfortunately, however, neither the mechanism of the development of ACS due to prothrombotic condition, nor any diagnosis for the occurrence of prothrombotic condition, which could be used as a warning to stave off the ominous event that may precipitate ACS has yet been identified. Investigations was carried out on the effect of various altitude heights on the plasma DCN-2 level, that it could be of useful in the diagnosis of prothrombotic condition at high altitude. It was found that the appearance of DCN-2 in high altitude actually precipitated AMI (Bank et al., 2014, Clinical Laboratory).
The death rate due to the occurrence of ACS or AMI in subjects with breast cancer is reported to be significantly higher than that in general female population at large. In this context, it has been reported before that while the death due to ACS or AMI in female population was 34%, the occurrence of breast cancer in the victims was found to increase the incidences of death due to ACS or AMI by ~50% (Darby et al., 2013). The role of DCN-2 in development of ACS in patients with breast cancer was investigated and reported in the article.

Finally, although cholesterol is reported to be a risk factor for ACS (Libby, 2005), no mechanism for increased ACS due to hypercholesterolemia is known. It was found that DCN-2, a double edged risk factor for both DM and hypertension could be involved in the increased occurrences of atherosclerosis in hypercholesterolemia. As such, DCN-2 could be determined as a global mediator of atherosclerosis.

In this chapter, a brief and general view of the involvement of DCN-2 in the pathogenic development of different conditions leading to ACS has been described herein.

2 Material and Methods

2.1 Ethical Clearance

Wherever appropriate the protocol described was approved by the Internal Review Board, Sinha Institute of Medical Science and Technology, Calcutta. All participants were asked to sign informed consent form. This study also used normal white Swiss albino mice (Mus musculus) and adult New Zealand rabbit. Appropriate permission was obtained from the IRB.

2.2 Selection of AMI Patients

All patients \((n = 29)\) between ages of 49 and 61 (median age 54 years) were admitted to the Intensive Care Unit of the Calcutta Medical College and Hospital, Kolkata. These patients met the following criteria of AMI: they had chest pain characteristic of myocardial ischemia for 30 mins or more and the electrocardiogram (ECG) showed ST segment elevation of at least two leads in the ECG reflecting a single myocardial region. The confirmation of the condition was determined by plasma troponin I. The sampling of blood was made within 6 h of the onset of the anginal attack before any therapy for the condition was initiated. Only those AMI patients who refused to ingest aspirin due to personal/religious beliefs served as “controls” when necessary.

2.2.1 Exclusion Criteria

(1) Patients with the history of diabetes mellitus, (2) showing the presence of bundle branch block or left ventricular hypertrophy in the ECG (3) or suffering from any severe infection, (4) took aspirin at least within 2 weeks, (5) hospitalized for any condition
within two months, and (6) took any cardiac medication including any antihypertensive drug within last 21 days were excluded from the study.

2.3 Collection of Blood

Blood samples (2–5mL), obtained from the participants by venipuncture by using 19-gauge siliconized needles, were collected in plastic vials and anticoagulated by gently mixing 9 vol of the blood with 1 vol of 0.13mM sodium citrate (Karmohapatra et al., 2003). The cell-free plasma (CFP) was prepared by centrifuging the blood sample from the participants at 30,000 g for 30 min at 0°C.

2.4 Preparation of DCN-2

DCN-2 was prepared from the blood of the subjects suffering from ACS by using SDS-Poly acryl amide gel electrophoresis as described before in detail (Ghosh et al., 2011).

2.5 Assay of DCN-2

The plasma DCN-2 was determined by Enzyme Linked Immunosorbent Assay (ELISA) using antibody raised against electrophoretically purified DCN-2 as the antigen in New Zealand white rabbit that has been described in detail before (Ghosh et al., 2011).

2.6 Assay on the Effect of DCN-2 on Hypertension and DM

All the assays regarding the effects of DCN-2 on hypertension both in humans (Ghosh et al., 2014, Cardiology Research and Practice) and in animal model (Ghosh et al., 2012, Thrombosis) and T1DM in human model (Ghosh et al., 2010, Ghosh et al., 2012, International Journal of Biomedical Science, Ghosh et al., 2011) and in animal model (Ghosh et al., 2012, Experimental Clinical Endocrinology Diabetes, Bhattacharya et al., 2013) and T2DM in animal model (Ghosh et al., 2014, Cardiology Research and Practice) has been described before.

