PUBLICATIONS

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1) **Bank S**, Jana P, Maiti S, Guha S, Sinha AK. Dermcidin isoform-2 induced nullification of the effect of acetyl salicylic acid in platelet aggregation in acute myocardial infarction. ***Sci Rep.*** 2014 Jul 24;4:5804.
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2) **Bank S**, Bhattacharya S, Maiti S, Bhattacharya R, Chakraborty D, Sinha AK. The impaired synthesis of insulin and its inability to inhibit platelet aggregation in cerebrovascular accident. ***Metab Brain Dis***. 2015 Oct;30(5):1309-18.
https://www.ncbi.nlm.nih.gov/pubmed/26188955

3) **Bank S**, Ghosh R, Jana P, Bhattacharya S, Sinha AK. The diagnosis of high altitude illness by the determination of plasma dermcidin isoform 2 levels by enzyme linked immunosorbent assay. ***Clin Lab.*** 2014;60(7):1187-91.
https://www.ncbi.nlm.nih.gov/pubmed/25134388

4) Ghosh R, **Bank S**, Maji UK, Bhattacharya R, Guha S, Khan NN, Sinha AK. The Effect of Acetyl Salicylic Acid Induced Nitric Oxide Synthesis in the Normalization of Hypertension through the Stimulation of Renal Cortexin Synthesis and by the Inhibition of Dermcidin Isoform 2, A Hypertensive Protein Production. ***Int J Biomed Sci.*** 2014 Sep;10(3):158-66.
https://www.ncbi.nlm.nih.gov/pubmed/25324696

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8) Manna E, **Bank S**, Maiti S, Jana P, Sinha AK. Neutralization by Acetyl Salicylic Acid of the Testosterone induced Impaired Maspin Synthesis Stimulated by Estriol in Neutrophils through Nitric Oxide Synthesis. ***Int J Biomed Sci.*** 2015 Dec; 11(4):176-84.

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10) **Bank S*,** Maiti S, Guha S, Sinha AK*. A TRIAD system (catalase, insulin and low dose aspirin) neutralizes the effect of dermcidin isoform-2 and prevent from the cell death in AMI and recurrence of the disease. ***Cardiol J.*** 2016;23(6):623-625. https://www.ncbi.nlm.nih.gov/pubmed/27976793

11) Bhattacharya S, Khan M, Ghosh C, **Bank S**, Maiti S. The role of Dermcidin isoform-2 in the occurrence and severity of Diabetes. ***Sci Rep.*** 2017 Aug 15;7(1):8252. https://www.ncbi.nlm.nih.gov/pubmed/28811499

12) **Bank S**, Jana P, Girish GV, Sinha AK, Maiti S. Expression of a Nitric Oxide Synthesizing Protein in Arterial Endothelial Cells in Response to Different Anti-Anginal Agents Used in Acute Coronary Syndromes. ***Protein Pept Lett.*** 2017;24(11):1066-1072. https://www.ncbi.nlm.nih.gov/pubmed/28901858

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14) **Bank S**, Bhattacharya S, Koschinski A, Zaccolo M, Maiti S, De SK, Khan GA, Guha S, Ghosh A, Ray U, Sinha AK. Stress-Induced Protein Dermcidin Develops Diabetes Targeting GLUT4/Insulin via NO/cGMP Inhibition (July 31, 2019). Available at SSRN: https://ssrn.com/abstract=3429875 or http://dx.doi.org/10.2139/ssrn.3429875.

- **(1, 2, 3, 7, 10, 12, 14 are thesis related publications)**

BOOK CHAPTER

Asru K Sinha, **Sarbashri Bank**, Debipriya Banerjee, Suman Bhattacharya, Pradipta Jana, Rageshwary Ghosh, Gannareddy Girish, Gausal Azam Khan. The role of dermcidin isoform- 2 in different conditions predisposing to acute coronary syndrome in humans. In book: **Detection and Diagnosis Methods of Diabetes 1st Edition**, ISBN 978-1-922227-584, Editors: **iConcept Press**.

PATENT

Bank S *et al*. A trypsin resistant oral milk-insulin protein complex preparation **Application number as 201631033559 (2016).**

ABSTRACT

1. **Bank S,** Mait S, Guha S, Sinha AK. "Dermcidin isoform-2 induced nullification of the effect of acetyl salicylic acid in platelet aggregation in acute myocardial infarction". *5th International conference in Clinical & Experimental Cardiology-2015, Philadelphia, USA.*
2. **Bank S,** Maiti S, Sinha AK. "The role of dermcidin isoform-2 in platelet aggregation in AMI and effects of acetyl salicylic acid on it." *26th annual symposium of Indian society for Atherosclerosis Research (ISARCON-2013).*
3. U. Ray, **S. Bank**, P. Jana, S. Bhattacharya, A. Sinha, Y. Byron, P. Roberts-Thomson. Dermcidin isoform-2 induced abolition of the effect of insulin in breast cancer being the casual pathophysiology behind the development of CAD through the nitric oxide

inhibition. AACC 2015 Annual Meeting Poster Abstract (A-380), page-S133, July 26-30, 2015, Atlanta USA.

4. **Bank S,** Maiti S, Sinha AK. "Trypsin resistant oral insulin preparation in alloxan induced type-I diabetic mice". Poster presentation in *EMBO Conference: Chemical Biology-2016, Germany.*
5. **Bank S**, Maiti S, Sinha AK**. "**LBPS 01-10 THE IMPACT OF A STRESS INDUCED PROTEIN DERMCIDIN ISOFORM-2 ON THE GENESIS OF DIABETES AND HYPERTENSION" *Journal of Hypertension* 34:e176-e177. DOI: 10.1097/01.hjh.0000500380.33656.a8.
6. **Sarbashri Bank***, Smarajit Maiti, Subrata Kumar De. Environmentally stress protein induced hyperglycemia in an animal model and predisposing to AIHD. *IISF-2018, Lucknow.*

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Dermcidin isoform-2 induced nullification of the effect of acetyl salicylic acid in platelet aggregation in acute myocardial infarction

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The aggregation of platelets on the plaque rupture site on the coronary artery is reported to cause both acute coronary syndromes (ACS) and acute myocardial infarction (AMI). While the inhibition of platelet aggregation by acetyl salicylic acid was reported to produce beneficial effects in ACS, it failed to do in AMI. The concentration of a stress induced protein (dermcidin isoform-2) was much higher in AMI than that in ACS. Incubation of normal platelet rich plasma (PRP) with dermcidin showed one high affinity (K_d = 40 nM) and one low affinity binding sites (K_d = 333 nM). When normal PRP was incubated with 0.4 μM dermcidin, the platelets became resistant to the inhibitory effect of aspirin similar to that in the case of AMI. Incubation of PRP from AMI with dermcidin antibody restored the sensitivity of the platelets to the aspirin effect. Incubation of AMI PRP pretreated with 15 μM aspirin, a stimulator of the NO synthesis, resulted in the increased production of NO in the platelets that removed the bound dermcidin by 40% from the high affinity binding sites of AMI platelets. When the same AMI PRP was retreated with 10 μM aspirin, the aggregation of platelets was completely inhibited by NO synthesis.

The aggregation of platelets by aggregating agents like ADP, *l*-epinephrine, collagen or thrombin is known to be an essential physiologic phenomenon in the life saving process of blood coagulation[1]. In contrast, excessive platelet aggregation particularly at the site of plaque rupture or fissuring on the wall of the major or medium size coronary artery is reported to result in the development of acute coronary syndrome (ACS) due to formation of thrombus (a micro aggregate of platelets embedded in fibrin mass) that led to the condition as a result of the blockade of the normal blood circulation in the heart musculature[2]. The blockade of normal blood circulation not only blocks the availability of the oxygenated blood but also interrupts the supply of water, nutrients, and metal ions those are essential for the normal function of the heart. Although there are several platelets aggregating agents, as described above, can aggregate platelets, it has been reported that ADP induced platelet aggregation played a critically important role in the formation of the thrombus in ACS in humans[3].

The excessive platelet aggregation is counteracted by several humoral inhibitors of platelets aggregation including prostacyclin[4], insulin[5], interferon α[6], and estriol[7] to achieve the systemic homoeostasis. In this context the pharmacological agents, acetyl salicylic acid (aspirin) is well known for its beneficial effect on the reduction of occurrence of ACS through its ability to inhibit platelet aggregation[8]. Although the aggregation of platelets on the arterial plaque rupture site may develop into ACS, the aggregation of platelets, sometime may also block the pericardial artery that might result in the cardiac cells death which appeared as dark patchy areas of infarcts developed due to the death of heart cells that ultimately could lead to acute myocardial infarction (AMI), which when massive, could result in the death of the victims[9]. Although >90% of the cases of AMI are the consequence of the development of the thrombus due to platelet aggregation[10], severe anemia, coronary artery spasm are also known to cause AMI[11].

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[8]. Unfortunately, however, aspirin has been reported to fail to inhibit platelet aggregation in AMI[12], and the use of aspirin in AMI is of little or no use in AMI[13]. 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 is currently available.

We have recently reported the appearance of a novel protein in the circulation of both ACS and AMI[14]. This protein has been determined to be a potent inducer of platelet aggregation, and was identified to be dermcidin isoform-2 (dermcidin), a stress induced protein of Mr 11 Kda[14]. This protein was reported to be a powerful inhibitor of all known forms of nitric oxide syntheses (NOS). The role of protein which was found to be present >5 folds in AMI plasma than that in ACS and >40 folds than in normal plasma, was studied to determine its possible contribution in creating the resistance of platelets to the inhibitory effect of aspirin in AMI.

We also present a unique and simple way to resensitize the platelets in PRP from AMI subjects to the inhibitory effect of aspirin by the stimulation of NOS in the platelets.

Results

Effect of acetyl salicylic acid (aspirin) on the ADP and other platelet aggregating agents induced platelet aggregation in PRP from normal volunteers and from AMI subjects. Preincubation of normal PRP with different concentrations of aspirin for 30 minutes at 37°C resulted in the maximal inhibition of platelets aggregation induced by 2 μM ADP at 80 μM of the inhibitor (fig. 1). In contrast, similar treatment of PRP from the AMI subjects with aspirin failed to inhibit the ADP induced platelet aggregation under identical condition (fig. 1). The use of aspirin for the inhibition of platelet aggregation in PRP from AMI subjects induced by either *l*-epinephrine (5 μM) or collagen (0.2 μg/ml) or thrombin (1 unit/ml) as aggregating agent instead of ADP also failed to produce the inhibitory effect of the compound.

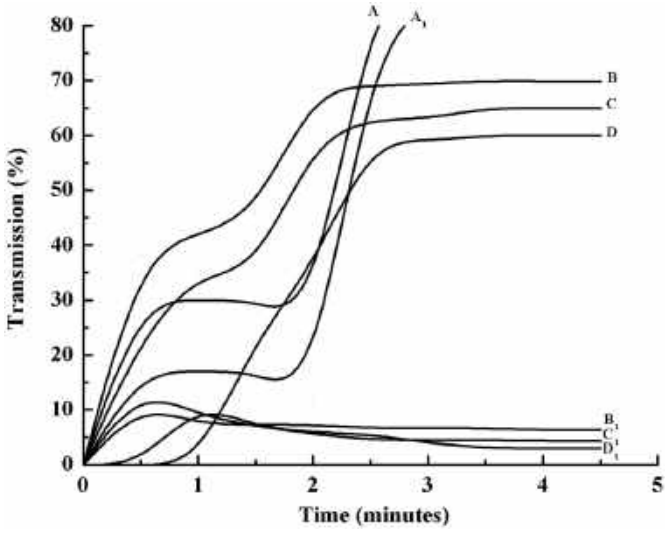

Figure 1 | The effect of aspirin on the inhibition of platelet aggregation in PRP from normal and AMI subjects induced by different platelet aggregating agents. The PRP preparations were incubated with 80 μM aspirin for 30 minutes at 37°C. After incubation, aggregation of platelets was initiated by adding different aggregating agents as indicated. (A) = The aggregation of platelets induced by thrombin (1.0 Unit/ml) in PRP from AMI subjects treated with aspirin. (A_1) = The effect of treatment of normal PRP with aspirin on the aggregation induced by thrombin. The upward increase of the transmission in both A and A_1 was due to the clotting of the PRP induced by thrombin. (B) = The aggregation of platelets induced by ADP (2.0 μM) in PRP from AMI subjects treated with aspirin. (B_1) = The effect of treatment of normal PRP with aspirin on the aggregation of platelets induced by ADP. (C) = The Aggregation of platelets induced by *l*-epinephrine (5.0 μM) in aspirin treated PRP from AMI subjects. (C_1) = The effect of treatment of normal PRP with aspirin on the *l*-epinephrine induced platelet aggregation. (D) = The aggregation of platelets induced by collagen (2 μg/mL) in aspirin treated PRP from AMI subjects. (D_1) = The effect of treatment of normal PRP with aspirin on the aggregation of platelets induced by collagen. Figures shown here are typical representatives of at least 20 sex and age matched normal volunteers and volunteers with AMI (n = 20, M = 10, F = 10).

The role of dermcidin in the development of resistance against the inhibitory effect of aspirin in the ADP induced platelet aggregation in normal PRP. To test this possibility the PRP from normal volunteers was incubated with 0.4 μM dermcidin (the concentration of dermcidin in AMI plasma was ≈0.4 μM, as described below) for 90 minutes at 37°C, and the dermcidin treated PRP was subsequently used to determine the inhibition by aspirin of the ADP induced platelet aggregation. It was found that the treatment of normal PRP with dermcidin resulted in the development of résistance of the platelets to the inhibitory effect of aspirin (line A) when compared to control experiment where the normal PRP was incubated with 0.9% NaCl for 90 minutes at 37°C (line B) (Fig. 2).

The concentrations of dermcidin in the plasma from normal volunteers and in the plasma of the subjects with AMI. As the PRP either from normal subjects or from the ACS subjects but not the PRP from AMI was found to be inhibited by aspirin in the ADP induced platelet aggregation, it was thought that the concentrations of dermcidin in the PRP samples might be related to the development of resistance to the inhibitory effect of aspirin. Determination of the plasma dermcidin concentrations demonstrated that while the dermcidin concentration in the normal plasma was 10 ± 3.6 nM, the dermcidin concentration in the cases of ACS as described before[14] and AMI were 80 ± 4.5 nM, and 400 ± 4.8 nM, ($p < 0.001$) respectively.

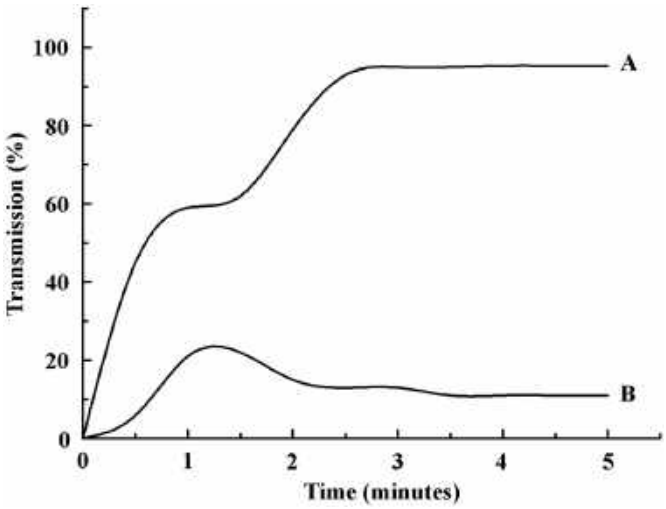

Figure 2 | The effect of incubation of normal PRP with 0.4 μM dermcidin on the aspirin treated platelet aggregation induced by ADP. PRP was prepared from normal subjects as described in Methods and Materials. The PRP was next incubated with 0.4 μM dermcidin for 90 minutes at 37°C. After incubation, the dermcidin treated PRP was incubated with 80 μM aspirin at 37°C and aggregation of platelet was initiated by using 2.0 μM ADP. Similar results were also obtained by using other platelet aggregating agents including thrombin, *l*-epinephrine or collagen as described under Figure 1 (not shown in the figure). (A) = ADP induced platelet aggregation in the dermcidin treated normal PRP in presence of aspirin, (B) = ADP induced platelet aggregation in normal PRP not treated with dermcidin in presence of aspirin. The figure is a typical representative of at least 10 different experiments using blood samples from 10 different volunteers.

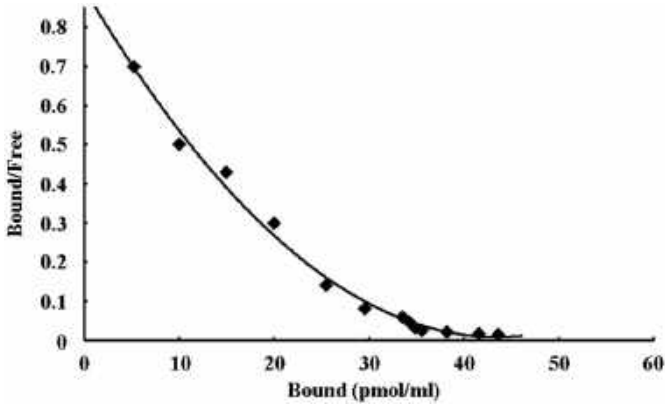

Figure 3 | Scatchard plot of the equilibrium binding of electrophoretically purified dermcidin to the gel filtered platelets. Gel filtered platelets were prepared from normal volunteers. The gel filtered platelets (3×10^8 platelets/mL) were incubated with 0.4 μM dermcidin for 90 minutes at 37°C. The unbound dermcidin was separated from the platelets bound dermcidin by using Millipore filtration as described in Methods and Materials. Platelets bound dermcidin was next released from the platelets by treated platelets with the 0.05% TritonX-100 and the dermcidin binding was quantitated by ELISA by using dermcidin antibody.

Scatchard plot analysis of the equilibrium binding of dermcidin to normal platelets. To determine whether the effect of dermcidin on the development of resistance to aspirin was mediated through the binding of the stress induced protein to the platelets, Scatchard plot analysis of the equilibrium binding of dermcidin to the normal platelets was carried out as described in the Material and Methods.

Scatchard plot of the equilibrium binding of dermcidin produced a curvilinear plot (Figure 3). The curvilinear nature of Scatchard plot suggested the presence of heterogeneous binding sites populations of dermcidin on the platelet surface i.e. one is high affinity-low capacity binding sites and another is low affinity-high capacity binding sites[30]. The analysis demonstrated the presence of one high affinity (K_d = 40 nM), with low capacity dermcidin binding sites (n = 128 × 10^3 molecules/platelet) and another low affinity (K_d = 333 nM) with high capacity binding sites (n = 189 × 10^3 molecules/platelet) in the normal platelet surface.

Effect of polyclonal antibody against dermcidin on the resistance of platelets from the AMI subjects in the inhibitory effect of aspirin. To determine the role of dermcidin further as the cause of resistance to the inhibitory effect of aspirin in platelets from the AMI subjects, the PRP from AMI subjects was incubated with dermcidin antibody for 90 minutes at 37°C. The dermcidin antibody treated PRP was subsequently challenged to the inhibitory effect of aspirin in the ADP induced platelet aggregation. It was found that the dermcidin antibody treated platelets from the AMI subjects yielded to the inhibitory effect of aspirin on the ADP induced platelet aggregation which was similar to that in the case of normal PRP (Figure 4, line B). Use of normal IgG at similar concentration had no effect on the aspirin induced inhibition on ADP due to platelets aggregation (Figure 4, line A).

The removal of the bound dermcidin from platelets from AMI subjects by the stimulation of NO in platelets. Dermcidin has been reported to be a potent inhibitor of nitric oxide syntheses (NOS) and the inhibition of NOS in platelets has been reported to aggregate normal platelets through the stimulation of thromboxane A2 synthesis even in the absence of the added ADP to PRP[15].

As such, the effect of the stimulation of platelet's NOS on the expulsion of the platelet bound dermcidin, and the consequent effect

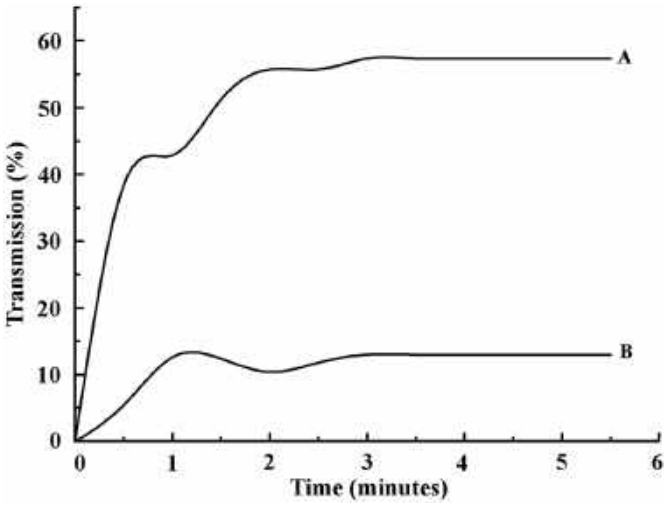

Figure 4 | Effect of incubation of PRP from AMI subjects with dermcidin antibody and the inhibitory effect of aspirin on the antibody treated platelet rich plasma. (A) represents the PRP from AMI subjects was incubated with aspirin for 30 minutes at 37°C and aggregation of platelets was induced by ADP. (B) represents the PRP from same AMI patients treated with dermcidin antibody and subsequently treated with aspirin under identical condition like A. Figures shown here are typical representative of at least 10 experiments using PRP from 10 different AMI patients (n = 10, M = 5, F = 5).

of the removal of dermcidin on the aspirin induced platelets aggregation in AMI platelets was studied.

