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Research Article

Inhibition of Rac 1 Protect Against Platelet-induced Liver and Kidney Injury in Diabetes Mellitus

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ABSTRACT

Diabetes mellitus (DM) both (Type 1 and Type 2) are one of the common causes for activation of platelet. Inflammation-induced abnormal platelet function contributes to chronic complications, which are the leading causes of death and morbidity among diabetics. Rac1 has been shown to regulate a variety of platelet functions; predicted Rac1could regulate platelet release of CXCL4, which leads to kidney injury in DM. DM' effect on Rac1 activation, a 21kD G-protein implicated in platelet activation, was investigated and platelet induced inflammation and kidney injury. Swiss albino male mice were pretreated with 5 mg/kg of a specific Rac1 inhibitor NSC23766 and injected with (45 mg/kg body wt.) streptozotocin, twice for 5 days. Moreover, the concentration of serum chemokines CXCL4 was assayed using ELISA and histology score for kidney was examined. Our results showed that DM was induced in mice by streptozotocin. In addition, platelet chemokines (CXCL4) were markedly higher in diabetic mice when compared to the sham (control) group. Moreover, pre-treatment with NSC23766 decreased liver and kidney injury assessed by histology score, $P < 0.05$. Our study reveals that Rac1 has a critical role in platelet chemokines secretion due to diabetes-induced inflammation in the liver and kidneys, targeting Rac1 could be a target for innovative treatment to control inflammation in diabetic individual. Targeting platelets involved in inflammatory pathways could be part of a strategy to control and manage diabetes and its consequences.

Keywords: Rac1, platelet, CXCL4, diabetes mellitus, liver and kidney injury

INTRODUCTION

Diabetes mellitus (DM) is a state in which set to be failure of the Langerhans islet of the pancreas to produce sufficient insulin, or for the insulin to be resistant.[1] The figures of people with diabetes are expected to soar by 550 million by the end of the year 2035.[2] Diabetes has detrimental consequences that are mainly because of it is micro and macroangiopathy which include several debilitating neurological complications and nephropathies in which serious glomerular complications develop leading to dropping the level of glomerular filtration rate (GFR) and eventually renal failure.[3,4] Determining factors for the increased levels of GFR in early DM have been observed utilizing different physiological experiments commencing factors and pathophysiology routes are particularly ambiguous to determine as the relation between form and function tense to be complicated microangiopathy complications in diabetes were found as renal lesions in Type 2 diabetes where hyperfiltration did not sound to occur while.[5,6]

Liver is a crucial organ in the regulation of lipid, glucose, and protein metabolism and is one of the numerous organs affected by this metabolic illness diabetes. The most specific markers of hepatic injury are alanine aminotransferase (ALT), and aspartate aminotransferase (AST), which are found in the hepatocellular mitochondria and cytosol. Diabetes is frequently accompanied by elevated serum levels of (ALT), (AST), and gamma glutamyltransferase.[7] Excess free fatty acids are directly toxic to liver cells in the insulin-resistant state, T2DM is thought to be a contributing factor in the development of liver diseases such as cirrhosis, non-alcoholic fatty liver disease, and non-alcoholic steatohepatitis, hepatocellular carcinoma will eventually develop.[8]

Pre-diabetic individuals or impaired glucose tolerance subjects have demonstrated lower levels of mean platelet

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volume (MPV) than diabetic people in several studies.[9] Higher platelet activity is frequently found to be more in diabetic patients, platelet glycation proteins promotion are directly link to platelet reactivity.[10] Diabetic patients are doomed to develop higher platelet reactivity, as both insulin resistance and deficiency levels up platelet reactivity.[11] Chronic diabetic patients in general are not an exclusion of the vicious circle in which hyperglycemia leads to high platelet activation: The latter is thought to be contributing in the producing the reactive oxygen species (ROS) in endothelium since this have been showed in several studies. Other than this ROS production in endothelium happens directly due to glucose metabolism and autooxidation indirect ROS production may also occur through the development of pro-inflammatory cytokine receptors for advanced glycation end products (RAGE).[12]

