CL-IBMECA ACTIVATED ADENOSINE A<sub>3</sub> RECEPTOR ELEVATES MRNA LEVELS OF ENOS AND NOX 4 IN DIABETIC MICE AORTA

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**ABSTRACT**

Rise of diabetes is one of the major threats to human health worldwide and is a high risk factor for many cardiovascular complications. The vascular damage results in functional, biochemical, and morphological abnormalities in the aorta. Some of these changes may be attributed to altered action of adenosine receptors which might be due to changes in the mRNA expression of nitric oxide synthase and NADPH oxidases. The present study investigates the role of adenosine A<sub>3</sub> receptor in mediating vasoconstriction response in wild type diabetic mice. Streptozotocin treated mice aorta were exposed to adenosine A<sub>3</sub> receptor agonist 2-Chloro-N6-(3-iodobenzyl)-N-methyl-5-carbamoyladenosine and antagonist 2,3-diethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate to measure the dose-response relationship. Further mRNA expression was done by real time polymerase chain reaction, and lipid peroxidation and total Nitrate/Nitrite levels were measured by colorimetry in control and diabetic groups. We observed an increased vasoconstriction response in diabetic group which was diminished in the presence of adenosine A<sub>3</sub> receptor antagonist. This study also explores the effect of adenosine A<sub>3</sub> receptor agonist on the mRNA expression of various nitric oxide synthase isoforms. The results suggest that adenosine A<sub>3</sub> receptor is responsible for promotion of vasoconstriction response in diabetes. There is alteration in the expression of endothelial nitric oxide synthase and NADPH oxidase-4 which might be mediated by adenosine A<sub>3</sub> receptor, which in turn may be responsible for vascular dysfunction during diabetes.
INTRODUCTION

Diabetes is a disorder of impaired carbohydrate, fat and protein metabolism which results from defective insulin secretion categorized as Type 1 or from a decreased sensitivity to insulin, recognized as diabetes Type 2 [1]. Development of diabetes is known to initiate hypertension and atherosclerosis [2-4]. It has been reported that diabetic patients are exceedingly predisposed to congestive heart failure [5] and the risk factor is hyperglycemia for this disease [6]. Diabetes-enhanced cardiovascular mortality is the consequence of increase in aortic stiffness [7]. Likewise, a number of clinical investigations have established that in patients with Type 1 or Type 2 diabetes, endothelium-dependent vasodilation is impaired [8-11]. Amid other significant alterations, an elevated adenosine concentration has been detected in diabetic heart [12].

Adenosine is a purine nucleoside which is endogenously generated in reaction to cell impairment and metabolic strain [13]. It adjusts vascular tone by direct or indirect mechanisms [14]. There are four recognized subtypes of adenosine receptors (ARs) known as A₁, A₂A, A₂B and A₃, each having an exclusive pharmacological outline, tissue dispersion and consequence of coupling. All four subtypes belong to the superfamily of G-protein-coupled receptors (GPCRs). Activation of A₁ receptors attenuates β-adrenoceptor stimulation [15] and delays ischemic contractures [16]. It has also been reported that in post ischemia reperfusion adenosine couples to adenosine A₂B receptor (A₂BR) and directs vasodilation and cardioprotection [17]. Overexpression of adenosine A₁ receptor (A₁AR) declines heart rate, conserves energetics and shields ischaemic hearts [18]. Low expression of A₁AR in the heart offers successful defense against ischaemic injury with no detectable undesirable consequences, although higher levels of A₁AR expression lead to the development of a dilated cardiomyopathy [19]. Ironically, targeted deletion of the A₁AR in mice also bestows resistance to myocardial ischaemic damage and does not check early preconditioning (20). It has been earlier confirmed that activation of A₁AR leads to endothelium dependent aortic contraction [21]. Further support for vasoconstrictor response of A₁ receptor comprises A₁AR dependent decline in cyclic AMP (cAMP) and adenylyl cyclase action in smooth muscle, and inhibition of cAMP accretion in cultured smooth muscle cells from aorta [22]. Therapeutic aiming of A₂B AR by agonist has provided an effective prospect to alter the development of diabetes induced cardiac and other A₁AR dysfunction diseases and to improve overall clinical outcome [23].