It was found that aspirin at 15 μM was able to stimulate NO in platelets[5,23] but did not inhibit platelet aggregation. On the other hand at that concentration aspirin was able to expel platelet bound dermcidin through NO from AMI PRP (figure 5). The use of 30 μUnit of insulin/mL to stimulate NO synthesis in platelets[5], instead of aspirin, was also found to remove platelets bound dermci-

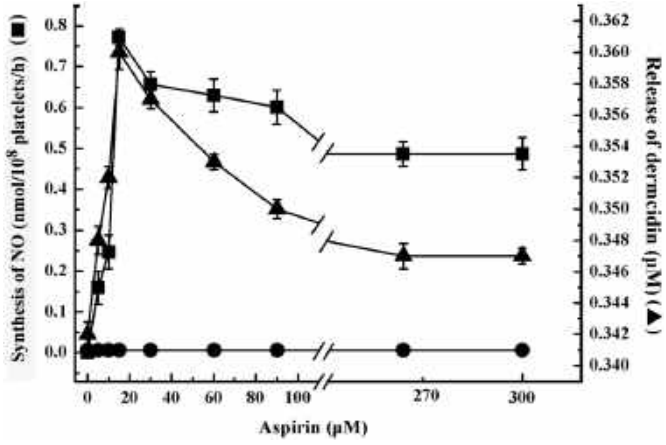

Figure 5 | The effect of treatment of PRP from AMI subjects with 15 μM aspirin on the increase of NO and the removal bound dermcidin from the platelets. Solid triangles (▲) represent the release of the bound dermcidin from AMI platelets treated with different concentration of aspirin as indicated. Solid squares (■) represent the synthesis of NO treated with different concentrations of aspirin as indicated. Solid circles (•) represent the aspirin treated PRP from AMI with 0.1 mM NAME (an inhibitor of NO synthesis) on the release of dermcidin from the platelets. Coefficient of correlation ("r") represents between increase of NO and release of dermcidin from AMI platelets from 0 μM to 30 μM aspirin was +0.967 and "two tailed p value" =0.006. The figure shown is a typical representative of at least 10 different subjects with AMI.

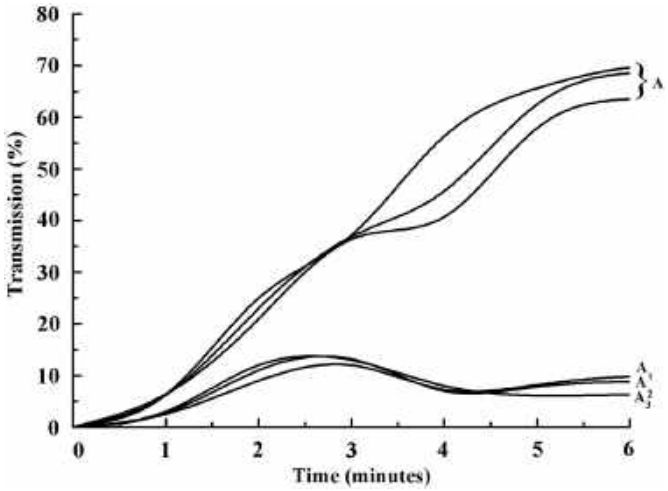

Figure 6 | Effect of reexposure of PRP from the AMI subjects to 10 μM aspirin on the inhibition of ADP induced platelet aggregation in the PRP pre-treated with aspirin or insulin or NO in 0.9% NaCl. The platelet rich plasma from the AMI subjects were incubated with either 15 μM aspirin for 30 minutes or 30 μUnit of insulin/mL for 2 hours or with 0.8 nM NO in 0.9% NaCl for 30 minutes at 37°C to remove the dermcidin bound to the platelets through the increase of platelet NO synthesis as described in the text. After incubation, the same PRP was treated with 10 μM aspirin for 30 minutes at 37°C and aggregation of the platelets was induced by using 2.0 μM ADP. (A) represents the aggregation of platelets in PRP from 3 different AMI subjects incubated with 80 μM aspirin for 30 minutes at 37°C followed by initiation of aggregation of the platelets using 2.0 μM ADP. A_1 = The PRP from the same AMI subjects preincubated with 15 μM aspirin for 30 minutes at 37°C followed by treatment of the PRP with 10 μM aspirin for 30 minutes at 37°C. After incubation, the platelet aggregation was studied. A_2 = The PRP from the same AMI subjects was incubated with insulin and after incubation platelet aggregation was studied by using 10 μM aspirin as described under A_1. A_3 = The PRP from the same AMI subjects was incubated with NO solution in 0.9% NaCl and after incubation the inhibition of platelet aggregation was studied by using 10 μM aspirin as described under A_1.

din in PRP from AMI subjects (not shown in figure 5). The preincubation of AMI platelets in the PRP with N^G-nitro-L-arginine methyl ester (L-NAME) (0.1 mM), an inhibitor of NOS[16], resulted in the nullification of the effect of aspirin both in the increase of platelet NO level and in the removal of the bound dermcidin in AMI platelets (Figure 5). Furthermore, the use of NO solution (0.8 nM) in 0.9% NaCl in the place of either aspirin or insulin was also found to be able to remove the bound dermcidin from AMI platelets. On the other hand, addition of *l*-NAME (0.1 mM) to the PRP which itself failed to negate the removal of the platelet bound dermcidin. The absence of the effect of the *l*-NAME on removal of the bound dermcidin from the AMI platelets suggested that it was not the *l*-NAME itself that removed the bound protein from the AMI platelets. It was also found that the optimal concentration of aspirin for the removal of the dermcidin in PRP from AMI subjects was in the range of 14–15 μM. The use of higher concentration of aspirin >15 μM actually resulted not only the production of less NO in the AMI platelets, but also resulted in the removal of less amounts of the platelet bound dermcidin and at 333 μM aspirin (equivalent to 300 mg aspirin/70 kg body weight), removal of platelet bound dermcidin in the PRP from AMI subjects was decreased by ≈40% compared to that in the presence of 15 μM aspirin.

The effect of removal of the bound dermcidin from the platelets in the PRP from AMI subjects on the inhibitory effect of aspirin in the ADP induced platelet aggregation. When the PRP from the

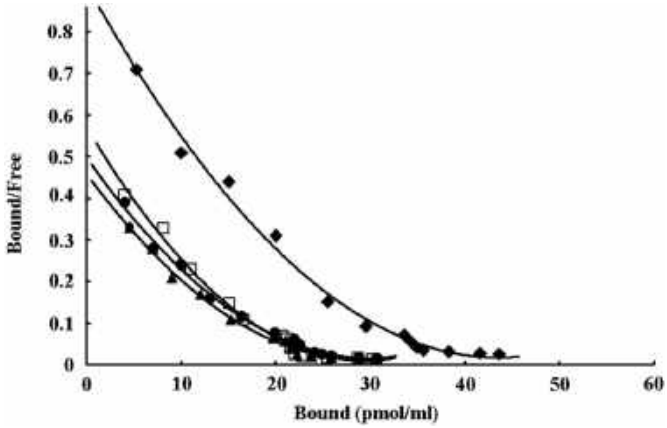

Figure 7 | Scatchard plot of the equilibrium binding of dermcidin on platelets from AMI subjects in presence of aspirin, insulin or NO in 0.9% NaCl. The solid squares (■) represent the binding of dermcidin on platelets in control (normal) experiment. Hollow squares (□) represent the binding of dermcidin in presence of 15 μM aspirin, solid triangles (▲) represent the binding of dermcidin in presence of 30 μUnit/mL insulin and solid circles (•) represent the binding of dermcidin in presence of 0.8 nM NO in 0.9% NaCl. In each case the binding of dermcidin to the platelets in presence of aspirin, insulin or NO was determined by ELISA as described in Method and Materials and the dissociation constant (K_d), the binding capacity (n = dermcidin binding sites/platelet) was determined. The Figure shown here is a typical representative of PRP from at least 6 different AMI subjects divided in 2 different groups with 3 AMI subjects in each group.

AMI subjects were preincubated with either aspirin (15 μM) for 30 min, or with insulin (30 μUnits/mL) for 2 hours or with 0.8 nM NO for 30 min at 37°C and the PRP was subsequently incubated with different concentrations of aspirin for 30 minutes at 37°C, and the ADP induced platelet aggregation was initiated by using 2.0 μM ADP. It was found that the ADP induced aggregation of platelets was inhibited maximally at 10 μM aspirin in all cases (Figure 6). That contrasted the maximal inhibition of platelet aggregation in normal volunteers at 80 μM aspirin under identical conditions (Figure 1). In other word, the platelets from AMI subjects became 8 folds more sensitive to the inhibitory effect of aspirin compared to that of the normal platelets when the bound dermcidin was removed from the platelets from AMI subjects.

The effect of preincubation of PRP from AMI subjects with aspirin, insulin or with NO in 0.9% NaCl solution on the binding characteristics of dermcidin to platelets in Scatchard plot. As the treatment of PRP from AMI subjects with aspirin, insulin or with NO in 0.9% NaCl solution all were found to restore the sensitivity of the platelets to the inhibitory effect of aspirin (figure 6), the effect of these agents on the binding characteristics of dermcidin to platelets from AMI was studied by Scatchard plot analysis (Fig. 7). It was found that the treatment of AMI platelets with the above mentioned agents decreased the binding sites (n) of dermcidin in the platelets in each case without changing the dissociation constant (K_d = 40 nM). It was found that the binding of dermcidin 128 × 10^3 molecules/platelet in the untreated AMI platelets decreased to 80 × 10^3 molecules/platelet and to 76 × 10^3 molecules/platelet and to 78 × 10^3 molecules/platelet dermcidin binding sites when the AMI platelets were treated with aspirin, insulin and NO solution respectively.

Plasma concentration of insulin in AMI subjects. As the presence of 30 μUnits/mL insulin in the PRP from AMI subjects restored the sensitivity of those platelets to the inhibitory effect of aspirin. It was

thought that the plasma insulin level in AMI could be insufficient to restore the inhibitory effect of aspirin in the absence of the added insulin to the PRP. When the plasma insulin level in the blood of AMI subjects was determined, it was found that the plasma insulin level was in the ranges from undetectable amount (near 0 to 5 μUnits/mL) by ELISA.

Discussion

Aspirin, through its ability to inhibit platelet aggregation by the inhibition of cyclooxygenase[17] as well as by the stimulation of nitric oxide synthase[24], is considered to be a corner stone in the therapeutic regimen both for the prevention and for the treatment of ACS that occurs due to the thrombus formation on the coronary artery. Although >90% of cases of AMI have been reported to be due to thrombosis[10], aspirin is reported to fail to inhibit platelet aggregation in AMI, and the use of aspirin in AMI is of little or no use[13]. And as such, it could be suggested that a third component in the circulation could be responsible for the resistance of platelets to the inhibitory effect of aspirin. We have reported before the appearance of a novel stress induced protein, identified to be dermcidin in the circulation in ACS[14]. Dermcidin was reported to be a potent inhibitor of all known forms of nitric oxide syntheses (NOS) and its systemic synthesis was determined to be due to the expression of dermcidin gene induced by environmental risk factors like tobacco extract or nicotine or alcohol or hypoxia[18]. As such, the appearance of dermcidin in the circulation of ACS was not a "metabolic expression" of the environmental stresses leading to the pathogenesis of the condition, but these environmental factors were actually capable of expressing the dermcidin gene in leucocytes, muscle, endothelial[18] and in hepatic cells and in the whole animal[19]. The synthesis of dermcidin was reported to be suppressed by insulin or by aspirin through the cellular increase of NO level[14]. The inhibition of NO synthesis by dermcidin in platelets resulted in the increased binding of dermcidin on platelet surface "receptors" site (Figure 3) that could be removed by increasing NO levels in platelets (figure 5) possibly due to the mobilization of Ca^{2+} in platelets[20]. Dermcidin, a potent inhibitor of platelet NOS, and a diabetogenic and hypertensive protein, was reported to be >40 folds more potent platelet aggregating agent than ADP through the inhibition of NO synthesis in platelet, that resulted in the increased thromboxane-A_2 synthesis in platelets, leading to the aggregation of platelets[14]. In the context of dermcidin as a potent inhibitor of NOS, we have recently reported that the systemic NO level was found to be reduced to ≈0 pmol/ml plasma due to the inhibition of systemic NO by dermcidin that could have cause the anginal pain both in ACS and AMI[21]. It might be the suggested that the anginal pain in these conditions might be consequence of the systemic appearance of dermcidin in the circulation.

The inhibition of NOS by dermcidin in platelets was found to be related to the increased binding of dermcidin on the platelet surface leading to the failure of aspirin to inhibit platelet aggregation in AMI. The increase of platelet of NO level by aspirin, insulin or by NO itself resulted in the removal of the bound dermcidin from its high affinity binding sites on the platelet surface, and as a result in the platelets from AMI subjects became more sensitive to inhibitory effect of aspirin by 8 folds (figure 6). As dermcidin is also present in the normal circulation[14], it could be suggested that the presence of dermcidin in the normal plasma might have rendered the normal platelets partly resistant to the inhibitory effect of aspirin.

As described above when the PRP from the AMI subjects that was first incubated with 15 μM aspirin for 90 min (to remove the platelet bound dermcidin) followed by the treatment of the same PRP with 10 μM aspirin for 30 min resulted in the complete inhibition of platelet aggregation (figure 6). As such, it could be argued that if the total amounts of aspirin used in these experiments was 25 μM in the inhibition of platelet aggregation in the PRP from AMI, why then the use of 300 mg aspirin/70 kg body weight, the usual therapeutic dose of aspirin used in ACS failed to inhibit platelet aggregation in PRP from AMI subjects in the first place? However, as it has been described under Figure 5, the aspirin stimulated NO synthesis in AMI platelets had a biphasic effect in that the maximal synthesis of NO that was achieved at 15 μM of the compound, and the use of higher doses of the compound actually resulted in the decreased production of NO, and at 300 mg aspirin/70 kg body weight (equivalent to 333 μM aspirin) reduced the synthesis of NO by 70% with corresponding reduction removal of the platelet bound dermcidin by 40%.

In conclusion to our results suggested that was not due to the aspirin itself but due to the presence of dermcidin causing aspirin to fail to inhibit aggregation through the inhibition of NOS. The presence of dermcidin in AMI results to the pathological components in the pathophysiology of deadly AMI. This protein nullified the effect of aspirin induced inhibition of platelet aggregation through the synthesis of prostaglandin. Where NO produce from aspirin[22], neutralizes the effect of dermcidin, as such the inhibition of aggregation of platelet by asprin has no effect in the inhibition of atherosclerosis, but inhibit the effect of dermcidin by the control of hypertension and hyperglycemia[18].

The protein has a diagnostic significance because we have performed some preliminary experiments about the aggregation of AMI PRP in presence of aspirin. This data might indicate the AMI even in the absence of Troponin-I determination in plasma.

If these results from *in-vitro* study using PRP from the AMI subjects could be extended to the subjects affected by AMI, it might be suggested that the use of the 300 mg aspirin is not the optimal dose, in that it might actually not only create problem but also could be harmful, perhaps the chronic use of 14 mg bolus of aspirin/70 kg body weight and after 30 min another 9 mg bolus of aspirin/70 kg weight as described (figure 6) might be helpful in expulsion of platelet bound dermcidin to inhibit aggregation and may improve situation (fig. 6). In respect to dermcidin effect, our study relate in the basic science in the pathophysiology of AMI and the failure of aspirin to inhibit aggregation but not a peripheral clinical study.

Methods

Ethical Clearance. The research project, "**Drmcidin isoform-2 induced nullification of the effect of acetyl salicylic acid in platelet aggregation in acute myocardial infarction**" required nominal amount of blood (2 mL) from patients with AMI and ACS. The **INSTITUTIONAL REVIEW BOARD, HUMAN & ANIMAL RESEARCH ETHICS COMMITTEE, SINHA INSTITUTE OF MEDICAL SCIENCE AND TECHNOLOGY**, Kolkata, India approved the study on the condition that followed the approved Human Ethics Protocol strictly in accordance with 1964 Helsinki declaration and no deviation in the study was allowed without the prior written permission of the board.

AMI and ACS patient volunteers who participated in the study must be over ages of 43 to 62 years. No mentally retarded, pregnant women or prisoner took part in the study. All the volunteers signed an informed consent form prior to their participation in the study. It was ensured that the AMI and ACS patients had no other life-threatening infection. Care was taken to see that none of the volunteers were hospitalized for any condition within the last 6 months.

Patients with AMI and ACS were selected for the study only under the strict supervision of a cardiologist. Their complete blood picture was studied intensely and only those patients, who were willing to participate, were selected. Nominal amount of blood samples were drawn under the supervision of the attending physician and nurses. Seepage of blood after withdrawn, the blood was controlled by appropriate technique if any. Written consent was obtained from each of the patients. The committee inspected the progress and problems of the current investigation routinely.

The animal care and all experiments were performed in accordance with the guidelines approved by the Ethics Review Committee for Animal Experimentation at **INSTITUTIONAL REVIEW BOARD, HUMAN & ANIMAL RESEARCH ETHICS COMMITTEE, SINHA INSTITUTE OF MEDICAL SCIENCE AND TECHNOLOGY**, Kolkata, India. This study used healthy white New Zeland Rabbit after being examined by a certified veterinarian (according to animal protocol no 14 B of the institute). A standard diet and sterile water were given *ad libitum*. Care was taken to ensure that no animals were unnecessarily harmed or were subjected to pain during the study and the studies were performed only in the presence of a member belonging to the Animal Right Group.

Chemicals. Goat anti–rabbit immunoglobulin G-alkaline phosphatase, human I_gG, *l*-epinephrine, collagen, thrombin and insulin were purchased from sigma Aldrich. Enzyme-linked immunosorbent assay (ELISA) Maxisorb plates were from Nunc, Roskilde, Denmark. Aspirin was obtained from Medica Zydus Healthcare. All other chemicals used were of analytical grade.

Selection of AMI patients. A total of 115 patients with chest pain lasting more than 120 min (n = 115; M = 92; F = 23) between the ages of 43 to 62 years, with characteristic chest pain of acute coronary syndrome (ACS) were admitted to the Intensive Coronary Care Unit of the Calcutta Medical College and Hospital, Calcutta. These subjects were further followed up for the occurrence of acute myocardial infarction as described below.

Exclusion Criteria. The patients with the history of diabetes mellitus or any life threatening infection were not included in the study. The subjects of ≥62 years old were also excluded. Also, as the pain due to pulmonary embolism, acute pericarditis, intestinal disorders, acute aortic dissection and other conditions that are known to simulate chest pain due to ACS, patients with these conditions, and the patients with severe anemia, coronary spasms were also carefully excluded. None of those patients had received aspirin or were undergoing thrombolytic therapy or the subjects who had been hospitalized for any condition within the past 6 months were excluded from the study. None of the female subjects had ever received any contraceptive medications.

Diagnosis of AMI. Only those patients who were suspected to be affected with AMI were followed up by the electrocardiographic manifestation of both non-ST elevated myocardial infarction (nonSTEMI AMI, n = 68) and the patients with ST elevated myocardial infarction (STEMI AMI, n = 47) were included in these study. In those subjects where the either occurrence of non-STEMI or STEMI could not be ascertained definitely by electrocardiography were not included in the study.

The patients included in the study characteristically had developing Q wave AMI. All non Q wave MI patients were excluded.

The occurrence of AMI was confirmed by the determination of the plasma Troponin I within 24 h of the hospitalization.

Selection of normal volunteers. An equal numbers of normal volunteers (n = 115; M = 92; F = 23) of similar ages participated in the study. None of these volunteers had the history of diabetes mellitus, hypertension, cardiovascular or cerebrovascular condition. None had any life threatening infections. None of these volunteers had been hospitalized for any condition in the past 6 months. All selected volunteers were asked not to receive any medication including aspirin at least for 6 weeks before they were requested to donate blood samples. Blood samples were collected from AMI subjects before the initiation of any cardiovascular therapy for their condition.

Preparation of platelet-rich plasma. The platelet-rich plasma (PRP) from the blood samples from either AMI subjects or normal volunteers were prepared by centrifugation as described before[22].

The platelet-free plasma (PFP) was prepared by centrifuging PRP at 10,000 g for 30 min as described before[22].

Aggregation of platelets. Unless otherwise stated, the aggregation of platelets was studied by using optimal concentration of ADP (2.0 μM) in a platelet aggregometer as described before[5].

Inhibition of platelet aggregation by acetyl salicylic acid (aspirin). If not otherwise indicated, the inhibition of the ADP induced platelet aggregation was studied by incubating PRP with 80 μM aspirin for 30 minutes at 37°C before the aggregation of the platelets was initiated by adding 2.0 μM ADP to the PRP. As described in the results the use of aspirin as in the case of ADP induced platelet aggregation was found to fail to the inhibition of aggregation, use of other aggregating agents including either *l*-epinephrine, collagen, or thrombin for the aggregation of PRP of AMI subjects was also found to be resistant to the inhibitory effect of aspirin. For this reason the aggregation of platelet was routinely performed using only 2.0 μM ADP.

Preparation of aspirin solution. Aspirin (acetyl salicylic acid) was dissolved in deionised water. The pH was adjusted to 7.0 by adding 0.1 M $NaHCO_3$ just before use and discarded after use.

Determination of nitric oxide. Nitric oxide (NO) was determined by methemoglobin method[23] by determining the spectral changes of the absorption maxima at 575 and 630 nm in a Beckman Spectrophotometer Model DU as described before[5]. The concentration of NO was independently verified by using chemiluminescence method[24].

Preparation of dermcidin. Dermcidin was prepared from the cell free plasma from the ACS patients by repeated electrophoresis on polyacrylamide gel first in the presence of sodium dodecyl sulphate (SDS), followed by the electrophoresis of the isolated protein in the absence of SDS[25]. The final preparation was dialyzed for 12 hours against 0.9% NaCl at 4°C as described in details before[26].

Production of dermcidin antibody. The polyclonal antibody against dermcidin was raised in Newzeland rabbits by using eletrophoretically pure dermcidin as the antigen as described before[27].

Enzyme linked immunosorbant assay (ELISA) for dermcidin and insulin. The details of the determination of dermcidin by ELISA have been described before[28]. The plasma insulin level was also determined by ELISA except that insulin antibody was the product of Santacruz Biotech.

Scatchard plot of the equilibrium binding of dermcidin to normal platelet suspension. Gel filtered platelets (GFP) suspension in Tyrod's buffer; pH 7.4 without Ca^{2+} was prepared by using sepharose 4 B column preequilibrated with the same buffer[29]. Typically, GFP (3×10^8 platelets/mL) in Tyrod's buffer was incubated with different concentrations of electrophoretically purified dermcidin for 90 min (optimal time required for the maximal binding of dermcidin to platelets determined in separate experiments). After incubation, 0.5 mL of incubation mixture was filtered over micro glass fibre membrane (GF/C, Sigma alrich) by using Millipore filtration unit as described before[30]. The use of GF/C membrane filtration allowed the platelets to remain adhered to the filter and the other constituents includig the incubation mixture including the free dermcidin passed through the filtrate under mild vacuum[30,31]. The membrane filter was washed with the 3 vol of the reaction buffer. After washing, the membrane filter was air dried and the bound dermcidin eluted from the filter by washing the filter with 1 vol of the buffer containing 0.05% TritonX-100. The filtrate was collected and dialysed against 0.9% NaCl for 12 hours at 4°C. The dialysed filtrate was used to determine the amount of dermcidin present in the filtrate by ELISA as described above.