2.7 Effect of DCN-2 on Aggregation of Platelets, HAI, AMI

Assays related to DCN-2 as an effective platelet aggregating agent (Ghosh et al., 2014, Cardiology Research and Practice), HAI [Bank et al., 2014, Clinical Laboratory] and in AMI [Bank et al., 2014, Scientific Reports] has been described before.

2.8 Selection of Subjects with Hypercholesterolemia

The blood samples were collected from the subjects with different degree of hypercholesterolemia ranging from 140mg cholesterol/dL of cholesterol (desirable) to 250mg cholesterol/dL (abnormally high). As the cholesterol levels in the participating subjects were intrinsically variable, the patients were divided into groups where each group
consists of five subjects (total = 70) whose cholesterol level differs by 10mg/dl either way.

2.9 Effect of Hypercholesterolemia on the Synthesis of Dermcidin mRNA

Leukocyte suspension was prepared from the venous blood which was withdrawn by using 19-gauge siliconized needle and was collected in plastic vial. The blood sample was anticoagulated by adding 1vol of sodium citrate to 9vol of blood as described before (Karmohapatra et al., 2003). Leukocytes were isolated from the buffy coat and purified by ficoll histopaque gradient (Klock & Bainton, 1976). The leukocyte preparation was suspended in Tyrode’s buffer pH 7.4 and was used as soon as possible.

Typically, the leukocytes suspension in Tyrode’s buffer pH 7.4, was treated with cholesterol and pure lecithin in the ratio 1:2, for 2h 30min at 37°C, the nucleic acids containing mRNA for DCN-2 were extracted by Trizol method (Ganguly et al., 2013), the nucleic acid extract was treated with the mixture of 1nM of all 20 different amino acids and 1.0µM ATP and the mRNA was translated by using plant leaf ribosomal particles (Ganguly et al., 2013). The synthesized proteins that also contained DCN-2 was determined by ELISA as described above.

In vitro experiments were performed to determine the effect of DCN-2 in goat artery endothelial cells incubated with 1:2 ratio of cholesterol and lecithin for 2h 30min at 37°C.

2.10 Selection of Breast Cancer Patients

Only female breast cancer patients 25 to 55 years of age (n = 1140) participated in the study. The breast cancer was diagnosed by mammogram followed by biopsy and was categorized by TNM classification at presentation. Those patients who were undergoing therapies including chemotherapy, radiation and even surgery was not included in the study. These volunteers were given appropriate legal counseling in the presence of their family members and legal counselors. All selected volunteers were asked to obtain judicial affidavit from the court of law and signed informed consent form. They were also advised to discontinue aspirin any time they wanted and they were at liberty to begin use of any therapy including chemotherapy, radiation or surgery for their condition without any consent from the investigators at any time.

2.11 In Vitro Translation of DCN-2 Synthesis in Leukocytes by Ethanol

Typically, the leukocytes suspension in Tyrode’s buffer pH 7.4, was treated with different amounts of ethanol, as described above. The synthesized proteins that also contained DCN-2 was determined by ELISA as described above.
2.12 Preparation of Cell Free Supernatant from the Disrupted Platelet Mass

The PRP was prepared from a single donor and centrifuged at 10,000g for 30min at 0°C. The platelet mass was resuspended in 1.0ml of Tyrode’s buffer pH7.4 and disrupted by freezing and thawing in liquid N₂. The disrupted mass was centrifuged at 30,000g, for 30min at 0°C. The supernatant was collected and used as the source of NOS.

2.13 Lineweaver-Burk Plot of the Nitric Oxide Synthase (NOS) Activity of the Supernatant from the Disrupted Platelet Mass

Typically 0.2mL of the supernatant of the disrupted platelet mass was treated with 2.0mM CaCl₂ in the presence or absence of 2.0µM ADP and different amounts of l-arginine (substrate of NOS) in a total volume of 1.0mL in Tyrode’s buffer pH7.4 and incubated for 5min at 37°C. The formation of NO in the supernatant was determined by methemoglobin method as described below.