Endothelium keeps balance between vasoconstriction and vasodilation, possibly regulated by endothelial derived mediators [24]. These mediators may be decreased in vascular disease, for instance hypertension and diabetes when there is an amplified discharge of contractile mediators [25,26]. There are three major isoforms of nitric oxide synthase (NOS): neuronal NOS (nNOS) or type 1 NOS, inducible NOS (iNOS) or type 2 NOS, and endothelial NOS (eNOS) or type 3 NOS which are encoded by separate genes on separate chromosomes.

Deficiency of eNOS gene results in hypertension [27] and amplification of vascular smooth muscle cell proliferation in reaction to vessel injury [28]. During inflammation, the generation of Nitric oxide (NO) in vascular tissue switches from eNOS to iNOS in the smooth muscles [29]. iNOS is very pertinent to diabetic pathophysiology. Recent studies have revealed that decrease in expression of eNOS is accompanied by an increase in the expression of iNOS and nitrotyrosine in the course of the progression of diabetes in rats [30].

NADPH oxidases (Nox) are the major source of Reactive oxygen species (ROS) in the vasculature having both physiological and pathophysiological roles in the modulation of vascular tone [31]. The NADPH oxidase family consists of seven members, Nox1–Nox5 and Doux1 and Doux2 [32]. Among these, Nox1, Nox2 and Nox4 are of significance in the cardiovascular system. Nox5 is not expressed in rodents due to gene deletion [33].

Presence of Reactive oxygen species (ROS) quenches NO, thus dropping its bioavailability [34]. Oxidative stress results due to disproportion between endogenous oxidants and antioxidants, a condition that plays a role in impaired NO bioavailability and vascular dysfunction. It is becoming increasingly clear that adenosine may exhibit some of its actions through modulating Nox activity [31, 35-38].

The aim of the present study is to explore the possible role of eNOS and NADPH oxidase (Nox4) in A₁AR mediated vasconstriction response in diabetic mice. To achieve this we have investigated endothelium mediated vascular dysfunction during diabetes promoted by A₁AR. To confirm the vascular dysfunction in diabetes is promoted by A₁AR, constriction experiments in the presence of specific A₁AR antagonist were carried out.

MATERIALS AND METHODS

Phenylephrine (PE), Streptozotocin (STZ), 2-Chloro-N6-(3-iodobenzyl)-V-methyl-5-carbamoyladenosine (CI-IBMECA), 2,3-diethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate (MRS1523), and apocynin were purchased from Sigma-Aldrich. Isoflurane was bought from HSB Veterinary Supply Inc., Sodium Chloride (NaCl), Magnesium Sulphate (MgSO₄), Potassium Dihydrogen Phosphate (KH₂PO₄), Sodium Hydrogen Carbonate (NaHCO₃), Glucose, Calcium Chloride (CaCl₂), Potassium Chloride (KCl), Sodium Citrate, Phosphate Buffer Saline(PBS), Sodium Dihydrogen Phosphate (NaH₂PO₄) were procured from Merck Chemicals and Hi-Media. All the chemical were of highest purity and of analytical grade.

Experimental/Methodology

Induction of diabetes in mice

Male C57BL/6J wild type (WT) mice aged 12-15 weeks were obtained from animal house of University Hospital Eppendorf, Hamburg. Diabetes was induced by a single intraperitoneal (IP) injection of Streptozotocin (STZ) (dissolved in 10mM citrate buffer) pH 4.5 at 180 mg/kg body weight as described by Revisin, et al [39].Control mice were injected with only sodium citrate buffer instead of STZ. Blood glucose level was measured from the blood drawn from mice tail vein after 48 hours of STZ administration using FreeStyle® blood glucose meter by Abbot Laboratories. Mice with a blood glucose concentration greater than 300 mg/dL were considered diabetic [39].
All animal experiments were approved by the institutional ethical committee (University Medical Center Hamburg-Eppendorf, Germany, Ref.Nr.39/12).