Scatchard plot of the equilibrium binding characteristics to the platelets was constructed and the dissociation constant (K_d), and the dermcidin binding number (n) on the platelet surface was calculated as described below. Platelet number was determined by optical microscopy.

Statistical Analyses. The results shown are mean ± standard deviation (SD); the significance (p) of the results was determined by student's "t" test. The coefficient of correlation ("r") was determined by Pearson test. The dissociation constant (K_d) and the number of the dermcidin binding sites (B_{max}) in the Scatchard plot analyses were determined by (Graphpad Prism software) and Microsoft Office Excel.

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Author contributions

S.B. and A.K.S. designed the project and wrote the manuscript. **S.B.** and **P.J.** performed all experiments and prepared all the figures. **S.B.**, **S.M.** and **A.K.S.** provided intellectual support and analysed data. **S.G.** and **A.K.S.** contributed reagents/samples. All authors reviewed manuscript.

Additional information

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RESEARCH ARTICLE

The impaired synthesis of insulin and its inability to inhibit platelet aggregation in cerebrovascular accident

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Abstract Both ischemic stroke (IS) and hemorrhagic stroke (HS) are reported to occur due to thrombosis on the arteries of the brain. As diabetes mellitus is a risk factor for strokes and insulin is reported to prevent thrombosis, the role of insulin in IS and HS was investigated. Forty eight stroke victims (IS = 22, HS = 26) and equal number of aged and sex matched normal volunteers participated in the study. Nitric oxide was determined by methemoglobin method. Insulin and Dermcidin isoform-2 (DCN2) level was determined by ELISA by using insulin and dermcidin antibody. Insulin binding to the platelet membrane was analyzed by scat chard plot. Treatment of normal platelet rich plasma (10^8platelets/ml) with 15μUnits insulin/ml produced 1.41 nmol NO. The PRP from the IS and HS victims produced 0.38 nmol NO and 0.08 nmol NO respectively. Pretreatment of PRP from IS or HS subjects with 15 μM aspirin followed by 15μUnits of insulin/ml resensitized the platelets to the inhibitory effect of insulin. Mice hepatocytes treated with 0.14 μM DCN2 abolished the glucose induced insulin synthesis by NO that can be reversed by using 15 μM aspirin. It can be concluded that presence of DCN2 in stroke causes a condition similar to type I diabetes and nullified the effect of insulin in the inhibition of platelet aggregation in both IS and HS. The effect was reversed by 15 μM aspirin.

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Introduction

Strokes, comprised of transient ischemic attack (TIA), ischemic stroke and hemorrhagic stroke, categorized under cerebrovascular disease, is the third most common cause of death and is the second most important cause of neurologic disabilities in humans (Thapa et al. 2013; Donnan et al. 2008). While TIA is reported to be due to embolism of thrombus or the atherosclerotic plaque from a distant part of the body (Mattioli et al. 1994) or from the brain itself, the ischemic stroke is reported to cause by thrombosis or by atheroma caused by thrombosis itself due to platelet aggregation in any one of the major arteries of the brain including cerebral, carotid and vertebral arteries that resulted in the stenosis of the affected artery leading to ischemic stroke due to the impaired blood supply in the brain although stenosis is very rare. However, the mechanisms behind ischemic stroke (IS) and TIA are generally considered to be the same. With respect to hemorrhagic stroke, IS may undergo hemorrhagic transformation but pure hemorrhagic stroke is generally not the result of rupture of atherosclerotic plaque. The most common causes of hemorrhagic stroke are hypertension and cerebral amyloid angiopathy (Lammie 2002; Vasilevko et al. 2010). It could be concluded that the aggregation of platelets leading to the thrombus formation (micro aggregate of platelets embedded in fibrin mass) on the site of atherosclerotic plaque on the arteries of the brain played a critical role in the pathogenesis of the strokes.

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

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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. Pathologically, these two atherosclerotic risk factors are also known to be the two major risk factors for the strokes. We have reported before that the insulin induced systemic synthesis of NO, was not only a potent inhibitor of platelet aggregation but NO can act as a thrombolytic agent in-situ due to the activation of plasminogen in the circulation (Karmohapatra et al., 2007). Also, the insulin induced NO production was reported to control essential hypertension (Ghosh et al. 2014). As the aggregation of platelets may result in the thrombus formation and consequently may lead to the strokes, studies were carried out to determine the possibilities whether the failure of systemic synthesis of insulin as well as the failure of the hormone itself to stimulate NO synthesis in platelets might be involved in the pathogenesis of strokes.

Methods

Ethics statement

Both normal and persons affected with hemorrhagic or ischemic strokes participated in the study. Appropriate approval from the INSTITUTIONAL REVIEW BOARD, HUMAN & ANIMAL RESEARCH ETHICS COMMITTEE, SINHA INSTITUTE OF MEDICAL SCIENCE AND TECHNOLOGY, Kolkata was obtained. Informed consent was obtained from all individual participants included in the study but the victims at presentation who were not physically fit to sign the informed consent form, but the withdrawal of blood samples was essential to exclude polycythemia, anemia and hypercoagulable state or the occurrence of myocardial infarction that may mimic stroke was necessary. The safety of obtaining blood samples from the stroke victims was decided by the attending physicians and by obtaining consent from the kins to the patients, and if necessary the consent of a legal councilor was also obtained. The experiment was strictly performed in accordance Helsinki declaration 1964. The committee had a permanent certified veterinarian whose duty is to ensure that the all the animals were free from any diseases as stipulated by the Animal Right Group. All animal related experiments were strictly performed in the presence of a member of the Animal Right Group and under the supervision of the veterinarian, and special care were taken to ensure that no animals were unnecessarily harmed or were subjected to pain during the study. After the termination of the study, the animals were sacrificed by euthanasia in a carbon dioxide chamber. White albino healthy mice (20-25 g each), Swiss strain, irrespective of gender were used for the study. These inbred animals were fed standard laboratory chow and sterilized water was given ad libitum. The animals were kept under 12 h cycles of light and dark at 23 °C. Appropriate permission was obtained from the INSTITUTIONAL REVIEW BOARD, HUMAN & ANIMAL RESEARCH ETHICS COMMITTEE, SINHA INSTITUTE OF MEDICAL SCIENCE AND TECHNOLOGY for the animal related studies.

The selection of patients with ischemic and hemorrhagic strokes

At presentation, all patients had neurologic disability affecting one or both limbs or were in semi or unconscious state and in most of cases were feverish. Total numbers of patients ($n = 48$, Ischemic stroke = 22 and Hemorregic stroke = 26; M = 28, F = 20) between the age of 35 to 83 years participated in the study who were admitted to the medical college and hospital, Calcutta for the condition. Care was taken to ensure that none of the subjects had any brain neoplasia or severe anemia or hypoglycemia, cranial nerve pain, migraine, subdural hematoma or cranial aneurysm that may mimic stroke. At presentation none of the participants had any history of diabetes mellitus and did not take insulin or acetyl salicylic acid (aspirin) at least 4 wk. before blood samples were withdrawn. None of the victims had been hospitalized for any condition for at least 2 months before they were included in the study. None of the participants had life threatening infections or venereal diseases. Most of the participants were found to be hypertensive and some of them were alcoholics and none had the history of substance abuse.

Although we intended to include TIA patients in our study, our effort to include these victims was not successful. Although there were TIA victims among the patients who came to the Calcutta Medical College and Hospital in Kolkata, a multinational large metropolitan city, we could not contact anyone of them as these patients did not believe they had any diseases and thought the "problem" was due to their "occasional" consumption of alcohol combined with self denial for the occurrence of any disease. For these reasons no study was conducted with TIA victims.

Selection of normal volunteers

An equal number of normal age and sex matched volunteers ($n = 48$, M = 28, F = 20) were also included in the study. None of the selected volunteers had history of systemic hypertension or diabetes mellitus or had any life threatening infections or venereal disease. They had not been hospitalized at least for 6 months before they were requested to donate blood. None of the volunteers received aspirin at least for 4 weeks before they participated in the study. None of the female volunteers had ever received any contraceptive medications.

Collection of blood and diagnosis for the occurrence of hemorregic stroke

Collection of blood samples (2 ml) were withdrawn by venipuncture using siliconized 19 gauze needle and collected in plastic vials and anticoagulated by mixing with sodium citrate as described before (Chakraborty et al. 2003). Most of the patients were in drowsy state, unconscious and stuporous state. Limb ataxia and facial palsy were found in most cases some of them were partially affected and did not response in commands. Most patients were in fasting condition and during the blood withdrawn process stasis was not applied. First sampling tube was discarded to avoid sampling artifacts at each time. The occurrence of hemorrhagic stroke was confirmed by computerized axial tomography (CAT).

Preparation of cell free plasma (CFP)

Cell free plasma was prepared by centrifuging the anticoagulated blood samples at 30,000 g for 15 min at 4 °C (Chakraborty et al. 2003). The platelet rich plasma (PRP) was prepared by differential centrifugation as described before (Chakraborty et al. 2003).

Aggregation of platelets

The aggregation of platelets was studied in an aggregometer as described before (Chakraborty et al. 2003). Unless otherwise stated the platelet aggregation was initiated by 2 μM ADP. The presence of anti hypertensive medications in the blood in usual therapeutic dose was found to be no contraindication in the platelet aggregation study.

Synthesis of insulin in the liver cells from adult mice

Mice hepatocytes were prepared from the liver of adult mice as described before (Bhattacharya et al. 2013). Typically, the freshly prepared liver cells homogenate suspended (10 mg protein/ml) in tyrodes buffer pH 7.4 was incubated in the presence of 0.02 M glucose with or without different concentrations of DCN2 (dermcidin isoform-2) or 0.1 mM NAME [N^Gmethyl l-alanine methyl ester], an inhibitor of nitric oixde synthse (NOS) for different periods of time at 37 °C. After incubation the synthesis of insulin in the reaction mixture was determined by enzyme linked immunosorbent assay described below.

Determination of glucose activated nitric oxide synthase (GANOS) in the liver cell suspension

Typically, the liver homogenate suspended in tyrodes buffer pH 7.0 containing 2.0 mM $CaCl_2$ with or without 0.02 M glucose for different periods of time at 37 °C as described before (Bhattacharya et al. 2013). The synthesis of NO was determined by methemoglobin method as described below. In some of the experiments 0.14 μM DCN2 or 0.1 mM NAME was also added to the reaction mixture to inhibit the synthesis of NO.

Assay of insulin

The plasma insulin was determined by enzyme linked immunosorbent assay (ELISA) using poly- clonal antibody against insulin as described before (Engvall and Perlmann 1972).

Assay of nitric oxide

Nitric oxide was assayed by methomoglobin method under N_2 in a Beckman spectrophotometer model DU as described before (Karmohapatra et al. 2007).

Determination of blood glucose level

The blood sugar level was determined by using glucometer (Behringer).

Scatchard plot of insulin binding to platelets

Gel filtered platelets were prepared from the PRP as described before (Dutta-Roy and Sinha 1987). Typically, $3X10^8$ platelets/ml in tyrodes buffer pH 7.4, was incubated with different concentrations of insulin for 2 h at 23 °C to reach equilibrium. The platelet bound insulin was separated from the free insulin by treating the platelet suspension with 0.05 % TritonX-100 in the same buffer. The platelet bound insulin was separated from the unbound hormone by filtration over GF/C micro glass fiber (Sigma) using Millipore filters (Kahn and Sinha 1990). The amounts of insulin were determined by ELISA as described (Engvall and Perlmann 1972). The dissociation constant (K_d) and the maximum binding (B_{max}) were calculated from the Scatchard plot analysis by using Graph pad Prism software.

Statistical analyses

The results shown are median of at least 10 different experiments using blood samples from different subjects. The significance ("two tailed"- *p* value) of the experiments was determined by student's paired "t" test. To demonstrate the comparison of two values, Wilcoxon sign rank test was performed and Spearman's rank correlation was analyzed to see the correlation. Whenever needed, the dissociation constant (K_d) and the number of the dermcidin binding sites (B_{max}) in the Scatchard plot analyses were determined by (Graphpad Prism software) and Microsoft Office Excel.

Results

The effect of insulin on the synthesis of nitric oxide in normal platelet-rich-plasma and in the platelet-rich-plasma from the subjects affected with ischemic or hemorrhagic strokes

When normal PRP was incubated with different amounts of insulin and incubated for different times at 37 °C, it was found that while as little as 1.0μUnits of insulin/ml was capable of stimulating NO synthesis in PRP when incubated for 2 h (optimal time), the maximal synthesis of NO was achieved when normal PRP was incubated at 15 μUnit/ml for same time at 37 °C. It was found that the maximal amount of NO production was 1.41 nmol/10^8normal platelets/h (median), ranging from 0.63 to 1.98 nmol/10^8 platelets/h (n = 48).

In contrast when the PRP from the ischemic stroke victims was treated with 15μUnits insulin/ml, the amount of NO synthesis was 0.38 nmol/10^8 platelets/h (median), ranging from 0 to 0.68 nmol/10^8 platelets/h (n = 48) under otherwise identical conditions (Fig. 1). In other word the insulin induced NO synthesis in normal volunteers and ischemic volunteers was analyzed from Wilcoxon signed rank test (z value = −4.1069, p value is 0, the result is significant at $p \leq 0.05$, two tailed). When PRP from the hemorrhagic stroke victims was used instead of normal or ischemic stroke, the amount of NO production was 0.08 nmol/10^8 platelets/h (median), ranging from 0 nmol/10^8 platelets/h to 0.30 nmol/10^8 platelets/h. In other word the insulin induced synthesis of NO in platelets from hemorrhagic platelets and ischemic platelet was analyzed by Wilcoxon signed rank test (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). IS and HS had different clinical characteristics (Table 1) like the different amount of NO production.

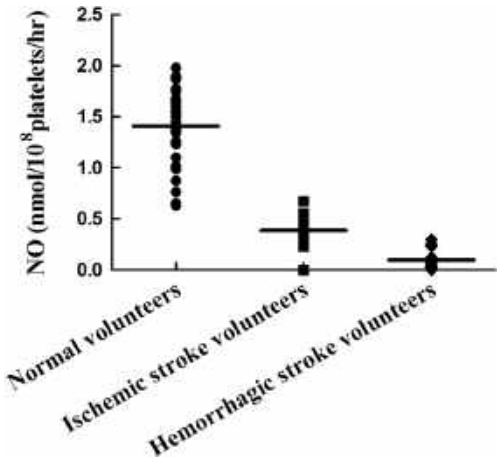

Fig. 1 Synthesis of NO in platelets from normal, ischemic stroke and hemorrhagic stroke volunteers in presence of insulin. PRP was prepared from normal volunteers and ischemic stroke and hemorrhagic stroke victims and was incubated with 15μUnits/ml insulin for 2 h at 37 °C. After incubation the synthesis of NO in presence of insulin was determined as described in methods and materials. This is a scattered graph of all 48 normal volunteers and 48 stroke victims, where middle line determines the median in all cases. Results shown here in each point is the median of NO production in platelets in all 48 subjects in each group

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

As described under Materials and Methods, at presentation, none of the participants selected for the study had diabetes mellitus, however when the plasma insulin levels were determined in the participants (victims) (Fig. 2), it was found that while the plasma insulin level in normal volunteers was 16.85 μUnit/ml (median) ranging from 9 to 25 μUnit/ml (n = 48). The plasma level of insulin in ischemic stroke victims was 1.3 μUnit/ml (median) ranging from 0 to 7 μUnit/ml (n = 22). In hemorrhagic stroke victims the plasma insulin was only 0.35 μUnit/ml (median), ranging from 0 to 7 μUnit/ml (n = 26). The significance "p" between the ischemic stroke victims and normal volunteers was ($p < 0.0001$). The "p" value between the hemorrhagic stroke victims and normal volunteers was ($p < 0.0001$).

Plasma dermcidin isoform-2 levels in ischemic and hemorrhagic strokes victims

We have recently described (Bank et al. 2014; Ghosh et al. 2011) the role of dermcidin isoform-2 (DCN2), an environmentally induced stress protein (molecular weight 11 kDa) in the circulation of persons affected with acute coronary syndromes (ACS) and as well as in acute myocardial infarction (AMI) and also in the systemic increase of arterial blood pressure through the inhibition of insulin induced NO production by DCN2 (Ghosh et al. 2014). This protein was reported to inhibit insulin synthesis in animal model (Ghosh et al. 2014). To assess the role of DCN2, if any, in the impaired NO synthesis in the stroke victims (Fig. 1) and on the inhibition of systemic insulin synthesis in the stroke victims by the stress induced protein as described above (Fig. 2), the plasma DCN2 levels were determined in ischemic and hemorrhagic stroke victims (Table 2). As described in the Table 2, the plasma DCN2 levels in both ischemic strokes victims and in hemorrhagic stroke victims was significantly higher than that in the normal counterpart.

Table 1 Clinical characteristics of stroke patients:

Characteristics	Ischemic stroke	Hemorrhagic stroke	Normal
a. BMI	BMI was greater than normal. It is a risk factor for ischemic stroke but the range was variable.	BMI was greater than normal. The risk factor range was not fixed, so that IS and HS could be identified by BMI.	BMI was in normal ranges (18-25 kg/m^2).
b. HbA_{1c}	$HbA_{1c} \geq 48$ mmol/mol i.e. 6.5 %	$HbA_{1c} \geq 59$ mmol/mol i.e. 7.7 %	$HbA_{1c} \geq 20$-41 mmol/mol i.e. 4–5.9 %.
c. CT/MR	In IS infarction was hemorrhagic	One entity, lobar and nonlobar hemorrhage cannot be differentiated.	No clinical mark was found.
d. nausea, vomiting, alcohol, smoking, headache	In ischemic stroke they were not so prominent. Ischemic heart disease patients were found to attack by IS.	They were significantly seen in hemorrhagic stroke. Smokers and alcoholics were attacked by HS.	Normals had no such specific syndromes.
e. Level of consciousness	Most were drowsy and few of them were alert.	Most of them were unconscious and stuporous condition.	Normals were in complete consciousness.
f. Commands (opening and closing eyes, grip and release nonparetic hand)	IS patients obeyed the one correctly.	Most of HS patients could not obey both; some patients were able to obey one.	All activities were normal.
g. Facial palsy (show teeth, raise brows, squeeze eyes shut)	IS patients were minor and partially affected and 2–4 patients were completely affected.	HS were found to complete facial palsy and a few were partially affected.	No facial palsy was seen.
e. Limb ataxia & speech clarity	Ataxia was in one limb and speech clarity was mild to moderate.	Ataxia was in two limbs and speech clarity was worse in most cases; some of HS had moderate clarity	Speech clarity was normal.

Effects of dermcidin DCN-2 on normal platelets in the insulin induced NO synthesis and in the inhibition of insulin induced inhibition of the platelet aggregation

While the incubation of normal PRP with 15μUnits/ml insulin resulted in the 1.41 nmol $NO/10^8$ platelets in 2 h, the pretreatment of the same PRP with 0.14 μM DCN2 for 90 min at 37 °C before the treatment of the PRP with the same amount of insulin produced only 0.2 nmol of $NO/10^8$ platelets after 2 h at 37 °C under otherwise identical conditions. In separate experiments when the normal PRP preincubated with 15 μUnits insulin/ml was treated with 2.0 μM ADP to initiate platelet aggregation, it was found that the pretreatment of the PRP with insulin resulted in the complete (100 %) inhibition of the platelet aggregation (Fig. 3c) compared to control PRP (Fig. 3b). In contrast when the same normal PRP was treated with DCN2 as described above and the PRP was subsequently exposed to 15 μUnits insulin/ml for 2 h, the ADP induced platelet aggregation could not be demonstrated (Fig. 3a). The aggregation curves of IS and HS were found like A. Here, the role of dermcidin protein was observed in normal platelet aggregation in which dermcidin played the main role in aggregation where only 15 μUnit insulin/ml could not inhibit the aggregation (line A). In this figure the level of dermcidin, was demonstrated in the stroke patients, was responsible for aggregation and effect of dermcidin was showed in normal platelets.

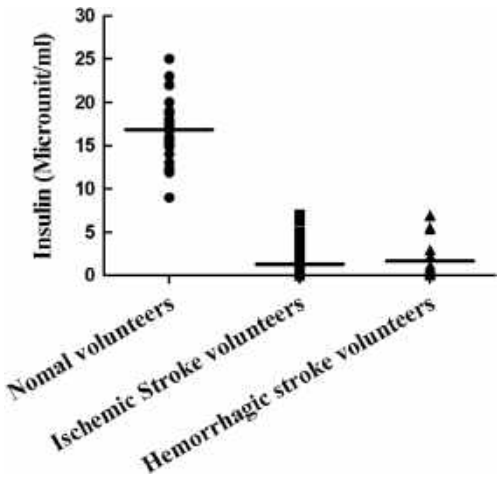

Fig. 2 Plasma insulin level in normal, ischemic stroke and hemorrhagic stroke volunteers. Insulin levels were measured by ELISA in each volunteers as described in methods and materials. Each point represents the amount of insulin (μUnits/ml) for the volunteers determined by at least 10 times in each 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)

Resensitization of the platelets to the inhibitory effect of insulin from ischemic and hemorrhagic stroke victims treated with acetyl salicylic acid in vitro in PRP

We have reported before that acetyl salicylic acid (aspirin) was capable of neutralizing the effects of DCN2 (Bank et al. 2014),

Table 2 The plasma dermcidin isoform2 level (nM) in normal volunteers, ischemic and hemorrhagic stroke victims:

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

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

to determine whether the pretreatment PRP either from ischemic stroke or from the hemorrhagic stroke victims with aspirin would be able to neutralize the inhibitory effect of DCN2 on the insulin induced inhibition of platelet aggregation was determined. When the above PRPs were treated with 15 μM aspirin, a concentration of aspirin (i.e. 15 μM aspirin) by itself had no effect on the inhibition of platelets aggregation (Fig. 4; line A, hemorrhagic stroke and line B, ischemic stroke). The pretreatment of PRP with 15 μM aspirin were able to neutralize the inhibitory effect of DCN2 on the insulin induced platelet aggregation (Fig. 4; line A_1, hemorrhagic stroke and line B_1, ischemic stroke).