2.14 Determination of NO

NO was determined by methemoglobin method as described before (Jia et al., 1996, Sinha et al., 1999). The amounts of NO formed was verified by independent chemiluminescence method (Cox & Frank, 1982).

2.15 Determination of Blood Glucose Level

The blood glucose level was determined by a glucometer (Behringer).

2.16 Determination of Blood Pressure Levels

The systolic and diastolic blood pressures were measured by using mercury sphygmomanometer.

3 Results

3.1 The role of DCN-2 in the Development of T1DMB in Hepatic Insulin Synthesis

We have reported before that the hepatic synthesis of insulin remained functional even when the pancreatic synthesis of the hormone was severely impaired as in the case of T1DM (Ghosh et al., 2010). As such, the issue remains “why there should be any T1DM if the glucose was capable of stimulating and secreting bio-active insulin in the hepatocytes in the liver?”. It was found that for the hepatic synthesis of insulin in the presence of glucose as well as the synthesis of NO is critically important (Ghosh et al., 2010). As
DCN-2 was found to be present in the liver and the protein was a potent inhibitor of nitric oxide synthases (Ghosh et al., 2010), the glucose induced hepatic synthesis and secretion of insulin in the hepatic cells was nullified in the absence of NO synthesis inhibited by DCN-2 (Ghosh et al., 2010). However, the inhibitory effect of DCN-2 could be nullified by increasing systemic NO synthesis either by applying sodium nitroprusside patch on the skin or by oral administration of aspirin (Ghosh et al., 2011) that effectively controlled the hyperglycemia in alloxan induced T1DM in animal model (Ghosh et al., 2012, Experimental Clinical & Endocrinology Diabetes) and in T1DMB in humans (Ghosh et al., 2012, International Journal of Biomedical Science) through the hepatic synthesis of insulin without the use of any external insulin.

Similar results were also obtained by the neutralization of the DCN-2 effect on the inhibition of insulin in hepatic cells by stimulating nitric oxide synthase by using nM quantity of estrogen or particularly progesterone which are reported before to be potent activators of NOS synthase (Bhattacharya et al., 2014).

We have reported before that at least 65% of the T1DM patients in India might be categorized as T1DMB and the plasma concentration of DCN-2 in these subjects were >124nM that contrasted the plasma DCN-2 of 15nM in non-diabetic age and sex matched volunteers (Ghosh et al., 2011). The increase of plasma DCN-2 was highly correlated to the hyperglycemia in these subjects (Ghosh et al., 2011).

### 3.2 The Role of DCN-2 in the Synthesis of Insulin in the Hepatic Cells and in the Pancreatic Islets of Langerhans

We have recently reported the existence of a glucose activated nitric oxide synthase (GANOS) in the liver. This enzyme (GANOS) was found to have critically important role in glucose activated NO synthesis in the production of insulin in the hepatic cells, not only in the glucose induced synthesis in the liver, but also in the secretion of insulin through the conversion of pro-insulin to bioactive insulin in the hepatic cells (Bhattacharya et al., 2013) by the activation of plasminogen in the circulation to plasmin by glucose induced nitric oxide synthase (GANOS). DCN-2 was reported to be a potent inhibitor of all known forms of nitric oxide synthases through its ability to be a competitive inhibitor of l-arginine, only known substrate for all forms of NO synthases. The liver GANOS was found to be very sensitive to the inhibitory effect of DCN-2, and as a result, the hepatic synthesis of insulin by DCN-2 resulted in the severe impairment of hepatic insulin synthesis leading to overt T1DMB (Bhattacharya et al., 2013) (Figure 1).

### 3.3 The Effect of Injection of DCN-2 in the Mice Circulation in the Synthesis of Insulin and on the Hyperglycemia

As discussed above, the cause for the development of T1DM remains obscure. However, when 0.2 μM dermcidin was injected in the tail vein of the test animals and the blood glucose and insulin levels were determined at different time intervals. The blood glucose level which was 98 ± 2.45mg/dL before the injection of dermcidin was found to be elevated to 350 ± 10.2 mg/dL at 160min after the injection of the stress-induced protein
with concomitant decrease of the plasma insulin level from $35.56 \pm 2.42 \mu$units/ml to $4.56 \pm 0.018 \mu$units/ml after the injection. Further studies demonstrated that the blood glucose level of $350 \pm 0.2$ mg/dL as well as the insulin level of $4.56 \pm 0.018 \mu$units/dL remained nearly unchanged for the next 120mins. However, after 24 h of dermcidin injection, both blood glucose levels and insulin levels were found to return to their normal ranges.