Organ bath experiments
Preparations of isolated mice aortic rings
The preparation of isolated mice aorta was done as described by Pomerleau, et al [40]. The age and weight matched mice were sacrificed under deep anaesthesia with isoflurane. The thoracic aorta was gently removed, cleaned of fat and connective tissues. The aorta was then cut transversely into rings measuring~3-4 mm in length.

Contraction experiments
The mice aortic rings were immediately suspended in organ bath containing 25 ml Krebs-Henseleit buffer. (The Krebs-Hanseleit buffer contained 118 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 11 mM glucose and 2.5 mM CaCl₂). The pH of the buffer was adjusted and maintained at 7.4. The organ bath temperature at 37°C was maintained with continuous bubbling with 95 % O₂ and 5 % CO₂. Individual aortic rings were mounted vertically between two stainless steel triangle wire hooks. The distal end of the hook was fixed to the bottom of the tissue bath, and the proximal end of the hook was attached to a force-displacement transducer that was coupled to a physiological recording system (AD instruments) and data was recorded on acquisition system. Aortic rings were equilibrated for 30 min with a resting force of 1.1 g as reported earlier [41]. The tissues were contracted with Phenylepherine (PE) (10⁻⁷ M) [38,41] to produce consistent submaximal (100%) response in our experiments. The integrity of the vascular endothelium was assessed pharmacologically by acetylcholine (10⁻⁷ M) to produce relaxation of PE precontracted rings. The tissues that did not elicit reproducible and stable contraction with PE (10⁻⁷ M) and relax <50% with acetylcholine (10⁻⁷ M) were discarded from the study. Only endothelium intact tissue was used in this study.

Isometric force measurement
The isometric force response was measured in each aortic ring stretched to resting tension of 1.1 g and was allowed to equilibrate for 30 minutes. At the end of equilibration period, the functional integrity of the vascular ring was verified by recording contraction after the addition of 50 mM KCl to the organ bath. Aortic rings that did not contract after the addition of KCl were eliminated from further studies. The changes in tension were monitored continuously with the fixed range precision force transducer connected to the differential amplifier (AD instruments). The data was recorded on a digital acquisition system and analyzed using Lab chart software [40].

Experimental protocol
To determine the vascular responses to adenosine A₁ receptor agonists, the aortic rings (with baseline resting tension of 1.1 g) were pre-contracted with 10⁻⁷ M Phenylepherine (PE). The concentration-response curves (CRCs) for aortic response in presence of specific A₁AR agonist 2-Chloro-N6-(3-iodobenzyl)-N-methyl-5-carbamoyladenosine (Cl-IBMECA) and antagonist 2,3-dihydro-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate (MRS1523) were run by cumulative addition of drug in the organ bath in pre-contracted rings with PE (10⁻⁷ M). The concentration of the agonists in the organ bath was increased in steps of 10⁻⁹ to 10⁻⁵ M. In all cases, agonists were added 30 minutes before the addition of agonist. CRC plots were generated in the presence and absence of antagonist and inhibitor.

Estimation of lipid peroxidation in control and STZ treated aorta.
The assay of lipid peroxidation (LPO) was done according to the method of Wright et al[ 42]. Aorta from control and diabetic treated groups were incubated in Cl-IBMECA for 30 minutes at 37°C, homogenized in homogenization buffer and then centrifuged. Pellet was discarded and supernatant was used for biochemical assay. The reaction mixture consisted of 0.58 ml of phosphate buffer (0.1 M, pH 7.4), 0.2 ml supernatant, 0.2 ml of ascorbic acid (100 mM) and 0.02 ml of ferric chloride (100 mM) in a total of 1 ml. This reaction mixture was then incubated at 37°C in a shaking water bath for 1 h. The reaction was stopped by the addition of 1 ml of 10% tri chloro acetic acid (TCA) followed by addition of 1.0 ml of 0.67% Thiobarbituric acid (TBA). All the tubes were then placed in a boiling water bath for 20 min. The tubes were shifted to an ice bath and then centrifuged at 2500 x g for 10 min. The amount of malondialdehyde (MDA) formed in each of the samples was estimated by measuring the optical density of the supernatant at 535 nm. The results were expressed as the nmol MDA /h/g tissue at 37°C by using a molar extinction coefficient of 1.56 x 10⁶ M⁻¹ cm⁻¹.