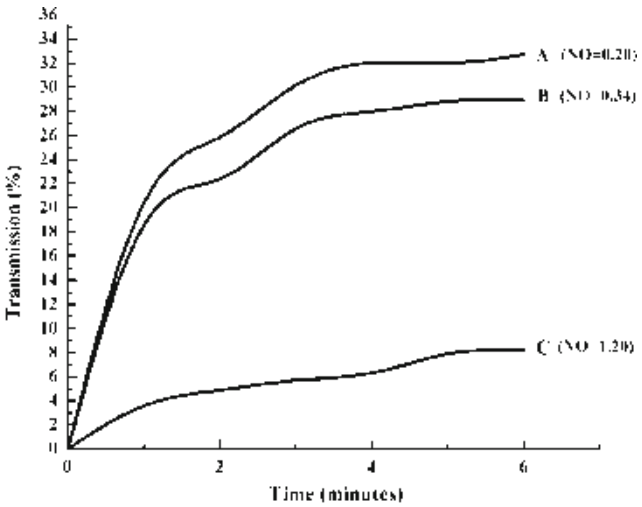

Fig. 3 ADP induced platelet aggregation from normal PRP in presence and absence of dermcidin. **a** represents the PRP from normal volunteers incubated with 0.14 μM dermcidin for 90 min, the same PRP was reincubated with 15μUnits/ml insulin for 2 h at 37 °C and the aggregation induced by 2 μM ADP. **b** represents the aggregation induced by 2 μM ADP of normal volunteers. **c** represents the preincubated normal PRP with 15μUnits/ml insulin at identical condition like (A) and aggregation induced by 2 μM ADP in the absence of DCN2. Figure shown here is a typical representative of 6 different experiments in each case that is equivalent to the other five cases

Scatchard plot analysis of the equilibrium binding of insulin to the platelets treated with aspirin from the ischemic and hemorrhagic stroke victims' in-vitro

As the treatment of PRP from either ischemic or hemorrhagic stroke victims with 15 μM aspirin resulted in the restoration of the insulin induced inhibition of platelet aggregation (Fig. 4, line A_1 & line B_1) when Scatchard plot of the insulin binding in the presence of aspirin was carried out by using PRP from the subjects, it was found that the treatment of these PRP in-vitro with aspirin restored the impaired insulin binding to normal ranges (Fig. 5), both for the high affinity (K_d = 2.19), low

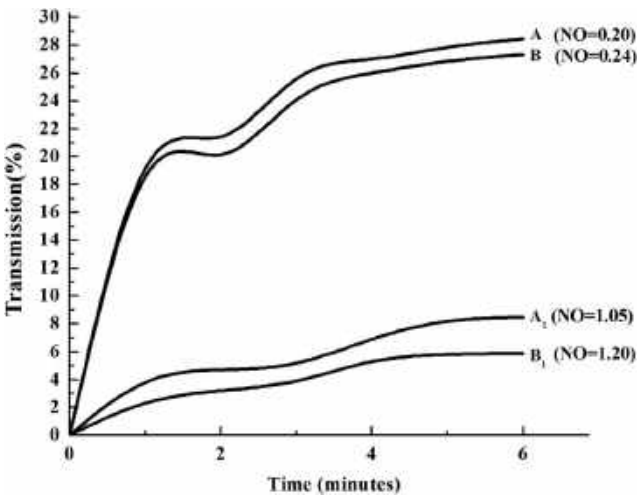

Fig. 4 ADP induced platelet aggregation of normal PRP, ischemic stroke PRP and hemorrhagic stroke PRP. **a** represents the PRP from hemorrhagic stroke victims incubated with 15μUnits of insulin/ml for 2 h at 37 °C and the aggregation induced by 2 μM ADP. **b** represents the PRP from ischemic stroke victims incubated with 15μUnits of insulin/ml under identical condition and the aggregation induced by 2 μM ADP. (A_1) represents the preincubated 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. (B_1) represents the preincubated ischemic stroke PRP with 15μUnits of insulin/ml for 2 h under identical condition like B and followed by incubation of 15 μM aspirin for 30 min at 37 °C

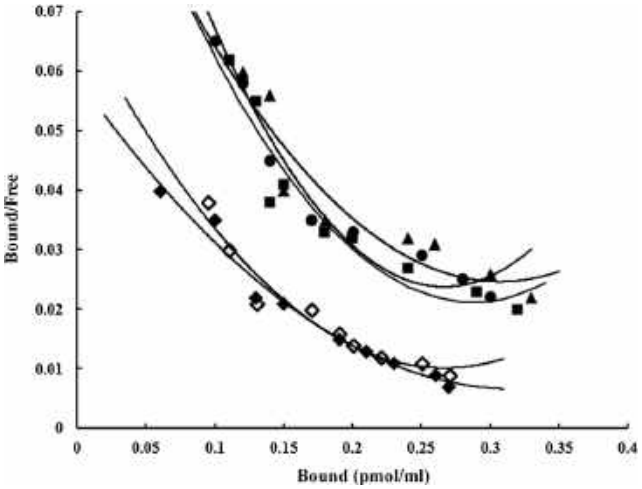

Fig. 5 Scatchard analysis of the equilibrium binding of insulin to the normal, ischemic stroke PRP and hemorrhagic stroke PRP treated with aspirin (♦) solid rhombi represent scatchard plot analysis of equilibrium binding of gel filtered ischemic PRP (10^8platelets/ml) preincubated with 15μUnit of insulin/ml for 2 h at 37 °C. (◊) hollow rhombi represent scatchard plot analysis of equilibrium binding of gel filtered hemorrhagic PRP (10^8 platelets/ml) preincubated with 15μUnit of insulin/ml for 2 h at 37 °C. (■) solid squares represent scatchard plot analysis of equilibrium binding of gel filtered ischemic PRP (10^8platelets/ml) preincubated with 15μUnit of insulin/ml under identical condition like (♦) and followed by incubation of 15 μM aspirin for 30 min at 37 °C. (●) solid circles represent scatchard plot analysis of equilibrium binding of gel filtered hemorrhagic stroke PRP (10^8platelets/ml) preincubated with 15μUnit of insulin/ml under identical condition like (◊) and followed by incubation of 15 μM aspirin for 30 min at 37 °C. (▲) solid triangles represent scatchard plot analysis of equilibrium binding of gel filtered normal PRP (10^8platelets/ml) preincubated with 15μUnit of insulin/ml under identical condition like above. From the scatchard plot, affinity (K_d) was analyzed from the slope and B_{max} was determined from the number of receptors binding site. Please note the curvilinear nature of insulin binding indicate heterogeneous binding site which may also indicate a negative cooperativity of insulin binding i.e. reported before

capacity insulin binding receptors ($n_1 = 1.10X10^3$) and for the low affinity ($K_d = 11.2$), high capacity insulin binding receptor ($n_2 = 2.45X10^3$). In other word, the treatment of platelets from ischemic stroke or from the hemorrhagic stroke victims with aspirin increased the number of insulin binding site on platelets from $0.74X10^3$ to $1.04X10^3$ molecules in the case of ischemic and from $0.70X10^3$ molecules to $1.01X10^3$ in the case of hemorrhagic from high affinity binding sites.

Role of DCN2 on the systemic inhibition of insulin synthesis

As described above not only the insulin induced stimulation of NO synthesis was impaired in the cases of platelets from either ischemic or hemorrhagic stroke victims, but the systemic synthesis of insulin itself was also severely impaired in these conditions (Fig. 2).

We have recently reported the role of a glucose induced nitric oxide synthase (GANOS) in the insulin synthesis in liver (Bhattacharya et al. 2013). Interestingly, contrary to the expectation very little or no GANOS was found to occur 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 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. 6).

Increased body temperature was not only due to the infection in stroke

In stroke, fever is due to pneumonia, catheter related sepsis, urinary tract infection, upper respiratory tract infection and gastritis where microorganism is the main culprit and infection depends on age and severity of stroke. But, in method section it was demonstrated that the infectious stroke patients were already excluded from experiment which may create complicacy in the whole process due to the generation of different molecule in infection. However, many stroke patients were found with fever. C-reactive protein was found not so elevated in stroke patients in this experiment. 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 at hospital. Fever due to infection was emerged at later time points; this suggested that if 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 subarchnoid hemorrhage (Oddo et al. 2009) because high temperature causes the transformation of ischemic penumbra into infarction, increases blood brain barrier breakdown (Reith et al. 1996.).

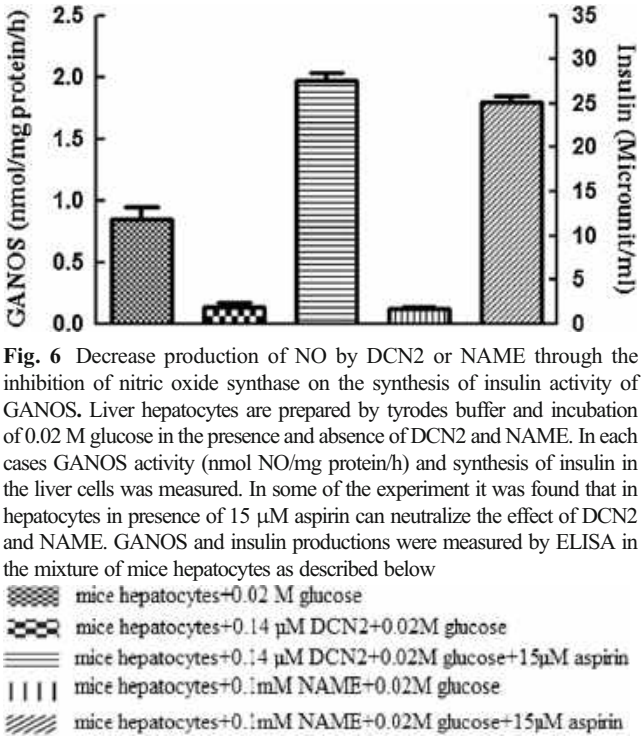

Fig. 6 Decrease production of NO by DCN2 or NAME through the inhibition of nitric oxide synthase on the synthesis of insulin activity of GANOS**.** Liver hepatocytes are prepared by tyrodes buffer and incubation of 0.02 M glucose in the presence and absence of DCN2 and NAME. In each cases GANOS activity (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 as described below

Stroke in alcoholics

Twelve stroke patients (M = 10, F = 2) were found to alcoholics among all strokes patients. The patients who were drinking alcohol >5 years (>3–4 drinks/day) were found to develop stroke. In older age alcohol consumption would be more risk in hypertension and diabetes (Kadlecová et al. 2015).

Discussion

These results suggested that although the development of Type I diabetes mellitus like condition was found to occur in both ischemic and hemorrhagic strokes, the systemic impairment of insulin synthesis was more acute in the cases of the hemorrhagic stroke than that in ischemic stroke. And, as at presentation none of these victims had the history of type I diabetes mellitus, the development of impaired systemic synthesis of the hypoglycemic hormone, essential for energy transduction due to the carbohydrate metabolism might explain the occurrence of the well known association of a fatigued condition in strokes that may continue even during the recuperation. Indeed severe hypoglycemia, which may precipitate impaired energy transduction, is known to mimic the symptoms of the strokes. However, not only the impaired systemic synthesis of insulin occurs in the strokes victims, but even the platelets from both of these groups, stroke victims were failed to found to produce NO when stimulated by insulin (Fig. 1). And, as such, both the impaired synthesis of the agonist as well as the desensitization of the platelets to the effect of the agonist which has been reported to be an antithrombotic humeral factor (Chakraborty and Sinha 2004; Sinha et al. 1999), was partially impaired in ischemic stroke and fully obliterated in hemorrhagic strokes, and thus these impairments might predispose the system to develop thrombosis in the strokes. Also, as insulin induced NO has been reported to dissolute even the formed thrombus on the arterial wall (Chakraborty and Sinha 2004), the impaired insulin induced NO synthesis in platelets in stroke victims might also facilitate the evolvement of the ensuing strokes. In this context it may be mentioned here that we have reported in 1994 a similar lack of insulin synthesis and the resistance of platelets to the antithrombotic effect of insulin also in the case of acute coronary syndrome in humans due to the increased platelet aggregation leading to thrombosis (Kahn et al. 1994). And, thus the failure of insulin to prevent thrombosis both in the case of acute coronary syndrome and in the case of the strokes was apparent from our results.

Indeed it was found that the stimulation of insulin induced NO synthesis in the platelets of hemorrhagic stroke was completely obliterated than the ischemic stroke and normal volunteers. In that it might be possible to differentiate hemorrhagic stroke victims from the ischemic stroke victims by determining the insulin activated NO synthesis in platelets from these subjects. In an effort to identify the stroke victims (ischemic or hemorrhagic) by the degree of insulin induced NO in platelets when the PRP samples from ischemic and hemorrhagic strokes were mixed randomized with the PRP from normal platelets it was found, in a double blind but preliminary study, that it was possible to differentiate hemorrhagic stroke victims from ischemic stroke victims by the insulin induced NO synthesis in platelets (unpublished data) that was subsequently confirmed by CAT scan studies.

The lack of insulin induced NO synthesis in platelets in strokes was found to be related to the diminished insulin receptor numbers on the platelets surface as determined by scatchard plot of the equilibrium binding of the hormone to the platelets from the stroke victims compared to the normal platelets (Fig. 5). It was further found that both the impaired systemic synthesis of insulin and the inability of the hormone to stimulate NO synthesis in the platelets from the stroke victims (Figs. 1 and 2) were due to the presence of DCN2 in the circulation (the correlation between the level of DCN2 and insulin induced NO-production was analyzed by Spearman's rank correlation Coefficient, $r = -1$ i.e. two variables are completely and inversely correlated), a gene expressed synthesis of a protein due to the environmental stresses i.e. not a metabolic expression of a protein (Ghosh et al. 2011). However, it was noted that the detrimental effect of DCN2 could be neutralized at least in-vitro by using 15 μM aspirin (equivalent to 14 mg aspirin/70 kg body weight) in the

reaction mixture would not only nullify the effect of DCN2 in-vitro but also reduced the synthesis of DCN2 to normal level (≈15 nM) in human volunteers affected with acute coronary syndrome that we have reported before (Bank et al. 2014). In this context it should be mentioned here that combination of aspirin and dipyridamole (extended release twice daily) reduces the risk of vascular death, stroke or MI compared with aspirin alone (Diener et al. 1996; Halkes et al. 2006). However, the recommended dose is reported to be 300 mg/day without providing any mechanistic reason, on the other hand our results suggested that only 14 mg/70 kg body weight would be sufficient for the restoration of both impaired insulin synthesis and for the impaired production of insulin induced production of NO in platelets in-vitro that led to the inhibition of aggregation through the inhibition of systemic DCN2, a double edges atherosclerotic risk factors that reported to be both a prodiabetic and a potent hypertensive agent (Ghosh et al. 2012). This protein was also reported to a potent platelet aggregating agent (Bank et al. 2014; Ghosh et al. 2011; Ghosh et al. 2012). It should also be mentioned here that DCN2 is the only environmentally induced stress protein currently known, that was capable of inducing both type I diabetes mellitus and type II diabetes mellitus like conditions in animal model within minutes after the injection of DCN2 in the circulation (Ghosh et al. 2014). This stress induced protein was also reported to inhibit insulin synthesis both in the pancreatic ß cells and in hepatic cells in adult mice by inhibiting glucose uptake and not by the expression of the proinsulin genes which is essential both for the synthesis and for the secretion of insulin through the activation of a GANOS (Bhattacharya et al. 2013). Interestingly the anti insulin effect of DCN2 can be also neutralized at least in-vitro by aspirin as a stimulator of NOS which induced synthesis of NO, this effect of aspirin has been reported to be independent of the well known inhibition of cyclooxygenase that inhibited prostaglandin (Kaplan and Jackson 2011) synthesis which has no effect either on the platelet NO synthesis or in the synthesis of insulin in the liver or pancreas. It should be mentioned here human liver cells have not been successfully cultured yet, we demonstrated all our experiment in mice hepatocytes homogenate.

In feverish patients who were not infected by bacterial infection, were severe cases where massive tissue necrosis can elevate body temperature (Reith et al. 1996) or blood in brain also one of the cause of temperature.

By taking all the results presented above, it is possible to suggest that the precipitation of strokes could be a special case of simultaneous but sudden onset of both type I diabetes mellitus (the lack of insulin synthesis) and type II diabetes mellitus (the lack of insulin effect in NO production in platelets) in the victims, albeit transient who were nondiabetic before beginning the acute episode of the strokes. These results also suggested the only 14 mg aspirin/70 kg body weight might correct both type I and type II diabetes mellitus in the stroke victims. Alternatively, the use of external insulin administration that would control both type I and type II diabetes mellitus might also be helpful in strokes at least to correct the acute type I and type II diabetic like conditions.

Conclusion

Above results demonstrated that the receptors of insulin were impaired because of the presence of dermcidin isoform-2, an environmentally stress induced protein was found in the circulation of AMI and ACS patients. And a type I diabetes like condition was found in the stroke patients. This protein DCN-2 inhibited the production of insulin through the inhibition of synthesis of NO, a stimulator GANOS enzyme which is essential for the secretion and synthesis of insulin. Our results also demonstrated that the use of 15 μM aspirin with insulin might overcome the effect of dermcidin isoform-2 and resensitized the effect of GANOS, thus prognosis of stroke could be prevented.

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Authors' contributions S.Bank, AKS designed the project and have been involved in drafting the manuscript; S.Bank performed all the experiments and made all the figures; S.B. carried out experiment on GANOS enzyme; S.M & A.K.S provided intellectual support and involved in critical data interpretation; R.B & D.C supplied the strokes samples. All authors read and approved final manuscript.

Competing interest Author declares that no competing interest exists.

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ORIGINAL ARTICLE

The Diagnosis of High Altitude Illness by the Determination of Plasma Dermcidin Isoform 2 Levels by Enzyme Linked Immunosorbent Assay

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SUMMARY

Background: **High altitude illness (HAI) is a cluster of syndromes which develops due to the injury of the central nervous system produced by the reduction of the partial pressure of O_2 in the atmosphere which disappears on decent. The HAI also results in a prothrombotic condition leading to acute coronary syndrome (ACS), which cannot be controlled on descent to the ground level. There is no diagnosis in HAI to forewarn of the impending ACS. A protein identified to be dermcidin isoform 2 (dermcidin), produced in the system due to environmental stresses, has been reported to be a potent diabetogenic agent. Investigation was carried out to determine the systemic stimulation of dermcidin synthesis at different levels of altitudes in normal adult male volunteers to assess the feasibility of developing a diagnosis for ACS in HAI due to dermcidin synthesis.**

Methods: **Normal, nondiabetic, normotensive male volunteers (25 - 35 years old, n = 16) participated in the study. The plasma dermcidin level was determined by enzyme linked immunosorbent assay (ELISA) and by *in vitro* translation of dermcidin mRNA. The plasma insulin level was determined by ELISA and blood glucose level was determined in a glucometer (Behringer).**

Results: **The plasma dermcidin level in the volunteers at ground level was 10 ± 2.10 nM and increased to 80 ± 4.62 nM at 15000 feet altitude. For each 1000 feet increase of altitude, the dermcidin level increased by 5.83 ± 0.21 nM with a Coefficient of Correlation "r" = +0.9405. The increase of plasma dermcidin level was found to be inversely related to the decrease of plasma insulin level from 23 µunit/mL to 5 µunit/mL from sea level to 15000 feet height ("r" = -0.9951) with concomitant increase of blood sugar level from 80 ± 3.6 mg/dL to 135 ± 2.01 mg/dL.**

Conclusions: **These results suggest the feasibility of a diagnosis of a prediabetic condition by determining the plasma dermcidin level in HAI by simple ELISA which may also be useful to forewarn of the possibility of developing an impending prothrombotic condition in HAI.**

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KEY WORDS

diagnosis of high altitude illness, dermcidin, insulin, leucocytes, *in vitro* dermcidin mRNA translation

INTRODUCTION

It is generally believed that high altitude illness (HAI) is a cluster of syndromes which develops in persons who are not acclimatized to the reduced partial pressure of O_2 in the atmosphere. While for syndromes such as thrombophlebitis at high altitude, water and electrolyte imbalance and increased capillary permeability lead to the accumulation of fluid at different locations of the body, the reduction of the partial pressure of O_2 in the ambient atmosphere is also known to cause central nervous dysfunction leading to dimmed vision and hemorrhages in nails, kidney, and brain. While these syndromes, although alarming in nature, usually disappear rapidly after decent to the plain, HAI is also known to

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cause the far more life threatening condition of prothrombotic disease due to the development of prediabetic/hypertensive consequences leading to acute coronary syndromes (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. The feasibility of developing such diagnosis for HAI induced ACS was attempted by our earlier studies that demonstrated a protein of 11 kDa, identified to be dermcidin isoform 2 (dermicidin) which was produced in the system due to various environmental stresses [1,2]. Dermcidin was found to result in the development of acute type 1 diabetes mellitus on the injection of the protein (0.2 μM final) in mice as a test animal [3]. Furthermore, dermcidin at the above concentration was also found to increase both systolic and diastolic pressures within 60 minutes of injection in rabbits [4]. This stress induced protein was also found to be a potent activator of platelet cyclooxygenase and was found to potentiate the ADP induced platelet aggregation by 70%. As platelet aggregation particularly by ADP [5] on the atherosclerotic plaque rupture site on the coronary artery has been reported to cause ACS inducing acute myocardial infarction, the role of dermcidin as a diabetogenic and hypertensive agent, as well as platelet cyclooxygenase activator could be of pathologic significance on the precipitation of ACS at high altitude.

The results of the investigation on the effect of various altitude heights on the plasma dermcidin level, show that it could be of useful in the diagnosis of prothrombotic condition at high altitude and are presented herein.

MATERIALS AND METHODS

Collection of blood samples from the volunteers

Blood was collected by venipuncture using 19 gauge siliconized needles and collected in plastic vials. The blood sample was anticoagulated by using 9 vol of blood with 1.0 vol of 13.3 mM sodium citrate and mixed by gentle inversion.

The preparation of cell free plasma and leucocytes

The anticoagulated blood samples were immediately centrifuged at 30000 g at 0°C and the cell free supernatant was collected and kept at -20°C. The leucocytes were prepared from the anticoagulated blood by hypaque-ficoll gradient centrifugation as described before [6]. The nucleic acids from the leucocyte preparation were immediately isolated by Trizol method [7].

The determination of the plasma dermcidin level

The dermcidin level in the cell free plasma preparation, as described above, was determined by enzyme linked immunosorbant assay (ELISA) using electrophoretically pure dermcidin prepared from the cell free plasma samples from a subject with acute ischemic heart disease as described before [1]. The details of the determination by ELISA and the "precision" of the assay using electrophoretically pure dermcidin have been described before [1].