### 3.4 The effect of DCN-2 on the Development of T2DM in Mice

As stated above, currently there is no mechanism known for the development of T2DM (Rathsman et al., 2012). Indeed, the effect of T2DM on the increased abdominal obesity has been recently confusingly claimed to be the cause of T2DM (Pal D., et al. 2012). On the other hand, as described below, it was found that the injection of 0.24µM DCN-2 in the circulation of adult mice caused acute increase of the blood glucose level to 320mg/dl from the basal 60mg/dl. However, it was also noted that the plasma insulin level simultaneously increased to 35µunits/ml from 10-15µunits of insulin/ml indicating that despite the glucose induced increased synthesis of insulin, the hypoglycemic hormone failed to control hyperglycemia (Figure 2), thus suggesting the development of systemic insulin resistance, a hallmark of T2DM. Furthermore, it was also found that insulin resistance could be overcome by injecting 25µunits of insulin in the system suggesting that the amount of insulin produced by the glucose in the animal was not ade-
Figure 2: The effects of DCN-2 on the blood glucose and plasma insulin level in adult mice.

Figure 2: The effects of DCN-2 on the blood glucose and plasma insulin level in adult mice.

quate to control hyperglycemia, and the injection of external insulin that made up for the inadequate availability of the systemic insulin “corrected” the hyperglycemia, a typical pathologic event in T2DM in man where the glucose induced insulin synthesis and release were impaired (Bogardus, 1993). T2DM could be also induced by feeding aqueous tobacco leaf extract or pure nicotine which was found to increase plasma DCN-2 level (Ghosh et al., 2014, Cardiology Research & Practice).

Adult mice (25–30gm) fed ad-libitum were injected with 0.24 µM DCN-2 in the circulation. At different times as indicated, after the injection of DCN-2, both the blood glucose and plasma insulin levels were determined. In control experiments, equal volume of 0.9% NaCl (vehicle for DCN-2) was used. Results shown are mean ± S.D. of 10 different animals. The solid circles (●) show the plasma insulin level. The open squares (□) represent the blood glucose level. In the same experiment, 25µunits of insulin/DCN-2 treated mice was injected at 50min in these mice (as instructed by ↓) and the blood glucose and plasma insulin levels were determined after the injection of the external insulin at different times as indicated.

3.5 The role of DCN-2 on the Increase of Arterial Blood Pressure in Hypertensive Human Subjects

We have reported before that in human hypertensive patients, the increased presence of DCN-2 in the plasma could be demonstrated, and the oral administration of acetyl salicylic acid (aspirin) normalized the increased plasma DCN-2 levels from 95nM to 15nM (normal concentration of DCN-2 in the patients suffering from ACS) within 3h of inges-
tion of the compound. In hypertensive subjects, the oral administration of aspirin decreased the systolic pressure from 165 mm of Hg to 125 mm of Hg with the simultaneous decrease of diastolic pressure from 95 mm of Hg to 80 mm of Hg and with decrease of the intensity of the DCN-2 band in the SDS-polyacrylamide gel, suggesting that in humans the aspirin induced decrease of the plasma DCN-2 level controlled the increase of both systolic and diastolic pressure (Ghosh et al., 2014, Cardiology Research & Practice). The effect of injection of DCN-2 in rabbits increased the systolic pressure from 155 ± 4.78 mm of Hg to 200 ± 10 mm of Hg with simultaneous increase of diastolic pressure of 57.5 ± 8.66 mm Hg to 125 ± 5.77 mmHg after 2 h of the injection (Ghosh et al., 2012, Thrombosis).

These results as described above strongly suggest that DCN-2 was a potent atherosclerotic risk factor through its ability to simultaneously cause both T1DMB and hypertension both in humans and in the animal model. As DM and hypertension are reported to be the two major risk factors for atherosclerosis leading to ACS, the environmentally induced stress protein DCN-2 could play a critically important role in the development of atherosclerosis.