Assay for estimation of total nitrate /nitrite level in diabetic and control aorta.
Mouse aortic tissues with intact endothelium were prepared and divided into two halves; one half was used for organ bath study and the other half for NOx assay as a measure of NO production. Total nitrate levels were estimated using Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical) as per manufacturer’s instructions.
Real-Time PCR for Nox4, iNOS and eNOS in mouse aorta

Purification of total RNA was performed using the RNeasy mini-kit (QIAGEN) according to the protocol of the manufacturer. RNA was eluted in 50 μl of nuclease-free water. Total RNA was isolated and reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit and included random hexamer primers (Life Technology Corp). The amplification was carried out in the StepOne instrument (Life Technologies Corp) using the TaqMan Fast Universal PCR Master Mix. Taqman inventories assays-on-demand gene expression products were purchased from Applied Biosystems. Mm00479246_m1 assay was used for Nox4 gene, Mm00440502_m1 assay was used for iNOS gene, Mm00435217_m1 assay for eNOS gene. Mouse GAPDH Endogenous Control NM_008084.2 (all Life Technologies Corp) was used for normalization. The fold change in copy number was calculated using the ∆∆Ct-method.

Statistical analysis

All the experimental values are presented as mean ± SE (n = number of animals). The comparison among different groups was analyzed by ANOVA followed by Tukey’s multiple comparisons test method as a post hoc test. Comparison between two groups was assessed by unpaired t-test. P < 0.05 was taken as significant. All the statistical analyses were performed using Graph Pad Prism statistical package (version 6.0, GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

Concentration dependent effect of specific A₃AR agonist CI-IBMECA in control/ diabetic mice aorta

Incubation of diabetic aortic rings with increasing concentration of CI-IBMECA enhanced the contraction in a concentration-dependent manner. At 10⁻³M concentration of CI-IBMECA, constriction in diabetic aorta increased significantly by 58.84%±9.06 (*p<0.05), as compared to control. CI-IBMECA produced concentration-dependent contractions with maximum contraction obtained at 10⁻⁷M concentration, in both diabetic and control mice aorta (Fig.1). The values express relative constriction at Emax. The statistical analysis was performed by an ANOVA for repeated measures. The data from the whole course of the curve instead of single data points were compared.

Figure 1: Vascular responses to CI-IBMECA in control and diabetic mouse aorta.

Effect of CI-IBMECA on contraction in control and diabetic mouse aorta. Data are expressed as mean ± SEM (n=9 for diabetic and n=8 for control). * p<0.05 compared to A₃ AR in control.

Concentration dependent effect of CI-IBMECA in control/ diabetic mice aorta in the presence of specific A₃AR antagonist (MRS1523)

The effect of 10⁻⁵M antagonist (MRS1523) was seen in control and diabetic mice aorta at different concentrations of CI-IBMECA. There was a decrease in the vasoconstriction in both control as well as diabetic aorta in the presence of antagonist. However the constriction in diabetic aorta induced by CI-IBMECA was significantly reduced by MRS1523. The inhibition response due to MRS1523 in control group was insignificant (Fig.2).
Figure 2: Vascular responses to MRS 1523 in Cl-IBMECA induced contraction in control and diabetic mouse aorta.

Effect of MRS1523 on Cl-IBMECA-induced contraction in control and diabetic mouse aorta. Data are expressed as mean ± SEM (n=3 diabetic and n=3 control). § p<0.05 compared to diabetic group in absence of antagonist.

Effect of apocynin (a specific NADPH oxidase blocker) on Cl-IBMECA mediated contraction in control and Diabetic mice aorta.