The synthesis of dermcidin by *in vitro* translation of dermcidin mRNA separated from the isolated leucocytes as described above, was carried out in parallel experiments and quantitated by ELISA as immediately as possible without storing the samples at cold temperature in a freezer.

The plasma insulin level was determined in the cell free plasma of the blood samples by ELISA as described before [1,2]. The blood glucose level was determined by using a glucometer (Behringer).

Statistical analyses

The effects of altitude both on the plasma dermcidin level as determined by ELISA and by *in vitro* translation of the mRNA of the nucleic acids from the leucocytes were studied in a "double-blind" technique. The "double-blind" technique was carried out by randomizing the collected samples in the absence of the knowledge of the assayer either of the altitude or the samples from the participating volunteers who were always at sea level during the study. Only after the completion of the experiment, were details of the volunteers made accessible to the investigators for statistical analyses.

Results shown are as mean ± SD of all (n = 16) volunteers at different altitudes at different days after the beginning of the study. The correlation between plasma insulin levels with the plasma dermcidin levels at different altitudes was performed using Pearson's test and expressed as correlation coefficient ("r").

RESULTS

Effect of increased altitude on the plasma dermcidin level in normal subjects who always lived at sea level

The participants (n = 16) who resided in the plains (sea level) had 10 ± 2.10 nM dermcidin/mL at 3000 feet above sea level on day 1. When the volunteers had gone up to 9000 feet and the plasma dermcidin level was determined, it was found that at day 3 the stress induced protein increased to 25 ± 4.97 nM at an altitude of 12000 feet above the sea level and the dermcidin level increased to 47 ± 5.01 nM on day 5. At 15,000 feet altitude, the plasma dermcidin level increased to 80 ± 4.62 nM on day 8. These results indicated an eight fold increase of the plasma dermcidin level at 15000 feet on day 8 compared to day 1 at 3000 feet in these volunteers (Figure 1). Coefficient of correlation ("r") between the plasma dermcidin level and the altitude was found to be

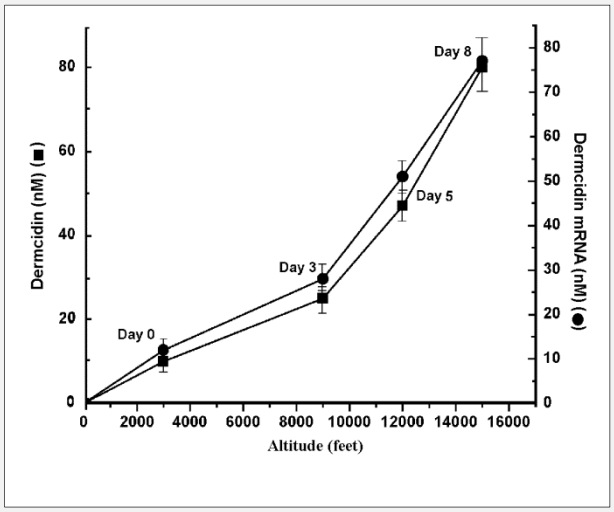

Figure 1. Increase of the plasma dermcidin level at different altitudes.

A group of normal male volunteers (n = 16) went from 3000 feet altitude to 15000 feet as indicated. The plasma dermcidin level was determined by ELISA at day 0 to day 8 as shown.
The results are mean ± SD of each sample done in triplicate by obtaining blood samples from all the 16 volunteers.
While the solid squares (■) represent the plasma dermcidin level by ELISA, the solid circles (●) indicate the synthesis of dermcidin in the leucocytes from the blood samples by the *in vitro* translation of dermcidin mRNA and quantitated by ELISA.

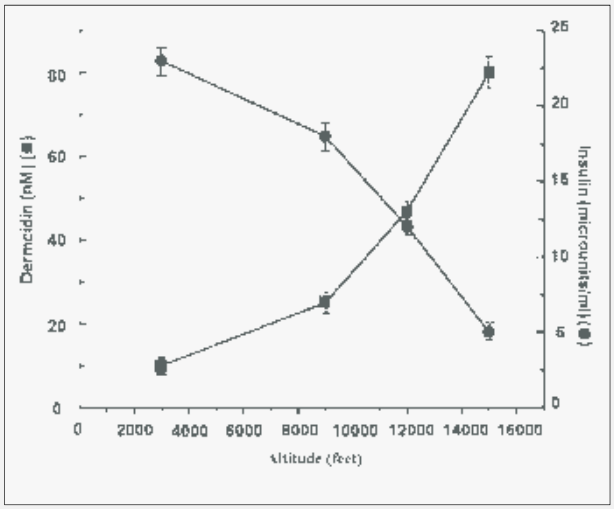

Figure 2. Relation between the plasma insulin and dermcidin levels at different altitudes.

The plasma dermcidin and insulin levels were simultaneously determined in the blood samples of the participating volunteers as described under Figure 1. The plasma dermcidin and insulin levels in the blood samples were determined by ELISA by using dermcidin and insulin antibodies.
Solid squares (■) represent the plasma dermcidin level in the participating volunteers at different altitudes.
Solid circles (●) indicate the insulin level in the volunteers at different altitudes.

+0.9405 suggesting a near perfect positive correlation between these variables. In a parallel experiment, when the *in vitro* translation of dermcidin mRNA was determined in leucocytes, the synthesis of dermcidin was identical to that obtained by ELISA of the stress produced dermcidin as described in the Materials and Methods.

The effect of a systemic increase of dermcidin on the plasma insulin levels and glucose at high altitude

As described under Figure 1, at 15000 feet above the ground, the dermcidin level was maximally increased to 80 ± 4.62 nM from 10 ± 2.10 nM at 3000 feet above the sea level. Dermcidin has been reported to inhibit pancreatic insulin synthesis through the inhibition of glucose uptake in the β cells of the pancreas [1]. As glucose is essential for both insulin synthesis and secretion in the pancreatic cells, the inhibition of the glucose uptake induced by dermcidin is reported to inhibit insulin synthesis without destroying the proinsulin gene. The stress induced synthesis of dermcidin was found to cause a decrease of plasma insulin level from 23 ± 3.5 μunits/mL insulin to 5 ± 2.3 μunits/mL insulin (Figure 2). Coefficient of correlation ("r") between the plasma dermcidin level and insulin was found to be -0.9951 suggesting a near perfect negative correlation between these variables.

As the decrease of the plasma insulin is known to lead to the development of hyperglycemia, when the plasma glucose level was determined, it was found that the fasting glucose level of 80 ± 3.6 mg/dL in these volunteers (n = 16) at 3000 feet above the sea level increased to 135 ± 2.01 mg/dL on the day 8 at 15000 feet above the ground level.

DISCUSSION

These results demonstrated that the increase of the plasma dermcidin levels in normal volunteers was an altitude dependent event that relates to the reduction of partial pressure of O_2 in the atmosphere due to the increase in the altitude. That the reduction of O_2 in the atmosphere was responsible for the increase of dermcidin level was supported by the fact that the incubation of human umbilical cord endothelial cells under hypoxic condition in tissue culture resulted in the expression of the dermcidin gene in the cells (unpublished).

The result presented in the Figure 1 demonstrated that the increase of altitude was highly correlated (the coefficient of correlation "r" was calculated to be +0.9405) at all altitudes where the plasma dermcidin levels were determined in the volunteers from the beginning of the study at day 0 to the end of the study on day 8 at 3000 feet and 15000 feet altitude, respectively. The dermcidin level was determined by ELISA of the cell free blood plasma or by the synthesis of dermcidin involving the *in vitro* translation of the dermcidin mRNA extracted from the leucocytes from the blood samples; indeed, the determination of dermcidin by two different methods produced nearly identical results.

Furthermore, the *in vitro* translation of the dermcidin mRNA demonstrated that the increase of the plasma dermcidin level as determined by ELISA was not merely due to the release of the preformed dermcidin in the circulation due to increased capillary permeability related to the reduction of the atmospheric O_2.

It could be argued that the increase of the dermcidin in the plasma with the increase of altitude (Figure 1) could also be related to the decrease of the ambient temperature which is known to decrease with the increased altitude. However, the internal body temperature is controlled by thermostatic mechanism, in that, the internal body temperature is nearly kept at ≈ 37°C despite the wild changes in the ambient temperature. Furthermore, storing normal leucocytes in plasma with 100 mg/dL glucose at 0 to 20°C did not affect the expression of dermcidin mRNA synthesis *in vitro* translation of dermcidin. Finally, staying at 10000 feet altitude in resorts in summer time with an ambient temperature of ≈ 20°C did not increase the synthesis of dermcidin compared to staying at the same height in winter time (the dermcidin level in both cases were ≈ 10nM).

The issue that the increase of dermcidin level in the blood might actually precipitate AIHD in the HAI victims cannot be addressed at present due to the fact that although the rupture of atherosclerotic plaques on the coronary artery can lead to AIHD due to platelet aggregation on the site of the injury, it is not yet possible to determine when, or if, the atherosclerotic plaque will rupture. However, the elevated level of dermcidin in the circulation of all subjects with AIHD has been reported before [1].

As it has been reported before, the development of atherosclerosis led to prothrombotic conditions [8], and diabetes mellitus both type 1 and type 2 are reported equally to cause atherosclerosis [9,10], and the increase of the dermcidin level in plasma was correlated to the reduction of systemic insulin synthesis (Figure 2). As such, the increased plasma dermcidin level in HAI might lead to AIHD due to prothrombotic conditions.

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Declaration of Interest:
No conflicts of interest exist.

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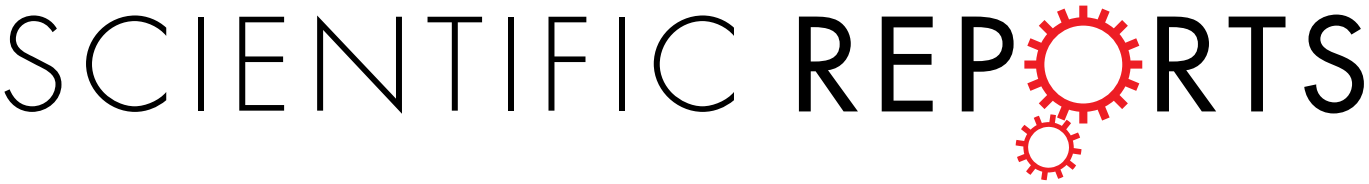

OPEN

The control of hyperglycemia by a novel trypsin resistant oral insulin preparation in alloxan induced type I diabetic mice

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A trypsin resistant oral insulin preparation was made by incubating insulin for 2 h at 23 °C with previously boiled cow milk at 100 °C that was coagulated with 0.6 M acetic acid. The precipitate was resuspended in the same volume of milk. The immunoblot analysis of the suspended proteins treated with 200 ng of trypsin/ml for 3 h demonstrated that the 80.1% of the insulin in the suspension survived the proteolytic degradation compared to 0% of the hormone survived in the control. The feeding of 0.4 ml (0.08 unit of insulin) of the resuspended proteins followed by 0.2 ml of the same protein to alloxan induced diabetic mice maximally decreased the blood glucose level from 508 ± 10 mg/dl to 130 ± 10 mg/dl in 7 h with simultaneous increase of the basal plasma concentration of insulin from 3 ± 1.1 μunits/ml to 18 ± 1.5 μunits/ml. In control experiment the absence of insulin in the identical milk suspension produced no hypoglycemic effect suggesting milk was not responsible for the hypoglycemic effect of milk-insulin complex. Coming out of insulin-casein complex from the intestinal gut to the circulation was spontaneous and facilitated diffusion transportation which was found from Gibbs free energy reaction.

Insulin, a hypoglycemic protein hormone discovered by Best and Banting[1] has an essential role in the carbohydrate metabolism in the transduction of energy for the survival of all forms of animal lives[2]; indeed there is no known alternative to insulin that can replace the protein hormone. According to IDF DIABETES ATLAS, (Sixth edition), the majority of the 382 million people with diabetes are aged between 40 and 59, and 80% of them live in low- and middle-income countries. All types of diabetes are on the increase, type 2 diabetes in particular: the number of people with diabetes will increase by 55% by 2035.

Despite the life saving properties of insulin, when there is a systematic deficiency either due to the systemic impaired insulin synthesis (type 1 diabetes mellitus) or the system itself became resistant to the hormonal effect (type 2 diabetes mellitus) the hormone must be injected in the system from external sources as insulin taken by mouth is rapidly destroyed in the digestive tract particularly due to tryptic digestion, and, as such the needed hormone is not adequately available in sufficient amount to make up for the systemic deficiency of the hypoglycemic protein. Although various efforts have been made to prepare orally active insulin for the control of hyperglycemia[3], the availability of oral insulin preparation has not yet been achieved.

We report herein an orally active trypsin resistant insulin preparation by using commercially available any kind of recombinant insulin by using a simple household procedure that can be carried out anywhere by using minimal facilities without using high technology or expensive instruments that requires trained personnel with expertise in protein chemistry.

Methods

Chemicals. Insulin was prepared and purified by chromatographic method[4] and the same effect was also found from insulin (Biphasic isophane insulin injection I.P.Human Mixtard) with different trade names from

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market, casein, trypsin, HRP-conjugated secondary antibody and primary insulin antibody were from Sigma-Aldrich, ELISA maxisorb plate from Nunc-roskilled. All the other chemicals were of analytical grade.

Ethical clearance. Ethical clearance for this experiment "**The control of hyperglycemia by a novel trypsin resistant oral insulin preparation in alloxan induced type I diabetic mice"** and all the experimental protocol were approved by INSTITUTIONAL REVIEW BOARD, HUMAN AND ANIMAL RESEARCH ETHICS COMMITTEE, Sinha Institute of Medical Science & Technology, Kolkata, India. Twenty mice were used for this purpose under the strict supervision of a licensed veterinarian. No deviation was permitted from the protocol. All the experiments were performed according to Helsinki declaration, 1964.

Preparation of oral insulin. 1 mL of cow's milk was boiled (100 °C) for 2 minutes and cooled to room temperature. Then 100 μlit of insulin (0.08 unit of insulin) was added to the cooled milk and incubated for two and half hours at room temperature (23 °C). After incubation, milk-insulin mixture was precipitated by 10 μL (0.6 M) acetic acid or by lemon juice and let it stand in that condition for 45 min. After precipitation, supernatant can be carefully decanted and added with same amount of cooled and boiled milk to the milk-insulin precipitated mixture to make up the volume 1ml (final).

Experimental animal. White albino Swiss adult mice (*Mus musculus*) (M-10, F-10) aged (3–4 months) wt. 25–30 gm were used in the study. A licensed veterinarian checked them to see that they were free of the disease.

Preparation of alloxan induced diabetic mice. Alloxan (Sigma Aldrich) was injected (150 mg/kg) to the fasting mice. Then the mice were kept with sufficient food so that they could not hypoglycemic. After 72 h, experiments were done by the diabetic mice.

Determination of the distribution of insulin in orally fed milk-insulin from different parts of the diabetic mice. The milk-insulin preparation (0.4 mL) as described above was fed to the alloxan induced diabetic mice. After 3 h and 7 h blood sample (0.1 mL) were collected from tail vein, left and right superior vena cava, hepatic vein, and femoral vein. At the same time blood sample was also taken from appropriate control diabetic mice where only insulin (i.e. not incubated with milk) was fed to the mice. To minimize the pain and discomfort of the mice, morphine was administered to the mice. The amounts of insulin was quantitated in each case by ELISA.

Preparations of intestinal loops filled up with milk-insulin preparation solution. Normal adult white mice (3–4 months) were killed by cervical dislocation and the small intestine was cut out. The intestine was cut into same size (1–1.5 inch) in five pieces. The inner portion of the each pieces of intestine was washed to clean well by Tyrode's buffer. One end of the piece of intestine was bound with silk cord and insulin-preparation solution bound to casein as described above was pipetted inside the loop then the other open side of the intestine was similarly bound tightly. These intestinal loops were next kept separately submerged in 2 mL of tyrod's buffer solution with glucose and both the milieu and the intestinal loop were mildly shaken at 16 Hz oscillation at 37 °C and at 1, 2, 3, 4, 5, 6, 7 h intervals a small portion (0.2 mL) of the milieu was withdrawn and the insulin in the milieu was determined by ELISA by using insulin antibody as described above.

Immunoblot analysis of milk-insulin mixture treated with trypsin. Presence of insulin in the trypsin treated milk-insulin mixture as described in the manuscript was analyzed by immunoblot using by insulin antibody[5]. The transfer buffer used in the experiment contains Tris, Glycine in 20% Methanol while TBST (TBS with 0.1% Tween-20) was used for washing. Incubation buffer was contained Tris and NaCl (TBS). The membrane was blocked with 5%BSA in TBS. Ponceau (1 gm/100 mL) was used to check the transfer of protein from gel to membrane.

Scatchard plot analysis of equilibrium binding of insulin with casein. Pure casein in PBS (phosphate buffer saline) was incubated with different concentration of insulin for 150 minutes (for maximum insulin binding optimal incubation time). 500 μL incubation mixture was filtered over glass fiber membrane (GF/C, Sigma Aldrich) by using Millipore filtration as described before[6]. The casein with the bound insulin remained adhere to the filter of GF/C membrane and unbound insulin passed through the membrane under mild vacuum condition[7,8]. The membrane filter was washed with 3 vol of reaction buffer. Next, the bound insulin was eluted from the filter by washing in 1vol of reaction buffer containing 0.05% TritonX-100. Amount of insulin in the filtrate was determined by ELISA using insulin antibody.

Scatchard plot analysis of equilibrium binding of insulin was constructed and dissociation constant (K_d) and number of insulin binding (n) to the casein was analyzed by using Microsoft Office Excel programme.

Statistical analyses. Glucose level and insulin concentration were shown here in mean ± standard deviation (S.D) of at least 10 different experiments. The relation between the decrease of glucose level and increase of insulin concentration was measured by Pearson's Correlation coefficient test determined by 'r' value. Scatchard plot analysis of the equilibrium binding of insulin was analyzed by using Graphpad prism software and Microsoft Office Excel were used to determine the dissociation constant (K_d) and the number of binding sites (B_{max}) in the casein molecule.

Results

When 400 μl and followed by after 3 h 200 μl of milk-insulin preparation solution was fed to the alloxan treated diabetic mice, the glucose level was found to decrease from 508 mg/dL to 130 mg/dL. When 400 μL of the insulin preparation (containing 0.08 U of insulin) in the milk as described

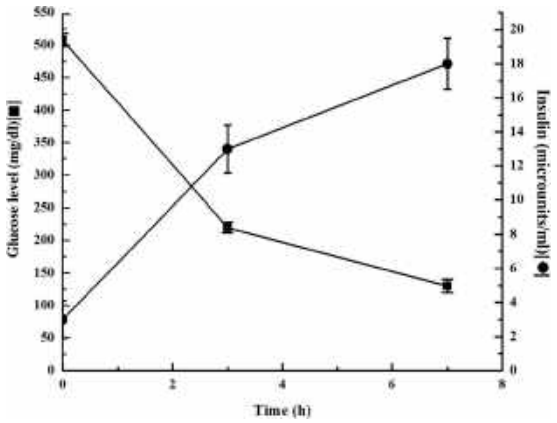

Figure 1. Level of glucose and insulin after oral ingestion of insulin preparation to the alloxan treated diabetic mice. After ingestion of 0.4 mL insulin preparation (containing 0.08 unit insulin) and followed by after 3 h, ingestion of 0.2 mL of insulin preparation to the alloxan treated diabetic mice, 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.

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

Table 1. Distribution of insulin in different arteries of the diabetic mice after feeding the milk-insulin to 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 in method and blood was taken 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.

before and followed by 3 h later 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[9]. It was found that the plasma glucose level of these mice which was 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 (Fig. 1) ($p < 0.001$, $n = 20$). Thus the feeding of milk-insulin preparation to the alloxan treated diabetic mice was found to control the hyperglycemic effect for 11 h. It was further noticed that insulin preparation did not produce any discernable pathological problems in mice; no sickness was determined by veterinarians. Insulin preparation did not produce any toxic effect at least for a month. Insulin preparation keeps its activity at least for 7 days at 4 °C but at room temperature, it was found to active only 3–4 h.

In a separate experiment, using the same mice, when the plasma insulin concentration was determined at different hours after the feeding of the insulin preparation as described under Fig. 1, it was found that the plasma insulin concentration in the alloxan treated mice which was 3 ± 1.1 μUnit of insulin/ml before the feeding of the insulin preparation was found to increase to 13 ± 1.4 μUnit of insulin/ml at 3 h and to 18 ± 1.5 μU of insulin/ml at 7 h (Fig. 1), the Pearson test for the correlation (r) between the decrease of plasma glucose level and the increase of the plasma insulin concentration was determined to be $r = -0.9946$, (two tailed p value, $p < 0.0001$) indicating the increase of insulin concentration was highly but inversely correlated to the decrease of plasma glucose level. As the alloxan treated mice were incapable of synthesizing insulin in the system[9], it was concluded that the increase of the plasma insulin concentration was due to the feeding of the milk insulin preparation from which the hypoglycemic hormone in digestive tract in these animals was able to enter into the circulation to produce the insulin effect in the control of hyperglycemia to normoglycemic level in the type I diabetic mice model. It was calculated that the almost 2.3% (in the 25 g mice) of the insulin that was present in the milk suspension would have entered into the circulation from the digestive tract in the animal.

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 1) at

different time intervals and enzyme linked immunosorbant 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 1). From the ELISA it was resulted that the demonstrated insulin was remained the more or less same.

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 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:

$$\Delta G_A = G_A(\text{in}) - G_A(\text{out}) \quad (1)$$

[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)

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

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

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

[as conc. of insulin in the external milieu $[A]_{out} = 0$, conc. of insulin in intestinal loop $[A]_{in} = 12 \times 10^4$ μU/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, $\Delta G_A = -2577\,J.\,mol^{-1}$
As $\Delta G_A < 0$, i.e negative value of ΔG indicated that the coming out of casein-insulin complex to the circulation (milieu here) was a spontaneous and energy independent process facilitated by diffusive transportation (natural entropy) (see Supplementary Fig. S1).