3.6 The Role of DCN-2 as a Potent Cyclooxygenase Activator in Platelets

We have reported before that DCN-2 was a potent platelet aggregating agent as a cyclooxygenase activator similar to that of the well known human platelet aggregating agent ADP (Ghosh et al., 2014, Cardiology Research & Practice). However, it was estimated that DCN-2 was at least 40-fold more effective platelet aggregating agent when compared to that of ADP.

3.7 The Effect of DCN-2 in the Development of ACS/AMI in High Altitude Illness

Personnel stationed in high altitude areas, persons travelling in airplane for prolonged period of time or sportsmen engaged in their sporting activities in high altitude areas are sometime reported to develop high altitude illness (Hackett & Roach, 2001). Although these high altitude illnesses are temporary conditions in nature, the high altitude induced illness usually and rapidly subsides when brought to the ground level. However, high altitude illness may also precipitate ACS/AMI.

Although few bureaucratic health official in certain countries, due to their lack of understanding on the pathogenesis of the prothrombotic condition leading to ACS or AMI, sometime claimed that high altitude illness is a “geographical disease”. We have recently reported that both ACS or AMI in the high altitude illness was due to the increase of DCN-2 in the circulation which was reported to help ACS to convert to dangerous AMI (Bank et al., 2014, Scientific Reports) please see below and when that happened, neither aspirin improve the condition nor bringing the victim at ground level can help them. And, as a result, the victims may die particularly due to AMI due to high altitude illness (Bank et al., 2014, Clinical Laboratory).
3.8 The Role of DCN-2 as the Cause of Severe Cardiac Pain in ACS and AMI

The development of characteristic cardiac pain in ACS and the development of even more severe cardiac pain in AMI (Everts et al., 1996) can be defined as the “hallmark” of these conditions in humans.

Unfortunately, however no acceptable mechanism of the severe cardiac pain in these conditions was available. We, recently have demonstrated, that the lack of systemic NO level was probably the cause of the cardiac pain in that the basal NO level in the plasma of AMI patients was found to reduce to undetectable ranges (~0nmol/ml) from 4nM in normal volunteers across the ethnic and geographical barriers (Ghosh et al., 2014, PloS ONE). The lack of systemic NO level was related to the increase of DCN-2 level in these conditions where the level of DCN-2 was 4 to 40 fold higher in AMI than that in ACS which inhibited the systemic synthesis of NO induced by all known forms of NOS (Ghosh et al., 2014, PloS ONE).

That the systemic increase of NO could rapidly control the cardiac pain in these conditions by using “nitro” compounds supported the role of NO in the control of cardiac pain in these conditions (Ghosh et al., 2014, PloS ONE). Both aspirin (Karmohapatra et al., 2007) and insulin (Bhattacharya et al., 2001) through their ability to increase systemic NO level also reported to control the cardiac pain in ACS and AMI (Ghosh et al., 2014, PloS ONE).

3.9 The Acute Effect of DCN-2 in the Nullification of the Aspirin Induced Inhibition of Platelet Aggregation in Acute Myocardial Infarction

Although the development of thrombosis in the arteries of the heart leads to ACS (Colman et al., 1987; Fuster et al., 1996), sometimes thrombosis interrupts the normal blood circulation in the heart. And, as a result of the blockage of blood circulation in the pericardium leads to the death of cardiac cells that caused acute myocardial infarction (AMI). The platelets from AMI unexplainably however became resistant to the inhibition of aggregation by aspirin, and as such the prevention of recurrences of AMI remains very difficult due to lack of suitable and effective inhibitor of platelet aggregation similar to that produced by aspirin.

We have recently reported that the binding of DCN-2 on its receptor sites on the platelet membrane in AMI, where the plasma DCN-2 level increased by 40 folds more than normal and more than 5 folds compared to ACS, resulted in the nullification of the inhibitory effect of aspirin (Bank et al., 2014, Scientific Reports). However, the platelet bound DCN-2 could be removed by increasing NO synthesis in the platelets that rendered the platelets from AMI to become “supertensive” to the inhibitory effect of aspirin in that the platelets from AMI subject could be inhibited (100%) by only 25μM aspirin.
3.10 Association of DCN-2 in Hypercholesterolemia

Hypercholesterolemia, also known as dyslipidemia, is a well known risk factor for ACS (Libby, 2005). Unfortunately however, the increased occurrence of ACS in hypercholesterolemia remains hypothetical. Furthermore, whether hypercholesterolemia may actually increase the platelet aggregation to increase thrombosis in the coronary artery leading to increased occurrence of ACS remains debatable.