Diabetic group in the presence of apocynin at $10^{-5}$M showed a 42.84% decrease in Cl-IBMECA induced A$_3$AR mediated response as compared to the diabetic in absence of apocynin (Fig.3). We observed no significant difference in the constriction response in the control group in the presence and absence of apocynin.

Figure 3: Vascular responses to Apocynin in Cl-IBMECA induced contraction in control and diabetic mouse aorta.

Effect of apocynin on Cl-IBMECA-induced ROS generation in control and diabetic mouse aorta. Data are expressed as mean ± SEM (n=6 diabetic and n=3 for control). $\$p<0.05$ compared to their corresponding diabetic aorta in the absence of apocynin.
Lipid peroxidation in diabetic aorta

Membrane damage in diabetes was assessed by estimating lipid peroxidation in mice aorta. Since malondialdehyde (MDA) formation was measured to demonstrate the oxidative damage in diabetic aorta. MDA level in control was found to be $4.36 \times 10^{-17} \pm 7.92 \times 10^{-18}$ nM/g/hr at 37°C while in diabetic aorta MDA was found to be $5.74 \times 10^{-17} \pm 6.95 \times 10^{-19}$ nM/g/hr at 37°C, *p<0.05 (Fig. 4).

![Figure 4: Lipid peroxidation in control and diabetic aorta stimulated by CI-IBMCEA.](image)

Effect of Streptozocin (180 mg/kg body weight) on MDA production, induced by CI-IBMCEA (10^{-7} M) in mouse aorta. Values are expressed as SEM (n=4 control=11 diabetic). *p<0.05 compared with control aorta.

Nitrate/Nitrite production in control and diabetic aorta

Total nitrate/nitrite levels were found to be 0.08491±0.0644 µM/µg of protein in control aorta and 0.16507±0.0845 µM/µg, (p>0.05) of protein in diabetic aorta (Fig. 5).

![Figure 5: Nox activity in control and diabetic aorta induced by CI-IBMCEA.](image)

Effects of Streptozotocin (180 mg/kg body weight) on Nox production, induced by CI-IBMCEA (10^{-7} M) in mouse aorta. Values are expressed as SEM (n= 3 for control=6 for diabetic). P > 0.05 compared with Control.

Nox4, iNOS, eNOS gene expression by Real-Time PCR in control and diabetic mice aorta

Relative measurements of eNOS expression in endothelium intact mouse aorta were carried out by using real-time PCR. Relative to the GAPDH mRNA expression level, eNOS gene expression levels were 0.6238±0.186 in diabetic group and 1.0006±0.036 in control group. Similarly for NOX 4 the fold change was 1.5384± 0.148 in diabetic group and 1.0001 ± 0.016 in control respectively.

The relative mRNA expression of Nox4 and iNOS in diabetic aorta increased to 53 % (*p<0.05) and 18% respectively, while there was a decrease of 40 %(*p<0.05) in eNOS mRNA expression (Fig. 6).
e major causes responsible for endothelial dysfunction. Since the presence of apocynin, a specific inhibitor of NADPH oxidase and general ROS inhibitor, which resulted in a concentration-

gate the role of another subtype of NADPH oxidase i.e Nox4 -ion in mRNA levels of iNOS, eNOS and Nox4 due to activation of

These results validate that the production of A -eNOS may be responsible for promot-

membrane damage in the diabetic aorta and lipid peroxidation is one of the.

It is well known that diabetes mellitus is involved in the enhancement of inflammatory processes including increased production of reactive oxygen species (ROS) [43, 44], which may have a significant role in causing and complicating the disease [45,46]. Therefore, to verify the role of ROS in promotion of vascular response we performed the A -AR mediated constriction experiments in the presence of apocynin, a specific inhibitor of NADPH oxidase and general ROS inhibitor, which resulted in a decrease in Cl-IBMECA induced response. Apocynin significantly reduced the contraction induced by Cl-IBMECA in diabetic group, suggesting A -AR activation involves ROS production in mouse aorta (Fig.3). We have recently demonstrated [41] that MPO has a role in promotion of A -AR mediated vascular dysfunction in diabetes; therefore it is likely that there may be relationship between Nox4 and MPO, since levels of both are altered during diabetes.