As $\Delta G_A < 0$, from the experiment, it was found that the rate of efflux of the insulin (K_1) from the intestinal loop to the external milieu (Tyrod's buffer) was 2.62 μUnits/ml/h where as on the other hand entering of insulin from the milieu in the intestinal loop was undectable (0 μUnits/ml/h) $K_2 = 0$, 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 μM each) and ATP (1 mM) as described[10]. 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. The above results also suggested that the added recombinant insulin in the milk suspension survived the tryptic digestion in the digestive tract of the animals. To determine whether insulin in the milk suspension was indeed resistant to the proteolytic degradation catalysed by trypsin, the insulin preparation in the milk suspension was incubated with 200 ng/ml pure trypsin (physiologic level)[11,12] for different time at 37 °C. After incubation, the extend of the proteolytic degradation induced by trypsin was determined by immunoblot analysis as described in the Method section. It was found that even after 3 h of incubation at 37 °C almost 80.1% of the insulin survived the tryptic digestion compared to the 0% insulin survived the trypsin effect (insulin alone) (Fig. 2).

Scatchard plot of the equilibrium binding of insulin to the casein. Scatchard plot analysis of the equilibrium binding of insulin to the pure casein produce a typical curvature plot similar to that of insulin receptors binding (Fig. 3), demonstrating that the interaction between the insulin and the casein followed a negative cooperativity as described in method between the casein and insulin binding sites in the casein. Analysis of scatchard plot binding of insulin to casein showed the heterogeneous binding sites of insulin in the casein molecule. A high affinity binding site ($K_{d1} = 12.36$ pM) with low capacity insulin binding site ($n_1 = 6.02 \times 10^{19}$molecules/casein) and a low affinity ($K_{d2} = 43.96$ pM) with high capacity binding site ($n_2 = 11.08 \times 10^{19}$molecules/casein) were found.

Discussion

It was found that the incubation of insulin with the milk at 23°C at least for two and half hours was essential before the milk-insulin was treated with 0.6M acetic acid to precipitate proteins when the insulin milk mixture

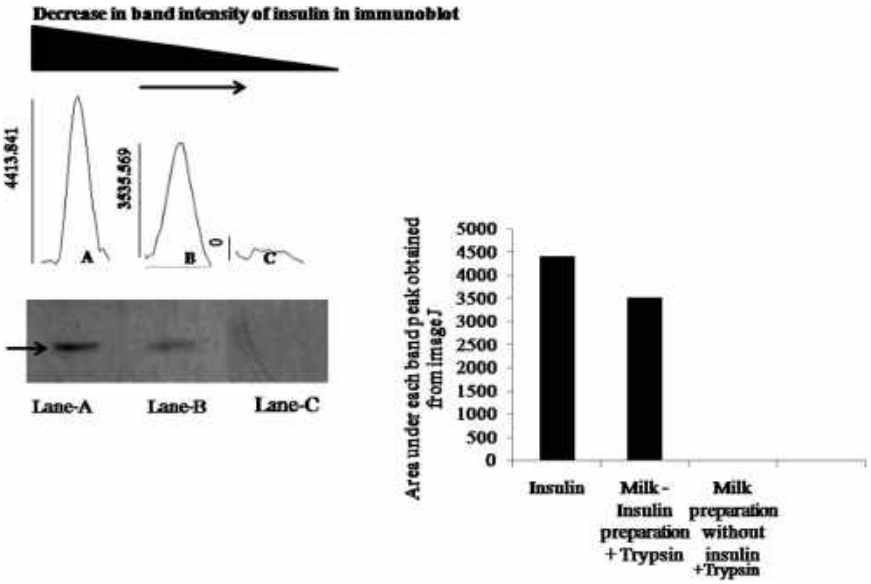

Figure 2. Western blot analysis of trypsin treated oral insulin preparation solution. This figure demonstrated the extension of insulin in trypsin treated insulin preparation solution. It was observed that trypsin was unable to degrade insulin in milk-insulin preparation solution. Here, Lane-A represented the pure insulin (insulin alone) band by western blot analysis where no trypsin was added and an arrow (→) indicated the positive band of insulin in Lane-A; Lane-B represented the insulin band 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 where as no positive band was found in Lane-C.

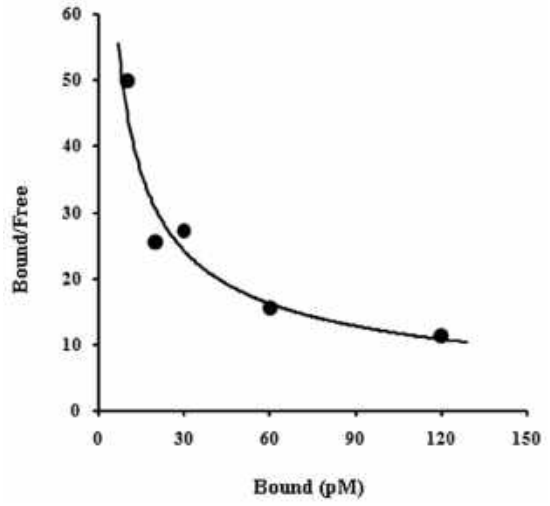

Figure 3. Scatchard plot analysis of equilibrium binding of insulin to the casein in presence of different concentration of insulin. Casein was incubated with different concentrations of insulin for 150 min. Unbound insulin was separated from casein bound insulin using by Millipore filtration unit as described in Methods and Materials. Bound insulin was released from casein in presence of 0.05% Triton-X-100 and the amount of insulin was analyzed by ELISA method by using insulin antibody. Dissociation constant (K_d) and binding capacity (n = number of insulin binding molecules/casein) was analyzed from Scatchard plot. From the analysis it was found a curvilinear plot of heterogeneous binding sites population of insulin on casein.

was treated with acetic acid without pre-incubation and the precipited to proteins were resuspended in the same milk preparation and fed to the alloxan treated mice no hypoglycemic effect on the insulin preparation as described above could be found. In control experiments the treatment of the milk with acetic acid alone had no hypoglycemic effect either when insulin was not added to the milk.

Furthermore it was found that the use of lemon juice (20 μL) instead of acetic acid in the milk and insulin mixture to precipitate the protein, produce hypoglycemic effect similar to the case of acetic acid.

That milk could have protective effect on the tryptic digestion in the digestive tract has been suggested before[13,14]. It has been suggested that mother's milk protects insulin against tryptic digestion in new born babies[15,16] our results as described concluded that casein due to its high affinity binding to insulin was able to co-precipitated with casein by acetic acid in the digestive tract of alloxan treated diabetic mice and the casein bound hormone protected from the tryptic digestion in the digestive tract of the animal. One of the most important steps of our experiment was the incubation of milk with insulin and precipitation by acetic acid. Protein must be precipitated as during the precipitation of protein, H-bonds in the casein molecules were collapsed and bound to insulin with high affinity (≈13pM) and as such serine protease could not get access to the insulin molecule for its enzymic effect.

Intestinal loop experiment also demonstrated that our oral insulin came out from the mice gut to the circulation due to the thermodynamically favored system which was based on the mechanism of Gibbs free energy (ΔG) of the reaction that was energy independent facilitated diffusion mediated by transport protein embedded in the cellular membrane.

The result presented in Table 1 clearly demonstrated that milk-insulin was evenly distributed in various arteries of the experimental mice. Insulin was thoroughly and uniformly distributed in all over the system of the animal. We preferred this method rather than tagged insulin by using I-125 insulin, because in our previous experience, I-125 insulin produced high background noise. In that sense ELISA assay of insulin is more appropriate and produce very low background noise.

Instead of painless modern injection, the inconvenience and uneasiness of insulin injection cannot be overlooked. However, as the diabetic person needs to repeat insulin injection continuously in life time and as such insulin injection might cause other problems specially bruising, soreness, infection, redness, irritation occurs at the site of injection. If it does not administer correctly, muscle cramp may cause. Lipohypertrophy, is one of the common side effect at the injection site, where overgrowth of fat cells often found that makes the skin look lumpy. Sometimes it can also look as scared tissue. We are sometime reluctant to change the site because we feel less painful in that area as hypertrophy can numb the area. Sometime injection may be more painful in that area not only, but abnormal cell growth can also limit the insulin absorption. In that sense our oral insulin could be safer and alternative to the insulin injection. From the above experiment it was found that our oral insulin is basal insulin which demonstrated from its duration of action.

As described above the oral insulin preparation contained only common food items and injectable recombinant insulin preparation available commercially. And, as such, the oral insulin preparation as described might be useful for the control of hyperglycemia in diabetes mellituses instead of injection of the hormone, uncomfortable and century old procedure both for the young and old victims with the condition particularly for the economically disadvantaged peoples in the poorer countries all over the world.

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Author Contributions

A.K.S & S.BANK designed the experiment; S.BANK wrote the paper and performed all experimental works; A.G. helped in western blotting; S.B. helped in insulin related works. S.M. and G.K. provided intellectual support.

Additional Information

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VIA MEDICA

LETTER TO THE EDITOR

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TRIAD system catalase, insulin, and low dose aspirin neutralize the effect of dermcidin isoform-2 and prevent cell death in acute myocardial infarction and recurrence of the disease

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Acute myocardial infarction (AMI) is a global leading cause of death in cardiovascular diseases. Stress is one of major factors predicting AMI [1]. The recurrence of AMI within a month after index hospitalization is very common in patients with cardiovascular diseases. Although the aspirin resistence may be associated with the diagnosis of a definite AMI [2], the low and precisely regulated dose of aspirin might be helpful in AMI through the inhibition of dermicidin isoform-2 (DCN-2) (Fig. 1 — f) i.e. expulsion of platelet bound DCN-2 from AMI plasma [3].

The atherosclerotic plaques are responsible for the initiation of cell death by inhibiting the supply of O_2, glucose, nutrients, and minerals to the site of injury [4]. In consequence, cells in the affected site start to die, being unable to continue core metabolic processes.

Free radical-induced oxidative stress is frequent in AMI patients. Catalase is a free-radical reductor that helps in the inhibition of platelet aggregation [5]. Different types of free radicals and H_2O_2 are generated at the time of platelet aggregation. Catalase acts as a crucial defender by destroying stress-induced free radicals, such as H_2O_2, helps in platelet aggregation inhibition, and subsequently saves the cells from death (Fig. 1 — g, g'). The prevention of cell death is also caused by the caspase inhibition via catalase activation (Fig. 1 — h) [6]. It has also been reported that DCN-2, an environmentally stress-induced 11 kDa protein, is involved in the prognosis of AMI. The concentration of DCN-2 protein is significantly higher in AMI circulation as compared to acute coronary syndrome patients and normal plasma [3]; it is a potent inhibitor of all forms of nitric oxide synthases (NOS) and an activator of platelet aggregation [3]. As a result, thrombus formation starts to progress, leading to cell death (Fig. 1 — i). Low-dose-aspirin and insulin are decisively considered to be inhibitors of DCN-2 [3]. Actually, DCN-2 was able to nullify the effect of aspirin and insulin. Thus, aspirin was unable to inhibit the platelet aggregation in AMI patients. This action is related to the respective synthesis rate/kinetics and local persistence of the endogenous substances *in vivo*, i.e. dermcidin-2 and insulin. In a severe stressful condition, a dominating concentration of DCN-2 may nullify aspirin and insulin effects. However, if we focus on the stepwise effects of DCN-2, i.e. binding on the platelet surface followed by the aggregation of platelets due to the inhibition of nitric oxide (NO) production, and accordingly antagonizing the effects stepwise manner, we could get substantially greater success. Hereby we show unique dosing of aspirin and insulin in a biphasic manner. The first dose of aspirin would be capable of removing the platelet-bound dermcidin from its high affinity binding sites through the production of NO, but it was incapable of inhibiting the platelet

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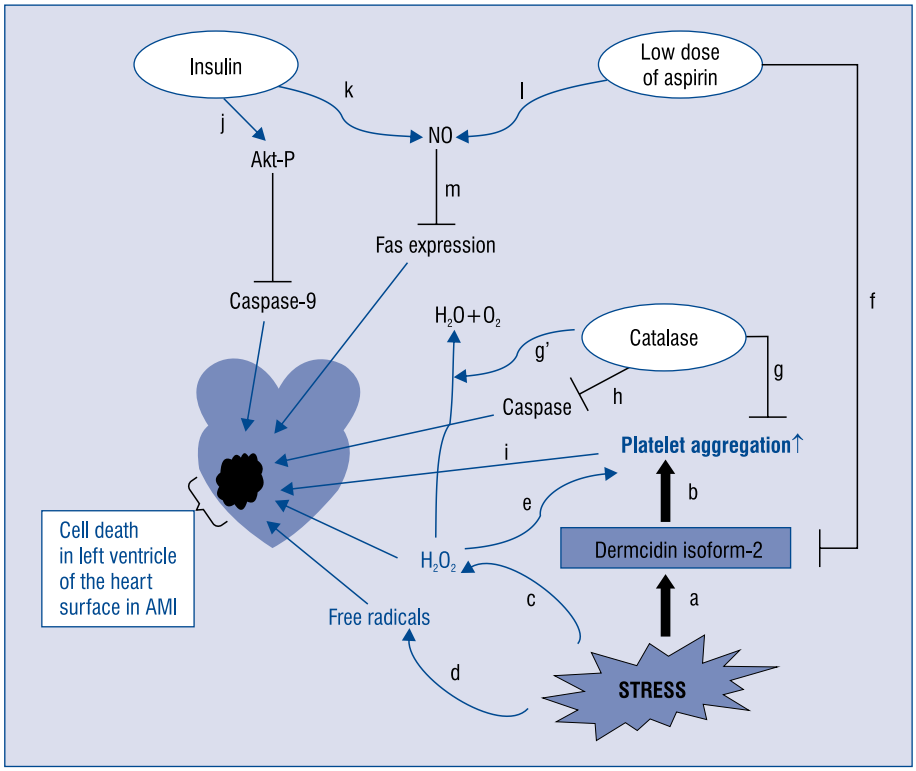

Figure 1. The figure demonstrates dermcidin isoform-2 and a stress protein-mediated platelet aggregation, followed by cell death in acute myocardial infarction (AMI) subjects. It subsequently indicates that the TRIAD system insulin, low dose aspirin and catalase would be capable of neutralizing the dermcidin effect mainly through nitric oxide (NO) pathway.

aggregation. Insulin or NO could be used instead of aspirin at first dose. After removal of dermcidin from the platelet surface, platelets become supersensitive to the aspirin and the second dose of aspirin is able to inhibit the platelet aggregation in AMI. Both of these organic (aspirin) and physiological (insulin) compounds were able to produce NO through the activation of aspirin-activated NOS [7] and insulin-activated NOS [8], respectively, and eliminated the effect of dermcidin [3]. It has also been reported before that insulin is an antithrombotic factor [9]. The high level of DCN-2 (0.4 μM) in AMI subjects is the cause of massive platelet aggregation. Due to the fact that DCN-2 is a stress-induced component, as H_2O_2 and other free radicals, it can directly activate platelet aggregation in AMI (Fig. 1 — a, b, c, d, e). Moreover, it becomes responsible for the cell death in the case of AMI (Fig. 1 — a, b, c, d) and also causes the failure of the aspirin-effect. Specific amounts of insulin and aspirin were able to neutralize the effect of DCN-2 through the production of NO and as such, cell death might be inhibited by this signaling molecule through the inhibition of Fas expression (Fig. 1 — k, l, m). We have already reported that exact amount of aspirin or insulin was able to produce NO. Precisely regulated psychological level of NO is a very important messenger molecule that plays an instrumental role in different cells and tissues of various functions. One of the essential roles of NO is to inhibit the unnatural cell death in AMI by impairing the Fas expression (Fig. 1 — k, l, m) [10]. Fas (Apo-1 or CD95) represents a well-known death receptor that is activated by its cognate — Fas ligand. Fas binding stimulates receptor trimerization and sequential recruitment of the adapter molecule Fas-associated death domain. The NO action impairs this signaling cascade.

On the other hand, if the availability of catalase in an AMI patient remains normal, cell death in AMI might be inhibited due to the reduction of free radicals during platelet aggregation. Therefore, by the interactive role of theTRIAD system (catalase, insulin, and aspirin), the cell death in AMI can be stopped and even the consequence of the recurrence of AMI due to the negative effect of DCN-2 could be prevented (Fig. 1). As a result, it can also be argued that if we were able to inhibit NOS in the heart by gene silencing through double stranded RNA-interference pathway, it would be better to

understand the efficacy of the TRIAD system on DCN-2.

In this context, our report explains the mechanism of cell death occurrence in AMI patients by the stress-induced protein DCN-2. This paper provides explanation how cell death can be prevented, and recurrence of the disease stopped, by a unique solution of maintaining catalase, insulin, and low-dose-aspirin in AMI patients.

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Conflict of interest: None declared

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RESEARCH ARTICLE

Expression of a Nitric Oxide Synthesizing Protein in Arterial Endothelial Cells in Response to Different Anti-Anginal Agents Used in Acute Coronary Syndromes

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Abstract: ***Background*:** Organic "nitro" compounds such as nitroglycerine, isosorbide dinitrate are useful in the control of chest pain in acute coronary syndrome. But the mechanism of it in pain regulation remains speculative. Here, increase of NO production was investigated by the possible regulation of constitutive nitric oxide synthase (cNOS) function from goat arterial endothelial cells. This protein was purified and sequence wise characterized as protein disulfide isomerase (PDI) in response to different nitro compounds.

***Method*:** The NO generating protein was isolated from arterial endothelial cells and prepared to homogeneity. NO was determined by methemoglobin method. Protein sequence was analyzed by (μLC/MS/MS).

***Results*:** A protein of Mr. ~57 kDa was isolated and found to be activated by not only "nitro" compounds but also by acetyl salicylic acid, insulin and glucose. The global BLAST of the protein sequence showed a significant alignment of the protein sequence with PDI. This protein trivially called pluri activator stimulated endothelial NOS (PLASENOS). The enzyme was stimulated by the above-mentioned activators in the presence of Ca^{2+}. Lineweaver-Burk plot of this NOS like activities were demonstrated with its specific substrate *l*-arginine as V_{max} = 5(nmol NO/mg of protein/hr) and $K_m \approx$ 0.5μM by the above activators. The enzyme activity was inhibited by the *l*-NAME, the specific inhibitor of NOS.

***Conclusion*:** The organic nitro compounds, acetyl salicylic acid, insulin and glucose were found to activate PLASENOS in the arterial endothelial cells for a continuous supply of NO to control the chest pain in acute coronary syndrome.

Keywords: Nitro compounds, insulin, nitric oxide, glucose, pain, PLASENOS.

1. INTRODUCTION

The development of severe chest pain in acute coronary syndrome (ACS) typically lasts for thirty minutes or longer [1] and in the cases of acute myocardial infarction (AMI) the pain is reported to be more intense and lasts longer than those in the case of ACS [2]. The presence of severe chest pain in ACS or AMI is considered to be one of the distinctive features for the occurrence of these conditions [3]. Despite its pathological implications, the mechanisms of the development of chest pain in these conditions remain obscured [4-7]. We have recently reported for the first time that the cause of cardiac pain could be related to the severe reduction of plasma NO level (as low as below detection level ≈ 0 nmol NO/mL compared to 4 nmol NO/mL) in normal plasma [8]. The NO is well-known for its ability to reduce pain [9] by the use of any of the organic nitro compounds that are converted to NO [10]. The use of acetyl salicylic acid is also reported to be an antianginal compound through the systemic stimulation of nitric oxide synthases [11]. Taken together, it was suggested that the systemic reduction of NO level was responsible for the cardiac pain [11]. In the above context, it should be mentioned here that nitro compounds, that are reported to be converted to NO for the control of cardiac pain, are extensively used for this purpose [10]. However, neither the 'site' for the conversion of a nitro compound to NO nor how NO forms is known. On one hand, how these are transported to the site of the pain in the heart remains elusive, as NO is converted to NO_2 in the presence of O_2 in the circulation within 10^{-8} sec, various theories have been proposed. On the other hand, the major source of physiologic NO production has been reported to be in endothelial layers [12]. The NO is called endothelial derived relaxing factor (EDRF) [12]. It perhaps misnomer and misleading in that endothelial cells, which do not produce NO by

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itself as there is no basal nitric oxide synthase (NOS) activity in these cells [13, 14]. The presence of a stimulator for the nitric oxide synthase is essential for the stimulation of NO in the endothelial cells [13, 14]. Unfortunately, the presence of nitric oxide synthase (NOS) in the endothelial cells has been demonstrated by an antibody assay only, but not by the actual synthesis of NO in the preparation. Moreover, NO is not only produced in the endothelial cells, but it is also produced in different cells including erythrocytes, neutrophils and in hepatocytes, as such NO as the EDRF may not be acceptable [15-17].

We herein report the presence of a constitutive NOS in endothelial cells of the goat carotid artery that can be stimulated by different and unrelated compounds ranging from the organic nitro compounds to insulin to acetyl salicylic acid (aspirin), and even by glucose (Graphical Figure). The purification and amino acid sequence of this endothelial NOS which is trivially called (PLASENOS) [pluri (many) activators stimulated endothelial NOS] are described herein. Nitro compound, aspirin, insulin and glucose-induced synthesis of PLASENOS mediated pain regulation in acute coronary syndrome is described in this manuscript.

2. MATERIALS AND METHODS

2.1. Chemicals

Nitroglycerin, isosorbide dinitrate, acetyl salicylic acid (aspirin) were from the Medica Zydus Health Company. Insulin, glucose, Triton-X-100, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) were purchased from Sigma chemical. All other chemicals were of analytical grade.

2.2. Preparation of the Goat Carotid Artery Endothelial Cells

Carotid arteries of goat were obtained from the local slaughterhouse and was used as immediately as practicable as described before [18]. Endothelial cells were isolated from the freshly killed goats' carotid arterial lumen by using a soft nylon bottle brush with the appropriate caliber and was dispersed in Tyrode's buffer, pH 7.4, and centrifuged for 10 minutes at 4ºC at 500 g (where g is the relative centrifugal force). The endothelial cells were washed appropriately two more times and suspended in the same buffer.

Factor V antibody was used to verify the properties of endothelial cells in this experiment as described before (19) with the help of the staining of the Weibel Pallade Body (WPB).

2.3. Preparation of Nitroglycerin, Isosorbide Dinitrate and Aspirin Solutions

Nitroglycerine and isosorbide dinitrate solutions were prepared with de-ionized water. Aspirin solution was also prepared in de-ionized water and the pH was adjusted to 7.4 by using 0.1(M) $NaHCO_3$ solution.