The determination of the plasma DCN-2 level in hypercholesterolemia subjects (ranging from 140mg/dl to 270mg cholesterol/dL) demonstrated, that while the DCN-2 level in the age and sex matched normal volunteers had ~15nM DCN-2, the atherosclerotic risk factor was increased to 130nM of DCN-2 ($n = 70$) in hypercholesterolemic subjects, which was found to be related to the increase of the plasma cholesterol level from 140 to 260mg cholesterol/dL in the participating hypercholesterolemic patients (Figure 3).

![Figure 3: The effect of increased cholesterol level in hypercholesterolemia on the increase of plasma DCN-2 level.](image)

As described in Methods and Materials, the mean of the cholesterol level of each group was used as shown in the x-axis of the figure. Results shown are mean ± S.D. as indicated above.

Furthermore, the treatment of goat carotid artery endothelial cells suspension in Tyrode’s buffer in the presence of cholesterol and pure lecithin (1:2) as described in materials and methods resulted in the increased synthesis of DCN-2 from the control (Lecithin only) 132pmol/ml to 190pmol/ml ($p < 0.0005$, $n = 50$).
3.11 The Role of DCN-2 in Breast Cancer in the Development of ACS in Female Breast Cancer Patients

The development of breast cancer in the female subjects has been reported to result in the increased incidences of ACS (Darby et al., 2013). In a pilot study, when the level of DCN-2 in female breast cancer patients was determined, it was found that the level of DCN-2 increased from 15.50 ± 0.64nM to 36.75 ± 0.85nM ($p < 0.001$) (Figure 4). As aspirin has been reported to neutralize the synthesis of DCN-2, in another study 1140 patients were asked to ingest 14mg aspirin/70 kg body weight for 2 years (The study was registered with the Clinical Trial Registration India (CTRI), trail registration no: CTRI/2014/12/ 005235), it was found that the rate of death due to ACS or AMI in breast cancer patients who had received 14mg of aspirin/70Kg body weight for 2 years was 10.43% that contrasted the death rate (50%) who didn’t receive aspirin. Z-test analysis between the groups were performed where the calculated Z value of the death rate due to ACS or AMI between the normal population and the breast cancer patients who received aspirin was 17.36 which was greater than the critical two-tailed z value 2.17. Also, the z-value of the death rates due to ACS or AMI between the breast cancer patients not receiving aspirin and the breast cancer patients using aspirin was 27.34 which was again greater than the critical two-tailed z-value 2.33. The P-value for the two-tailed test in all the cases was found to be 0 which was less than the alpha value of 0.5 (i.e. $P < 0.5$). Thus Z-test analysis of the death rate due to ACS or AMI in normal, breast cancer patients and the breast cancer patients who received aspirin demonstrated that the use of aspirin could be useful for the prevention of death rate due to ACS or AMI in female breast cancer patients.

![Figure 4](image_url): Quantitation of DCN-2 in plasma in breast cancer subjects and in age matched normal female volunteers.
Citrated blood was collected from breast cancer volunteers and normal female volunteers and the plasma DCN-2 was determined by ELISA using antibody raised against electrophoretically pure DCN-2 as the antigen. The values shown are represented as mean ± S.D. of twenty eight different breast cancer subjects and equal number of age matched normal female volunteers. The significance of the increase of plasma DCN-2 level was determined by Student’s t-test where \( p < 0.01 \). Symbol (●) represents breast cancer volunteers and symbol (▲) represents normal volunteers.