NADPH oxidases are involved in A -AR mediated vascular dysfunction [38]. As A -AR activation involves ROS production [38,41], we have tried to explore the role of A -AR activation on the level of Nox4 in aorta of diabetic and control mice. To study the Nox production due to activation of A -AR by Cl-IBMECA, we determined total nitrate/nitrite levels (Nox). We found an alteration in the Nox levels in diabetic group as compared to control, possibly due to the elevated levels of A -AR (Fig.5) but the difference is not significant. Previous studies [38] have shown that NADPH oxidase is involved in A -AR vasoconstriction response which is possibly mediated by Nox2. Taking that in consideration we have tried to investigate the role of another subtype of NADPH oxidase i.e Nox4 in endothelium mediated vasoconstriction by the activation of A -AR in diabetic mice. It is known that there is an alteration in mRNA levels of NOS isoforms during diabetes. To correlate the alteration in mRNA levels of iNOS, eNOS and Nox4 due to activation of A -AR induced by Cl-IBMECA in diabetes, we performed RT-PCR experiment. We observed an increase in mRNA expression of Nox4 while there was a significant decrease in the mRNA expression of eNOS(Fig.6). We already know that NO is a well known vasorelaxant [47] and expression of eNOS is altered in disease such as atherosclerosis, diabetes, and hypertension. Therefore we can infer from our study that the decrease in eNOS may be responsible for promotion of vasoconstriction response which is augmented by Cl-IBMECA induced activation of A -AR .

Endothelial dysfunction is believed to be the key feature in the pathogenesis of vascular disease in diabetes mellitus [48-50] and lipid peroxidation is one of the major causes responsible for endothelial dysfunction. Since the end products of lipid peroxidation are reactive aldehydes, such as malondialdehyde, measurement of MDA was done to assess the extent of LPO. To assess membrane damage in the diabetic aorta we estimated levels of MDA in diabetic mice aorta due to A -AR activation by Cl-IBMECA and found that diabetic mice aorta has higher lipid peroxidation as compared to control (Fig. 4). As A -AR is responsible for ROS production [38] elevated levels of A -AR may be the reason for high MDA production in diabetic group. We have earlier reported the level of SOD (Superoxide dismutase) a marker of excessive ROS in diabetic mice aorta [41].

DISCUSSION

This study demonstrates that Nox4 and eNOS have role in A -AR mediated vascular dysfunction in diabetic mice aorta. Our findings suggest that A -AR activation in the presence of specific A -AR agonist Cl-IBMECA results in alterations in mRNA levels of Nox4 and eNOS in diabetic mice aorta possibly due to altered expression of A -AR . We have earlier reported that there is overexpression of A -AR in diabetic mice aorta which leads to vascular dysfunction [41].

In the present study we found an increased A -AR mediated vasoconstriction response in diabetic group as compared to control (Fig1). A specific A -AR agonist Cl-IBMECA produced concentration-dependent contraction in control and diabetic mice aorta, which was completely abolished by A -AR specific antagonist (MRS 1523) (Fig.2). These results validate that the Cl-IBMECA induced contraction was due to the fact that Cl-IBMECA is a specific A -AR agonist, and suggest that contraction of aortic rings was primarily due to activation of A -AR by Cl-IBMECA ; A -AR being a key element in mediating contraction action of Cl-IBMECA in mice aortic rings.

To confirm the agonist mediated response of A -AR, we performed the constriction experiments with antagonist MRS1523. In the presence of antagonist we observed that the Cl-IBMECA mediated A -AR response was almost completely inhibited in control group. The diabetic group showed response at low doses of Cl-IBMECA, while at higher concentrations of Cl-IBMECA, there was complete abolition of vasoconstriction response (Fig.2).

Relative expression of eNOS, iNOS and Nox4 mRNA was measured in STZ treated and control mouse aorta. The values are expressed as SEM for eNOS (n=6), Nox4 (n=6) and iNOS(n=5).*P<0.05 compared to control aorta.

Figure 6: Effect of in vivo treatment with Cl-IBMECA on expression of eNOS, iNOS, and Nox4 by real-time PCR.