2.4. Incubation of Endothelial Cell Suspension with Different Nitro Compounds, Acetyl Salicylic Acid, Insulin or Glucose

Endothelial cells were incubated with these agents for 30 min at 37°C for the maximal activation of endothelial cells as determined by previous but identical experiments. The incubated cell suspension was frozen next and thawed for 30 min in liquid N_2. The cytosolic supernatant was obtained by centrifugation at 30,000 g at 0°C for 30 min. The remaining pellet (that would contain membranes) was mixed and incubated with 0.05% Triton X-100 to release the membrane bound proteins and NOS. The Triton X-100 treated cell suspension was centrifuged at 30,000 g for 60 min at 4°C and the supernatant was used as a membrane protein source. In the control experiments, endothelial cells were not incubated with the stimulators but the supernatant was similarly prepared and the NOS activity was determined.

2.5. Nitric Oxide Assay of Cytosol and Membrane Fraction

Nitric oxide activity was determined in the cytosol and in the membranes by the methemoglobin method using a Beckman spectrophotometer (DU) model and observing spectral changes between 575 and 630 nm as described [20]. Chemiluminescence was simultaneously carried out to confirm the synthesis of NO [21]. Nitric oxide production was found to be occurring with greater amounts in the membranous fraction containing protein than that in the cytosolic protein fraction.

2.6. Gel Electrophoresis of Membranous Protein Fraction Treated with Triton X-100

Ten percent SDS-polyacrylamide gel electrophoresis was done for the NOS containing membrane protein treated with Triton X-100 in the presence and absence of reducing agents. It was found that the molecular weight of a NOS protein was 57 kDa. The nitric oxide activity of the sliced and triturated protein in 0.9 % NaCl solution was confirmed. From the different protein bands, the maximum NO activity was found at 57 kDa. The gel slice with the highest NOS activity was further cut into 0.1 mm slices and each slice was separately triturated with 0.9% NaCl at 4°C. The triturated material was centrifuged at 30,000 g for 30 min to clarify. The clarified supernatant was re-electrophoresed in poly acrylamide gel without SDS. The protein band was cut out of the gel and was lyophilized and prepared for protein sequencing analysis. In control experiments, no bands with NOS activity were observed.

2.7. Protein Sequencing Analysis

Sequence analysis of the purified protein was performed at the Harvard Mass Spectrometry and Proteomics Resource Laboratory, FAS Centre for Systems Biology, Northwest Bldg Room B247, 52 Oxford St, Cambridge MA by

microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) on a Thermo LTQ-Orbitrap mass spectrometer.

2.8. Stimulation of NOS Activity of the Electrophoretically Purified 57 kDa Protein by Different Stimulators

Typically, 0.08 mg/mL of the purified protein was incubated with different agents, including 10 μM nitroglycerine or 10 μM isosorbate dinitrate or 25 μUnit insulin/mL or with 15 μM aspirin or with glucose (0.02 M) at pH 7.0 with 2.0 mM $CaCl_2$ and different concentration of arginine in a total volume of 2.5 mL. After incubation of the reaction mixture for different times at 37°C, the synthesis of NO was determined. It was found that the endothelial NO was maximally stimulated after 30 min of the incubation at 37°C. A Lineweaver-Burk plot was constructed from the rate of NO production during the maintenance of the first order reaction rate (*i.e.* within 1 min). The K_m and V_{max} were calculated from the Lineweaver-Burk plot.

3. RESULTS

3.1. The Amino Acid Sequence of the Purified PLASENOS

The arterial PLASENOS from the goat carotid artery was purified to homogeneity gel as described in Materials and Methods. The purified protein was repeatedly electrophoresed in the presence and absence of SDS (Figure **1a**). After multiple levels of purification, the specific enzymatic activity of this protein was found to be very high compared to that of its crude preparation. This suggests that the degree of purification was satisfactory. The final product was electrophoresed through a polyacrylamide and its molecular weight was determined as 57 kDa and was 69,444 folds purified over the starting endothelial homogenate (Table **1**). According to sequence analysis, the protein comprised 264 amino acid residues as shown in Figure **1b**. The global alignment and multiple sequence alignment with peptide sequences in protein data bank demonstrated that the enzyme was a member of the protein disulfide isomerase gene family [22]. This enzyme catalyzes and regulates the formation-breakage of disulfide bonds between cysteine residues within proteins. This ensures that proteins would find proper arrangement and conformation with the disulfide bonds in their fully folded state. In continuation, it will help the protein to perform its optimized functions at its exact folded state.

3.2. The Characteristics of PLASENOS

The enzyme was found to be Ca^{2+} dependent for the stimulation of NO production. A certain isoform of NOS is known to be essentially dependent on the presence of calcium for its function. The arterial NOS was found to have no

Figure 1a. Purification of PLASENOS

PLASENOS was purified from membranous fraction of goat carotid artery endothelial cells at multiple SDS-PAGE rounds as described in Materials and Methods. In the figure, PANEL-A demonstrates the electrophoresis of TritonX-100 treated membrane protein and PANEL-B demonstrates the sliced, triturated and electrophoresis of the 57 kDa band of PANEL-A in the absence of SDS. In each case, the target band was identified by Coomassie brilliant blue staining. The figure is a typical representative of 10 similar experiments using membrane protein 10 times. The arrow indicates the molecular wt. of PLASENOS protein. M denotes the molecular wt. marker protein.

Figure-1b. Amino acid sequence of the electrophoretically purified PLASENOS as described in the figure-1

After obtaining a single band from the gel electrophoresis, the protein band i.e 57 kDa was sequenced and analyzed. Standard one letter abbreviation of the amino acids are: G glycine, P proline, A alanine, V valine, L leucine, I isoleucine, M methionine, C cysteine, F phenylalanine, Y tyrosine, W tryptophan, H histidine, K lysine, R arginine, Q glutamine, N asparagine, E glutamic acid, D aspartic acid, S serine, T threonine

Table 1. Summary of the purification of PLASENOS.

Protein	Concentration of Protein (mg/ml)	Specific activity (nmol/mg of Protein/hr)	Folds of Purification
Crude homogenized endothelial mass	187	0.009	1
Cytosolic fraction	62	0.0885	9.8
0.05% TritonX-100 treated membrane fraction	14	2.214	246
Purified protein after gel electrophoresis in the presence followed by in the absence of SDS	0.08	625	69,444

Table 1 explains the purification of our target enzyme in terms of nitric oxide synthesis activity [Specific activity (nmol/mg of protein/h) i.e. Production of nitric oxide/mg of protein] and folds of purification is indicated here by comparing the specific activity of the protein in each stage [specific activity of the protein in each stage/ground stage (crude homogenized endothelial mass)].

Endothelial cells were prepared from goat carotid arteries as described in Materials and Methods section. Cytosolic fraction was separated by centrifugation at 30,000 g. The membrane bound proteins were extracted from Triton X-100 treated (TritonX-100 does not interrupt in the assay) membranous fraction. The purified protein was obtained from the membranous fraction by electrophoresis in presence and followed by in the absence of SDS as described in figure-1. Specific activity (NOS activity) of both fractions were determined by the synthesis of (nitric oxide) (mg protein)$^{-1}$ present in the fractions.

basal enzymatic activity, even in the presence of its substrate arginine alone [23]. The enzyme found to be activated in the presence of different stimulators as described under the Figure **2**. Some of these selected stimulators (eg aspirin) are clinically used, but their NOS related action was not obvious.

Endothelial cells of goat carotid arteries were treated with nitro compounds (10 μM), aspirin (15 μM), insulin (25μUnits/mL) and glucose (0.2 M) at different time intervals. The maximum NOS activity (nmol/mg of protein/h) was found at 30 min for each of the stimulator (Figure **2**). Therefore, in each case, different stimulators were required to activate nitric oxide synthase in endothelial cells and it was endothelial derived constitutive nitric oxide synthase. From the figure, it was found that at 30 min NOS activity (nmol/mg of protein/h) was maximized. The results shown here are mean ± SD of 10 different experiments using separately purified PLASENOS for each of the stimulators.

3.3. Activation of PLASENOS by Different Stimulators of the Enzyme

Although the purified enzyme had no basal enzyme activity for the synthesis of NO even in the presence of arginine, the arterial cells were found to be activated by different and unrelated compounds which were demonstrated in the above experiment. This suggests that therapeutic responsiveness at emergency situations might be beneficial and at the normal physiological condition, this NOS activation might not be a prime requirement. This provision of NOS-like activation may be regarded as an adaptive response during the severe pathological conditions.

The electrophoretically purified enzyme from each sample (different nitro compounds, aspirin or insulin or glucose) was added to the different concentration of arginine and the synthesis of nitric oxide was determined. It was found from the Lineweaver-Burk plot that the purified and activated PLASENOS protein showed V_{max} of 5 nmol NO formed/mg protein/h in all the cases where this protein was activated by different stimulators ((A) Nitroglycerine, B) Isosorbide dinitrate, C) Insulin, D) Aspirin, E) Glucose)) (Figure **3**). It was also noted that the NO synthesis activity of PLASENOS was completely inhibited in the presence of 0.1 mM NAME (N^G-Nitro-L-arginine methyl ester hydrochloride) in the reaction mixture. The constant and sharp increase in the NOS like activity by different NO generating compounds suggests a new pathway and activation procedure of NO production (Figures **2** and **3**). In pathological conditions this NO source could have been extremely beneficial. In addition to

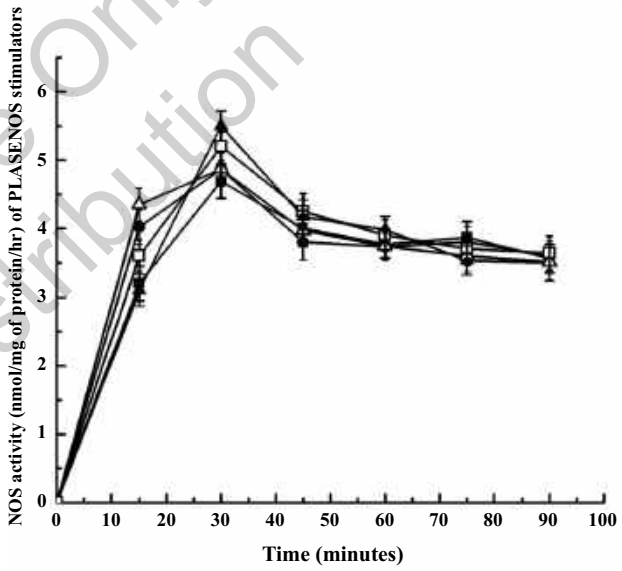

Figure 2. Stimulation of nitric oxide synthase activity (nmol/mg of protein/h) of different PLASENOS stimulators

This figure demonstrates the NOS activity of purified PLASENOS in presence of different stimulators [nitroglycerine (▲), isosorbate nitrate (□), insulin (Δ), aspirin (●) and glucose (■)]. Endothelial cells from goat carotid arteries were incubated with these stimulators at different time intervals. After incubation the NOS activity *i.e.* the synthesis of NO was characterized by the methemoglobin method. The results shown here are mean ± SD of 10 different experiments using separately purified PLASENOS for each of the stimulators.

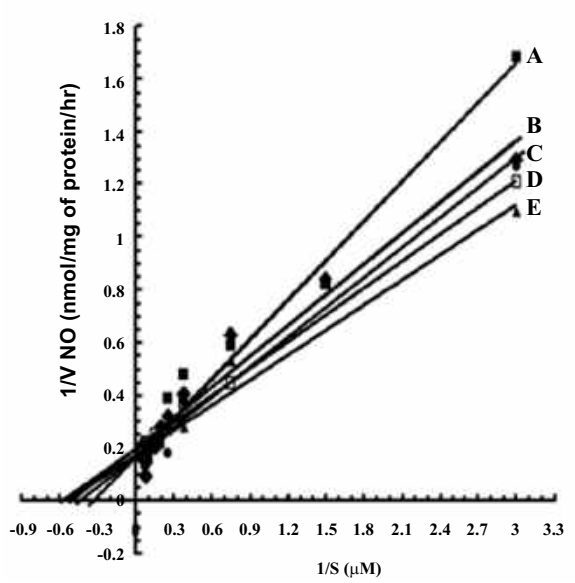

Figure 3. K_m and V_{max} analysis of PLASENOS in presence of stimulators

Lineweaver-Burk plot of purified PLASENOS protein in presence of **A**) Nitroglycerine, **B**) Isosorbide dinitrate, **C**) Insulin, **D**) Aspirin, **E**) Glucose. The L-B plot was constructed by adding the different concentration of arginine and the velocity was the production of nitric oxide at different arginine concentration with the stimulators. From the figure it was found that in all cases V_{max} was almost same i.e. 5 (nmol/mg of protein/h). The K_m of nitroglycerine, isosorbide dinitrate, insulin, aspirin and glucose were 0.4 μM, 0.47 μM, 0.45 μM, 0.4 μM and 0.5 μM respectively ($K_m \approx 0.5$ μM).

synthetic compounds, some physiological natural molecules are found to be potent in this regard. This indicates a possible natural therapeutic management of this disease.

Interestingly, the electrophoretically purified enzyme, unlike the whole endothelial cells that are reported to need glutathione for the conversion of organic nitro compounds (nitroglycerine or isosorbide dinitrate) into NO, did not require the presence of glutathione for the enzymatic activity of the PLASENOS. The only requirement for the *in vitro* enzymatic activity of the arterial enzyme was 2 mM $CaCl_2$. The flexibility in the requirement the thiol substances for the activation of the PLASENOS may indicate the enzyme operates in a redox independent manner and might be beneficial in different pathological conditions. Graphical Figure is the brief demonstration of the steps of the actions of several NO-activating molecules. Some synthetic compounds like acetyl salicylic acid and nitroglycerine and biomolecules like glucose and insulin are shown here in the process of receptor-mediated activation in the arterial endothelial cell membrane. As a result, the activation of membrane bound NOS-like proteins to utilize the substrate arginine to produce citrulline and NO. The NO can perform therapeutic activities in cardiac pathological conditions.

4. DISCUSSION

These results demonstrated the presence of a unique NOS activity in goat carotid-artery endothelial cells, which was activated by 5 different stimulators of the enzyme, including nitroglycerin, isosorbide dinitrate, aspirin, insulin and glucose. All of these agents, except glucose have been reported to control anginal pain through the activation and synthesis of NO. While acetyl salicylic acid (aspirin) is reported to control all syndromes in ACS [24], we have reported before that insulin was capable of controlling angina pain even in unstable angina through the synthesis of NO [25]. We have also reported that the aspirin-induced pain control in ACS could be due to the activation of NOS by the compound instead of its well-known effect, as an inhibitor of cyclooxygenase [20]. We have also reported that glucose was able to stimulate NO synthesis in the hepatic cells [17]. The effect of glucose to stimulate NO synthesis that might also control angina pain without the use of any nitro compound needed however, to be explained. The presence of thrombus itself in the arterial wall in the heart might not allow the plasma glucose to reach the site of pain in the heart. As it has been reported, the development of thrombus not only blocks the availability of O_2 in the heart muscle, but the thrombus will also block the availability of water, nutrients (including glucose), minerals that are essential for the cardiac cell functions and consequently may lead to ACS. As such, it can be hypothesized that the presence of thrombus in the arterial wall that blocks the availability of glucose for the production of NO catalyzed by PLASENOS is itself responsible for the anginal pain. Insulin can directly help in NO synthesis [25] and promote the transportation of glucose which also stimulates NO synthesis.

As discussed above in the text, the site of conversion of nitro compounds to NO remains speculative in that it has been claimed that a thiol compound in the endothelial layers reacts with the nitro compounds for the transportation of anti-anginal agents to the site of the pain [26]. However, a simple chemical calculation of the nitro compounds after being reacted with a thiol group of the protein (that result in the conversion of these nitro agents to NO) would show that the amounts of NO production in the milieu would be exiguous (≈ 1-2 pmol/mL) even in the absence of O_2 (anoxia) in the circulation. As such, the direct conversion of the nitro compounds to NO to act as anti angina-agent may not be adequate.

Our results demonstrated that the presence of PLASENOS would provide a continuous supply of NO for the anti-anginal purpose, provided the substrate arginine was available in adequate quantities in the circulation. The well-known development of tachyphylaxis due to continuous use of nitro compounds in ACS victims could be related to the feedback inhibition of the NOS activity by the product i.e. NO itself [26]. This might hamper the sustained supply of NO to the affected areas.

In the context that PLASENOS may continuously supply NO which is believed to control cardiac pain, it should be mentioned here that the development of hyperglycemia particularly in acute myocardial infarction reported resulting in the worst prognostic outcome of the condition [27]. The presence of NO with glucose was reported to stimulate systemic insulin synthesis and to control hyperglycemia in ACS [28]. The failure of proper functioning of insulin due to the diabetic conditions leading to hyperglycemia synergistically

worsen the situation by nullifying the role of intracellular glucose in NO generation and insulin synthesis. The arterial PLASENOS might be involved not only in the control of anginal pain but might be helpful to improve the prognosis of AMI through the control of systemic hyperglycemia.

From this experiment, it was found that different stimulators activate nitric oxide synthase and could regulate NO i.e. they all could be able to regulate pain through PLASENOS. Nitroglycerin and isosorbide dinitrate produced NO and it was through PLASENOS enzymatic activation. We also reported that aspirin and glucose were able to produce NO [20, 27, 17] and from this experiment it was found that was occurring through PLASENOS. It has been previously reported that insulin can also directly activate the purified protein through its binding to protein [29]. In another experiment, it was also found that insulin was able to stimulate NOS activity in different tissues and NO was capable of performing carbohydrate metabolism like insulin, and NO can also stimulate tyrosine kinase and PI-3 kinase; so, it was demonstrated that nitric oxide is the second messenger of insulin [30]. Therfore, it could be argued that nitro compounds, insulin, aspirin, and glucose all were able to produce NO and they were somehow capable of doing that through protein disulfide isomerase expression. From the Lineweaver-Burk plot it was found that all were possessed nearly the same maximal velocity (V_{max}) of enzymatic reaction of PDI.

The amino acid sequence of PLASENOS demonstrated that this NOS was a member of the protein disulfide isomerase families that have been reported to have an important contribution in both health and disease [22]. The global BLAST of the protein sequence showed a significant alignment of the protein sequence with PDIs from different species i.e. rodents, primates and higher order mammals, including human and goat (result not shown). Interestingly, protein disulfide isomerase has been reported to be involved in the protection in different cardiovascular conditions. Nitric oxide is an important messenger molecule which plays the critical role in a) vascular relaxation b) inhibition of platelet aggregation c) neural transmission d) immune modulation and cytotoxicity. The synergistic effects of more of these could have been beneficial in concerned pathophysiological conditions. In our experiment, we actually have shown that different types of nitro compounds and even glucose, insulin and aspirin were able to express the PDI protein, which was able to regulate itself the production of nitric oxide through the mechanistic pathway of nitric oxide synthase (Graphical abstract). This has been demonstrated by the present experimental protein from the similar pattern of reaction kinetics, substrate utilization and inhibition by specific inhibitor usage as can be noticed during NOS catalysis.

We have found that nitric oxide is responsible for the pain regulation in coronary artery diseases. Here, this experiment demonstrated that nitro compounds, insulin, glucose which can inhibit the pain in CAD, could able to express the PDI in goat arterial endothelial cell membrane and help producing NO *in-vitro*. Thus, it can be assumed that expression of PDI is somehow related to pain regulation at least through the NO pathway.

CONCLUSION

So, from the above experiment it can be said that insulin, acetyl salicylic acid, nitro compounds and even glucose were able to express PLASENOS which was identified as PDI protein in arterial endothelial cells, was able to produce NO i.e. PDI was involved in some way in the regulation of nitric oxide and regulate pain. Therefore, above mentioned drugs might work through the PLASENOS pathway not only in the normalization of pain in ACS or AMI but also protect from the life threatening heart diseases.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The ethical clearance of the experiment was obtained from the Internal Review Board of Sinha Institute of Medical Science & Technology (according to the animal protocol no 16E of Institute), Kolkata, India.

HUMAN AND ANIMAL RIGHTS

No humans were used and all animals were used according to international, national, and institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were strictly followed by the ethical standards of the institution and no deviation from the protocol.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Research Article

Insulin resistance in prostate cancer patients and predisposing them to acute ischemic heart disease

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Lack of insulin or insulin resistance (IR) plays a central role in diabetes mellitus and makes diabetics prone to acute ischemic heart disease (AIHD). It has likewise been found that many cancer patients, including prostate cancer patients die of AIHD. Previously it has been delineated from our laboratory that dermcidin could induce anomalous platelet aggregation in AIHD and also impaired nitric oxide and insulin activity and furthermore dermcidin was also found in a few types of cancer patients. To determine the role of this protein in prostatic malignancy, a retrospective case–control study was conducted and blood was collected from prostate cancer patients and healthy normal volunteers. So, we measured the level of dermcidin protein and analyzed the IR by Homeostasis Model Assessment (HOMA) score calculation. Nitric oxide was measured by methemoglobin method. HDL, glycated hemoglobin (HbA1c), BMI, hs-cTroponin-T were measured for the validation of the patients' status in the presence of Dermcidin isoform-2 (DCN-2). Multiple logistic regression model adjusted for age and BMI identified that the HOMA score was significantly elevated in prostate cancer patients (OR = 7.19, $P<0.001$). Prostate cancer patients are associated with lower level of NO and higher level of both proteins dermcidin (OR = 1.12, $P<0.001$) and hs-TroponinT (OR = 1.76, $P<0.001$). From the results, it can be interpreted that IR plays a key role in the pathophysiology of prostate cancer where dermcidin was the cause of IR through NO inhibition leading to AIHD was also explained by high-sensitive fifth generation cTroponin-T (hs-cTroponinT) and HbA1c level which are associated with endothelial dysfunction.