4 Discussion

The development of atherosclerotic plaque in the coronary artery that resulted in the prothrombotic condition is reported to be the leading cause for the development of ACS in humans (Colman et al., 1987; Fuster et al., 1996) while atherosclerosis itself known to be a natural condition, mechanism for the condition remains obscure. Perhaps, more importantly, atherosclerosis once developed on the coronary artery; there is no known non-invasive way to remove the plaque from the wall of the coronary artery. In this context, it must be mentioned here that extensive trial with aspirin in human volunteers has demonstrated that this compound was capable of reducing the incidences of ACS, a major killer disease of the human race (Pollack, 1995). The mechanism of aspirin effect on the reduction of the occurrences of ACS has been reported to be due to the inhibition of platelet aggregation (Pollack, 1995).

At present, only known way to prevent atherosclerosis is to control DM (both T1DM and T2DM) and hypertension (Sowers et al., 2001). These two independent risk factors are however were capable of influencing each other, and considered to be the two major risk factors for atherosclerosis (Kosiborod, 2008; Goldberg et al., 2007; Sowers et al., 2001). Even dyslipidemia including hypercholesterolemia that can cause atherosclerosis and deregulated thrombolysis all are reported to be the consequences of T2DM (Goldberg, 2001). And as such, the inhibition of platelet aggregation by aspirin through the inhibition of cyclooxygenase cannot control either hypertension or diabetes mellitus (either type 1 or type 2). The effect of aspirin has been reported to be due to the activation of a constitutive form nitric oxide synthase that has been purified to homogeneity (Karmohapatra et al., 2007). Nitric oxide thus formed was capable of neutralizing the effect of DCN-2 as a atherosclerotic risk factor through its ability to control both hypertension (Ghosh et al., 2014, Cardiology Research and Practice) and diabetes mellitus (Ghosh et al., 2012, ECED; Ghosh et al., 2012, International Journal of Biomedical Science).

Although hypercholesterolemia is decidedly a potent risk factor for ACS, its mechanism of action remains hypothetical and even enigmatic, and the molecule itself is an essential component of cell membrane as well as a precursor molecule of all steroids. As described above, the increase of plasma cholesterol that led to the increased synthesis of DCN-2 in the endothelial cells strongly suggested, that the increase of plasma cholesterol level similar to other environmentally induced risk factor for atherosclerosis, led to the increased synthesis of DCN-2, a potent double edged atherosclerotic
risk factor (Ghosh et al., 2012, Thrombosis). Interestingly, in a preliminary study, it was found that the presence of aspirin in the reaction mixture inhibited the synthesis of DCN-2 through its ability to stimulate NO synthesis in the endothelial cells [unpublished], suggesting possible use of aspirin in hypercholesterolemia to control increased DCN-2 synthesis and consequently atherosclerosis.

DCN-2, an environmentally induced stress protein which was found to be simultaneously an inducer of both T1DM as well as T2DM (Ghosh et al., 2012, Thrombosis; Ghosh et al., 2014, Cardiology Research & Practice) was also found to be associated with hypertension T2DM (Ghosh et al., 2012, Thrombosis; Ghosh et al., 2014, Cardiology Research & Practice), and as such, the protein could be an all-round atherosclerotic risk factor. The plasma level of this protein was found to be increased in different and sometimes unrelated diseases (including DM, hypertension, hypercholesterolemia or even breast cancer) all of which are previously demonstrated to cause ACS in the victims through increased atherosclerosis. It should be mentioned here, that the increase of DCN-2 level in the reaction mixture as in the case of hypercholesterolemia as described above or in the cases of different environmentally induced stresses including hypoxia, nicotine aqueous extract of the tobacco leaf or alcohol (Ghosh et al., 2014, Cardiology Research & Practice), was not due to the release of preformed DCN-2 from the cells in the mixture, but was related to the synthesis of DCN-2 in each case, was demonstrated to be actual synthesis of the protein by in vitro mRNA translation (Ghosh et al., 2014, Cardiology Research & Practice) due to appropriate gene expression.

5 Conclusion

The protein DCN-2 was found to be involved in the development of T1DM, T2DM, hypertension, AMI, high altitude illness, hypercholesterolemia and ACS in breast cancer, thus indicating all round detrimental role of DCN-2 in ACS in both human and animal model. Interestingly, the use of aspirin has shown to inhibit systemic DCN-2 synthesis through the stimulation of mRNA by the activation of aspirin activated nitric oxide synthase leading to the synthesis of NO.

References


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