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We can thus presume that in diabetic mice there is an increased vasoconstriction response mediated by A_3AR, which may be responsible for vascular dysfunction, one of the causes for cardiovascular disease in diabetics. A common feature of endothelial dysfunction is reduced bioavailability of NO in the vasculature. Possible mechanisms for impaired NO availability include a reduction in eNOS mRNA or protein expression levels [51], which is what we too have observed in our real time PCR experiment. Our results also comply with the previous studies [30] that with decrease in expression of eNOS there is an increase in iNOS expression which may be responsible for vascular inflammation marked by membrane damage as we have shown high lipid peroxidation in diabetic aorta.

As already acknowledged, that there is an excessive ROS/RNS production during diabetes [52,44]. To consider the role of ROS/RNS in A_3AR mediated response in diabetes, we carried out the constriction experiments in the presence of general ROS and NADPH oxidase inhibitor, apocynin. We observed that there was a decrease in A_3AR mediated constriction response in diabetic group. A_3AR is engaged in ROS production [38,41] and there is a significant increase of A_3AR expression during diabetic in mice aorta [41]. Therefore an upregulated A_3AR results in an elevated vasoconstriction response which may be due to the altered mRNA levels of Nox4 and eNOS in diabetic mice aorta.

A limitation of this study is lack of knowledge of mechanism by which A_3AR overexpression alters the mRNA levels of Nox4 and eNOS. Therefore further studies are needed to confirm the changes in the protein expression of Nox4 and eNOS in diabetic mice aorta.

CONCLUSION

Therefore in conclusion, we suggest that decrease in mRNA expression of eNOS may result in alteration of A_3AR expression in diabetes, possibly mediated by upregulation of Nox4, which is responsible for promotion of vasoconstriction resulting in endothelial dysfunction during diabetes.

The future prospects of this study aim at exploring the pathways which might be responsible for altering the expression of mRNA levels of Nox4 and eNOS mediated by A_3AR. Moreover with the availability of both selective ligands and animal models, several new roles of the A_3 receptors that are ambiguous will emerge in the near future. This will allow the chemistry and pharmacology of the A_3 receptor agonist/antagonist to be utilized clinically for the development of selective molecules for this important target (A_3 receptor agonist/antagonist) that may improve the outcomes of patients with a number of cardiovascular diseases.

Authors’ Statements
Shamama Nishat, Luqman A Khan and Seemi F Basir participated in research design, experiment were Conducted by Shamama Nishat, data analysis was conducted by Shamama Nishat and Seemi F Basir; Shamama Nishat and Seemi F Basir wrote and contributed to writing of manuscript, Seemi F Basir revised and finalise the manuscript. This work was supported by DAAD and UGC PhD scholarship grant secured by Shamama Nishat. Authors want to thank Anna Klinke for providing lab facility to carry out some experiments. University Hospital Eppendorf Hamburg, Germany.

Competing Interests
The authors declare no conflict of interest.

List of abbreviations

A_3AR Adenosine A_3 Receptor
ARs Adenosine Receptors
cAMP Cyclic AMP
cDNA Complementary DNA
Cl-IBMECA 2-Chloro-N6-(3-iodobenzyl)-N-methyl-5-carbamoyladenosine
CRC Concentration Response Curves
eNOS Endothelial Nitric Oxide Synthase
GAPDH Glyceraldehyde 3-Phosphate Dehydrogenase
GPCR G-Protein-Coupled Receptors
iNOS inducible NOS
LPO Lipid peroxidation
MDA Malondialdehyde
MRS1523 2,3-diethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate
nNOS neuronal NOS
NO Nitric Oxide
NOS Nitric Oxide Synthase
Nox NADPH Oxidases
PE Phenylepherine
RNS Reactive Nitrogen Species
ROS Reactive Oxygen Species
RT-PCR Real Time Polymerase Chain Reaction
SOD Superoxide dismutase
STZ Streptozotocin
TBA Thiobarbituric Acid
TCA Tri Chloro Acetic Acid

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