Introduction

Malignant neoplasm is one of the leading causes of mortality across the world, both in developing and the developed nations. Approximately 9.6 million deaths have been found to occur with cancer in 2018 according to the World Health Organization and approximately 1.8 million of them are prostate cancer [1–3]. Prostate cancer is the fourth leading cause of cancer deaths in men in the world [1–3] and it is the most common cancer for men over 55 years of age [4]. In Australia, there is a chance of one in five men developing prostate cancer before the age of 55 [4]. In North America, this figure is approximately one in seven men [5]. There are many epidemiological evidence-based studies in the literature indicating that prostate cancer is inherited in many cases [4,6]. It is an already known fact that the BRCA1 and BRCA2 genes which are involved in breast cancer [7], ovarian cancer [8] and pancreatic cancer [6] have a pivotal role in the growth of prostate cancer [5,9–11] as well. However, the mechanism behind the problem

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remains obscure in that area. It has been found that 30% prostate cancer at the age of 50 and 80% are at the age of 70, so it is well known that prostate cancer is the old-aged disease. It has been also identified that many of the prostate cancer patients die from cardiovascular diseases [12,13]. It has been reported that the risk of coronary artery disease (CAD) is increased during first 6 months after prostate cancer diagnosis, and metastasis is associated with an increased risk of CAD [14]. Hypertension, diabetes mellitus, chronic kidney diseases and malignancy play the central roles in the pathophysiology of acute ischemic heart disease (AIHD) in these cardinal non-communicable diseases in the modern civilization [15–17]. In prostatic malignancy, patients die of AIHD, urinary tract infection, chronic kidney failure and cerebrovascular stroke. Metabolic syndromes are the risk factors of the prostate cancer [18] but the mechanism of insulin resistance (IR), elevated insulin-like growth factors and even hyperinsulinemia have not been fully elucidated in the setting of prostate cancer.

CAD and cancer share common risk factors, such as smoking, and there is a moderately increased risk of tobacco-related cancers among survivors of myocardial infarction (MI). CAD may predate the development of cancer from the common pathophysiology of IR or may result from treatment of cancer itself. We reported from our laboratory that insulin is an antithrombotic humoral factor for the prevention of CAD through the production of nitric oxide [19]. Therefore, it could be speculated that IR might be the important issue in prostate cancer through the suppression of nitric oxide synthesis. We have likewise reported that the production of nitric oxide is greatly perturbed in cancer patients [20]. Dermcidin isoform-2 (DCN-2), an 11-kDa small, environmentally stress-induced protein, plays the main role in IR and produces atherosclerosis in the pericardial arteries of the heart and as a result cardiac cell death and acute MI (AMI) develop [21,22]. DCN-2 protein functions as an inhibitor of all forms of nitric oxide synthases and inhibits the action of insulin in AIHD [21]. If there is an acute myocardial injury or necrosis, there is sharp elevation in hs-TroponinT level in the circulation of the patients. In the present study, we looked into the basic biochemical phenomenon which takes on an important pathophysiologic part at the molecular level to precipitate the AIHD in prostate cancer patients. Cardiovascular disease was found to occur in many cancer patients and here we considered only the prostate cancer patients to observe the IR by Homeostasis Model Assessment (HOMA) analysis. With the elevated IR condition, we also found some other increased level of risk factors of AIHD in prostate malignancy. And we found here dermcidin protein, which contributes one of the central roles in the regulation of other risk factors of vascular diseases. So, in this clinical experimentation, we have unveiled that these factors can control AIHD in prostate cancer wherever chemo, radiation or hormonal therapy-induced carcinogenic shock/dysfunction would be expected. Our objective was to evaluate if there is any correlation between IR (through the mediation of dermcidin protein, NO, HOMA score, Insulin, HDL) and status of prostate cancer outcome which is evaluated as health status. We were also intended to observe the relation of NO level with cancer outcome because our primary objective was to verify the cancer state and its influence on cardiac outcome.

Methods

Chemicals

Goat anti-rabbit immunoglobin-G alkaline phosphatase was purchased from Sigma–Aldrich. Enzyme-linked immunosorbent assay (ELISA) plates were from Nunc Roskilde, Denmark. Dermcidin primary antibody was from Abcam. High-sensitive fifth generation cTroponin-T (hs-cTroponinT). All the chemicals were of analytical grade.

Selection of prostate cancer patients

The present study was based on a subset of patients drawn from a retrospective case–control study of prostate cancer patients who were seen at the Royal Hobart Hospital, Tasmania, Australia and Sinha Institute, India. Patients were eligible for the study if they met all of the following criteria: age > 40 years; have not taken any aspirin-like medicine at least 1 month before the blood withdrawal. Those patients were included who were initially diagnosed with cancer and before the commencement of their formal treatment/medication blood sample was collected. We were able to avoid the influence of other disease and any medications. So, no cancer medicine was taken by the patients previously and before starting the cancer treatment, blood sample was withdrawn from the prostate cancer patients. Patients were excluded if any of the following criteria were encountered: diabetics and on insulin, nitrates and non-steroidal anti-inflammatory medications (NSAIDs), suffering from any life-threatening diseases; heart-failure and taking different kinds of medicines. A total of 27 prostate cancer patients (n=27) was considered and a total of 25 healthy control subjects who were free from any type of disease complicacy (subjects had no high blood pressure and had normal lipid profiles) and were not taking any kind of medicine since last 1 month, were included in the study.

Diagnosis of prostate cancer

All the suspected prostate cancer patients were diagnosed for its confirmation. The cancer patients who had PSA levels > 10 ng/ml were included in the experiment. Prostate cancer was confirmed by biopsy of prostate tissue (here it is noted that biopsy was not done in our experiment, but for the treatment of the cancer patients, we just took the data), both pre-operatively and post-operatively were taken into consideration and noted [Gleason score was (4+3), i.e., grade-3] otherwise doubtful samples were excluded from the study.

Collection of blood

A total of 1.5 ml of blood was collected by vein puncture in citrate solution (1 vol. of citrate solution: 9 vol. of blood sample) from the study participants, including both prostate cancer patients (here it was noted that blood was drawn from the patients to know the parameters of the sample for their treatment purpose, we took a little amount of blood from the withdrawal sample) and healthy control subjects [23].

Preparation of plasma

Plasma was prepared from whole blood of study participants by centrifuging at 5000 rpm for 12 min as described previously [23].

ELISA for dermcidin

Plasma level of dermcidin was quantified by ELISA by using dermcidin antibody as described before [24].

Assay of nitric oxide

Production of nitric oxide was measured by the methemoglobin method by using Beckman spectrophotometer model DU by the spectral changes (575–630 nm) as described in [25,26] and the NO assay was confirmed by Chemiluminescence method [27].

HOMA score analysis

For the estimation of IR, HOMA-estimated IR (HOMA-IR) calculation was used for the study. In prostate cancer patients, HOMA score was analyzed by glucose level and insulin level. HOMA estimates the β-cell function and insulin sensitivity [28]. It was analyzed by means of the multiplication of the fasting plasma glucose (FPG) by fasting plasma insulin (FPI) and then divided by the constant 22.5 (the resultant value below 2.5 is normal). Insulin was measured by the Chemiluminescence assay in Immulite system and glucose was measured by Abbott Architect c-8000 by using the hexokinase method.

HDL measurement

The plasma HDL level of the study participants was measured by using Architect ci8000/Cobas-6000 through immunoturbidimetric assay method.

hs-cTroponinT measurement

Cardiac troponin-T is used for the diagnosis of AMI. hs-cTroponinT is able to measure very low level of troponin in AMI and was used here for the diagnostic accuracy of AMI. hs-cTroponinT was measured by Cobas 6000 Immuno-analyser.

Glycated hemoglobin measurement

Bio-Rad's mini-column D-10 equipment using high performance liquid chromatography (HPLC) ion exchanger with mobile phase (gradient) and spectrophotometric detection was used to obtain glycated hemoglobin (HbA_{1c}) measurements.

Statistical analysis

Both descriptive and inferential statistical analyses were applied to the collected data using the R statistical software [29]. Continuous variables were expressed by the mean value and the corresponding 25th and 75th percentiles. The Student's *t* test was used for comparison of continuous variables. Multiple binary logistic regression model was applied to assess the association of the HOMA score, dermcidin, NO levels and hs-cTroponinT with the prostate cancer status, adjusting for the demographic variable age and the clinical variable BMI. A bias-reduction method that was

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Table 1 Characteristics of the study population

Characteristics	Case (n=27)[1]	Control (n=25)[1]	P-value[2] (95% C.I.)
Age (years)	70 (66–77)	58.7 (55–65)	<0.001 (6.4, 16.3)
BMI (kg/m^2)	27.4 (26.5–28)	26.2 (25–26.8)	0.001 (0.5, 1.9)
HbA_{1c}	6.2 (6.2–6.5)	5.4 (5.3–5.5)	<0.001 (0.8, 1.0)
HOMA score	5.2 (4.2–6.2)	2.3 (2.0–2.3)	<0.001 (2.2, 3.5)
Glucose	5.7 (4.9–6.5)	4.8 (4.4–5.2)	<0.001 (0.4, 1.4)
Insulin	21.8 (19.7–24.0)	10.4 (9.5–11.5)	<0.001 (9.4, 13.5)
HDL	0.9 (0.8–1.0)	1.3 (1.2–1.4)	<0.001 (−0.5, −0.3)
Dermcidin (nM)	71.5 (59.8–82.4)	17.9 (8.5–27.8)	<0.001 (45.9, 61.2)
NO (nmol/10^8 platelets/ml)	0.2 (0.1–0.3)	1.4 (1.3–1.7)	<0.001 (−1.4, −1.1)
hs-cTroponinT	20.4 (14.8–21.5)	4.9 (3.4–5.9)	<0.001 (12.3, 18.7)

[1]All the values are medians with 25th and 75th percentiles in parentheses.
[2]P-values were determined from the Student's t test.

Table 2 Bias-reduced logistic regression results after adjusting for age and BMI

Characteristics	Adjusted odds ratio[1]	P-value
Dermcidin (nM)	1.12 (1.04, 1.21)	0.003
NO (nmol/10^8 platelets/ml)	0.02 (1.35E-03, 1.85E-01)	<0.001
HOMA score	7.19 (2.25, 22.94)	<0.001
hs-cTroponinT	1.76 (1.18, 2.60)	0.005

[1]Odds ratios are adjusted for the demographic variable age and clinical variable BMI. 95% confidence intervals are given in parentheses.

proposed in [30] was considered when fitting the logistic regression models using the R software package *brglm* [31]. Bias-reduced methods are well-suited for small sample studies, which will have lower standard errors for the estimated parameters as compared with the traditional maximum likelihood estimates and it accounts for the complete or quasi separation issues in logistic regression [30,32]. A two-tailed P-value of less than 0.05 was considered to indicate a statistically significant difference for all the analyses performed. All statistical analysis was performed using R statistical software [33].

Results

In Table 1, we summarize the characteristics of the two groups of subjects in the study. We noticed a highly significant difference between prostate cancer patients and control group in demographic and clinical characteristics. Patients' group had a higher level of results compared with the control group in all the characteristics, except for HDL and NO.

Dermcidin level in prostate cancer patients and corresponding nitric oxide level

As most of the prostate cancer patients die because of cardiac diseases, so we wanted to detect the DCN-2 protein and nitric oxide if any, in prostate cancer patients because NO was also reported to involve with DCN-2 protein in cardiovascular diseases [21,22]. It was found from the ELISA experiment that the average dermcidin level in prostate cancer patients was 71.5 (59.8–82.4) nM. For the healthy control group, the average dermcidin level was 17.9 (8.5–27.8) nM. Binary logistic regression analysis of the dermcidin against the prostate cancer status (reference group: healthy participants) shows that there is a positive association with dermcidin and the prostate cancer status (Table 2). Therefore, on average, dermcidin level is higher in prostate cancer patients as compared with the healthy participants of the study.

In contrast with a weak positive correlation with the healthy control group, we observed a moderate negative correlation between NO and dermcidin levels in prostate cancer patients (Table 3). Hence, on an average the level of NO in prostate cancer patients is lower than the healthy participants' of the study. The average production of NO in prostate cancer patients was 0.2 (0.1–0.3)[1] nmol/10^8 platelets/ml. However, the average production of NO in healthy group

[1]The 25th and 75th percentiles are given in the parentheses.

Table 3 Pearson's correlation coefficient values for the prostate cancer patients group and the healthy control group (in parentheses)

	Age	BMI	HbA1c	HOMA	Glucose	Insulin	HDL	Dermcidin	NO	hs-cTroponinT
Age	1	0.020 (−0.105)	0.097 (0.298)	0.156 (−0.134)	−0.002 (−0.061)	0.398[1] (0.129)	−0.395[1] (0.289)	−0.090 (0.457[1])	−0.022 (0.149)	0.193 (0.041)
BMI		1	0.136 (−0.084)	−0.327 (0.172)	0.066 (−0.306)	−0.306 (0.069)	−0.189 (0.017)	−0.224 (−0.288)	−0.084 (−0.001)	−0.055 (−0.018)
HbA_{1c}			1	0.211 (0.253)	0.521[2] (0.269)	0.010 (0.105)	−0.196 (0.266)	0.178 (0.390)	−0.043 (−0.032)	0.217 (0.054)
HOMA				1	0.331 (−0.178)	0.791[2] (0.347)	−0.157 (0.199)	0.287 (0.070)	0.008 (0.047)	−0.244 (0.030)
Glucose					1	0.002 (−0.786[2])	−0.006 (−0.243)	0.163 (0.222)	0.107 (−0.073)	−0.153 (0.069)
Insulin						1	−0.272 (0.249)	0.361 (0.037)	−0.095 (0.103)	−0.038 (0.038)
HDL							1	−0.277 (0.402[1])	0.101 (0.294)	−0.285 (0.125)
Dermcidin								1	−0.503[1] (0.187)	0.054 (0.005)
NO									1	−0.148 (0.176)
hs-cTroponinT										1

[1]Significance at 5% level.
[2]Significance at 1% level.

was 1.4 (1.3–1.7)[1] nmol/10^8 platelets/ml. Binary logistic regression model (adjusting for age and BMI) indicated that there is a negative association with NO and the prostate cancer status.

Determination of HOMA-IR score analysis in prostate cancer patients and simultaneous detection of hs-cTroponin level

From the epidemiological studies, it has been reported that IR is found in malignancy [34] and as IR is well known in cardiac disease, so troponinT might be elevated. So, to test the hypothesis of IR in prostate cancer HOMA-IR was performed. In general, HOMA was found to increase in the prostate cancer patients, which indicates IR in prostate cancer patients (Table 1). The average HOMA score for the prostate cancer patients was 5.2 (4.2–6.2)[2] and for the healthy control group it was 2.3 (2.0–2.3)[2]. There was a significant difference (P-value <0.01) in HOMA score values between the two groups (Table 1). Furthermore, the binary logistic regression model (adjusted for age and BMI) confirmed that there is a significant positive association with the HOMA score and the prostate cancer status (Table 2).

hs-cTroponinT is an important marker protein to determine the level of AMI patients [35,36]. In the present study, it was found that this marker protein amplified in prostate cancer patients with the simultaneous increase in HOMA score levels. There was a significant difference (P<0.001) in hs-cTroponinT levels across the healthy and the cancer group of patients (Table 1), where the level of hs-cTroponinT is higher in prostate cancer patients as compared with the healthy group. Furthermore, the logistic regression model after adjusting for the age and BMI suggested there is a positive association with hs-cTroponinT and the prostate cancer status.

Discussion

Herein it has been found that the risk factors of cardiovascular diseases are prevalent in the prostate malignancy due to IR which has been explained by HOMA score analysis and diminution of nitric oxide synthesis. From our previous experiment, we found that the death rate of the cancer patients is increasing due to AMI where NO is crucially involved [20]. The plasma level of NO, that has been reported to possess various anti-neoplastic properties [37–39], was found to be diminished due to the impairment of insulin-activated nitric oxide synthase (IANOS) as a result of the appearance of a novel antibody against the enzyme in the circulation in various cancers compared with normal control [37]. It was reported that the resumption of NO synthesis through the neutralization of antibody resulted in

[2]The 25th and 75th percentiles are given in the parentheses.

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favorable modifications of various cancer-associated pathophysiologic consequences [37]. We have reported before that restoration of nitric oxide in physiological level might be helpful to prevent the MI death *in vitro* [21].

In some instances, it has been reported that higher degree of testosterone somehow might be the causative agent in prostate malignancy, though the exact mechanism was speculative [40]. From our previous experiment, we reported that in the presence of testosterone (male androgen hormone), aggregation of platelets was higher compared with control, whereas only ADP-induced platelet aggregation is a normal phenomenon [41]; so, prostate cancer patients are prone to AIHD in such cases. Most of the AIHD or CAD patients would have an IR and nitric oxide synthesis anomaly in their system, which ultimately results in a failed fight against inflammatory and ischemic events. Eventually the situation arises where platelets aggregate in the coronary arteries or cerebral arteries culminating an AMI or cerebrovascular stroke, respectively. It has been reported that dermcidin is a cancer cachectic and proteolysis inducing factor (PIF) [42]; it was also found to overexpress in malignant proliferative cells [43]. This stress-induced protein, which was found in the circulation of AMI and ACS patients, was the platelet aggregator and the inhibitor of the aspirin effect through the impairment of nitric oxide [21,22]. We reported from our laboratory that dermcidin-induced hyperglycemia was due to the impairment of NO [44,45] which causes endothelial dysfunction relating to the elevated HbA1c [46,47]. Insulin, which is an anti-thrombotic factor, plays a very important role in the inhibition of AIHD [19,48] through the production of nitric oxide [49] by the expression of eNOS gene [50]. Insulin shows its thrombolytic activity by synthesizing plasmin from plasminogen and helped in the breakage of fibrinogen bond [51]. Insulin also imparts in the skeletal muscle vasodilation through the release of nitric oxide [52], as such nitric oxide is the second messenger of insulin [53]. In different types of cancer, the production of nitric oxide was found to be zero or significantly diminished [20] and it was also found that the low level of nitric oxide was the responsible factor for the creation of acute ischemic cardiac pain, so nitro compounds (nitroglycerin, isosorbide dinitrate) when administered can help in the neutralization of this acute chest pain through the expression of nitric oxide regulating protein [54]. So, it can be argued that diminished nitric oxide might be the factor in prostate cancer patients predisposing to AIHD. In case of IR in prostate cancer patients, heterogeneous binding of insulin was inhibited on the platelet surface and that is why the level of insulin was found to be low and here DCN-2 might impart the major role in the reduction or impairment of insulin action in the condition as described before [21]. It was also reported that DCN-2 was involved in different types of cancer progression and metastasis; expression of dermcidin gene was involved in the development of micro-environment of prostate cancer in hypoxia [55] and consequently it was also demonstrated before that the protein was responsible for the aggregation of platelets and inhibition of the effect of aspirin in AMI patients [21].

In the present study, we observed that the dermcidin concentration was higher in prostate cancer patients with the simultaneous low production of NO, while in healthy participants' nitric oxide production was found to be normal. Physiological level of nitric oxide is very significant because it acts as a messenger molecule in several cellular functions. Furthermore, HOMA score was found to be elevated in prostate cancer patients. This explains the IR in those patients. We also observed that the hs-TroponinT levels were significantly higher in prostate cancer patients. But the fallacy is that the androgen deprivation therapy (ADT) [56] or radiation/chemotherapy is employed in the prevention of prostate malignancy but these agents can induce cardiotoxicity and many of the prostate cancer patients die due to cardiac diseases not from cancer. So, our experimental approach showed that dermcidin might regulate other parameters which are involved in cardiac diseases and if we aware of DCN-2 regulated IR (HOMA score analysis), NO, HbA_{1c} and cTroponinT level, then our experiment might be helpful for the prostate cancer patients (patients are already in the IR state and prone to AIHD development) to whom different types of cardiotoxic therapy would be exposed, because the timing and dosages of therapeutic agents then would be exposed in a controlled manner and cardiotoxicity might be decreased at least. Not only the abovementioned way of exposure, but also the aspirin therapy might be helpful through the inhibition of DCN-2 activity by aspirin's low and specific dose [57,21].

Though our experimental sample size is not large, but has an important clinical outcome which is explained above. A larger study with more primary cases is required to further validate the research findings of the present study. Furthermore, the case–control study was not an age-matched study. However, to account for this the demographic variable, age was adjusted in the multiple logistic regression models along with the clinical variable BMI. So, despite the small sample size, it can be assumed from the above found results of clinical experimentation that dermcidin could be one of the responsible factors for the creation of prostate malignancy and concomitantly IR here. Previously, it was found that dermcidin impaired the insulin activity through NO inhibition [22,44] and the found result of elevated HbA1c level and lower level of NO are the indication of endothelial dysfunction which actually precipitates cardiovascular disease. Chest pain is the distinctive feature of AMI and low level of NO might be the causative factor of pain in AMI, and cTroponon is the well known marker of the occurrence of AMI. So, from the results it can be inferred that IR in prostate malignancy plays the critical role by the induction of dermcidin protein and other risk

factors, which help in the construction of micro-environment of acute cardiac disease in prostate cancer patients and it can be presumed that various anti-cancer therapies might worsen the situation. We found a highly significant correlation between severity of prostate cancer status and cardiac failure outcome. And in this association the role of dermcidin via dysregulation of NO has been very prominent. The oxidative stress condition initiated due to cancer causing factors was the major determinant of DCD production and NO inhibition. That finally resulted in cardiac anomalies.

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Ethics Statement

Selection of subjects with their consent from Sinha Institute of Medical Science and Technology, Calcutta and Human Research Ethics Committee of Tasmania, Royal Hobart Hospital in Australia with the existing history of prostate cancer and superimposed Acute Coronary Syndrome (ACS). Ethics approval: Human Research Ethics Committee (Tasmania) Network, Approval Ref. No: H11427. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. It was confirmed that patient consent was written and obtained from all individual participants included in the study.

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Author Contribution

Udayan Ray and Sarbashri Bank conceived the study, developed the design of the study and coordination, performed experiment, collected data and drafted the manuscript. Madawa W. Jayawardana performed all the statistical analyses, interpretation of the results and helped to draft the manuscript. Jahar Bhowmik provided statistical analysis support and helped to draft the manuscript. Frank Redwig, Suman Bhattacharya, Pradipta Jana and Emili Manna assisted in study design and data collection. Subrata Kumar De, Smarajit Maiti, Phil Roberts-Thomson and Venkat Parameswaran helped in the study and intellectual support. All authors reviewed the manuscript.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Abbreviations

ACS , acute coronary syndrome; AIHD, acute ischemic heart disease; AMI , acute myocardial infarction; BMI , Body Mass Index; BRCA, Breast Cancer gene; CAD , coronary artery disease; C.I. , confidence interval; DCN-2, Dermcidin isoform-2; ELISA, enzyme-linked immunosorbent assay; eNOS , endothelial nitric oxide synthase; HbA1C , glycated hemoglobin; HDL , high-density lipoproteins; HOMA, homeostasis model assessment; HOMA-IR, HOMA-estimated insulin resistance; hs-cTroponin T , high-sensitive cardiac troponin T; IR, insulin resistance; MI, myocardial infarction; OR , odds ratio; PSA , Prostate-specific antigen.

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