Cytosol of platelets embed pro-inflammatory derivatives like CXCL4 which is sent to be important mainly produced from RNA and the fate is to be spilled on platelet activations. This pro-inflammatory derivative is released upon platelet activation during cecal ligation and puncture mice which in its turn leads to expression of CXCL4 and this may cause aggregation of other immune cells and exaggerate inflammation during abdominal sepsis.[13,14] Rac1a small guanine nucleotide-binding proteins (G protein) is a key signaling molecule that coordinates intracellular transduction pathways that lead to NADPH oxidase activation.[15] Similar to other small GTPases, Rac1 must be isoprenylated to move from cytosol to the plasma membrane, where its presence facilitates the assembly of some NADPH oxidase subunits.[16,17] This assemblage is a very important step for the enzymatic activation of the ROS-producing NADPH oxidase system. As a result, Rac1 activation may be a key mechanism in hyperglycemia-induced vascular oxidative stress. Rac1 a small GTPase protein playing a crucial role in DM as a signaling molecule.[18] Furthermore, platelet secretion of CXCL4 in sepsis is also controlled by Rac1.[13,14,19] Numerous theses have emphasized the principal contribution of Rac1 in sepsis, lamellipodia formation, as well as the activation of phospholipase, granule secretion, and clot retraction in platelets. So far, the molecular and cellular mechanisms contribute to the inflammation and complication effects of diabetes are only partially recognized; hence, the aim of the present study was to investigate the Rac1 role in releasing CXCL4 from platelets in DM and the inhibition of Rac1 to protect against liver and kidney damage due to DM.

MATERIALS AND METHODS

Animals

Swiss albino male mice were used in all experiments 8–9 weeks of age (weight: 20–25 g) The Department of Pharmacy Hawler Medical University, Iraq, is in accordance with animal welfare standards legislation, and the Regional Animal Experimentation Ethical Committee has given its approval. The animals were housed in a pathogen-free environment with a 12–12-h light–dark cycle. Water and food were provided twice daily clean and fresh drinking water provided specific nipple used to access ad libitum. The trials were conducted after a 7-day acclimatization period. Animals were sub-grouped in to three groups sham (injected with saline only), vehicle (injected with streptozotocin), and treatment group which pretreated with NSC23366 and streptozotocin (NSC+STZ), each group contains five mice. With environment enrichment, the mice were maintained in cages with no more than five mice per cage in each group.

Materials

Streptozotocin (STZ; Glentham Life Science. Ltd., U.K.) is the chemical that uses for induction of DM.[20] NaOH the buffer that uses for preparation of Streptozotocin was made by dissolving (10.7 g) of sodium citrate in (200 ml) of distilled water and (9.6 g) of citric acid added and the volume completed to l000 ml with distilled water. By adding (NaOH) to the solvent, the pH of the solution was adjusted to (4.5). NSC23766 (N6-[2-[[4-(Diethylamino) -1-methylbutyl] amino] -6-methyl- 4-pyrimidinyl] -2 methyl--4, 6-quinolinediamine trihydrochloride, Chem Cruz, Santa Cruz Biotechnology, California). The Accu-Chek Active blood glucose meter was used to monitor the blood glucose level in the study.

Animal Experiments

(5 mg/kg) of Rac1 inhibitor (NSC23766) was administered intraperitoneally to the animals based on past research, this dose of NSC23766 was chosen.[19,21,22] After 30 min, the animals were treated using multiple (i.p.) STZ (45 mg/kg body weight) was injected intraperitoneally into the experimental mice to produce DM. STZ was dissolved in a buffer of 0.01 M sodium citrate (pH = 4.5) and given to mice for 5 days in a row. This dose of STZ is selected on the base on the previous study.[23] To minimize hypoglycemia produced by STZ, after the injection, the animals were given a glucose solution (5% w/v) to drink overnight. Sham mice were given an equivalent dose of vehicle (citrate buffer) only. For 5 days, STZ and NSC+STZ treated mice were housed in normal settings after dosing was completed. In fact, after these time period mice developed diabetes by measuring fasting blood glucose levels were approximately 11.1 mmol/l. Blood samples from the tail vein of NSC+STZ and STZ-treated mice were taken after a 12-h fast to assess blood glucose levels. Diabetes was defined as fasting blood glucose levels higher than 11.1 mmol/l in diabetic mice.[23] Moreover, hence, they were selected for further studies. Intravenously, sedation was achieved by administering 75 mg ketamine hydrochloride (HoffmanLa Roche, Basel, Switzerland) and 25 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium) kg-1 body weight. The animals were harvested and blood took from Vena Cava. The serum was allowed to coagulate at room temperature for 10–20 min. Before being centrifuged for 20 min at 2000–3000 RPM. The serum was extracted from the supernatant and kept at 80°C for use in an ELISA test later. For histopathology, the kidney was preserved in formaldehyde.

Biochemical Determination

Before the start of the procedure, all experimental animals' blood glucose levels were monitored. Fasting blood glucose levels were checked on a regular basis until diabetes was confirmed. Mice with a fasting blood glucose level of 11.11 mmol/l or above were classified as diabetic. Blood was collected from the tail veins of all experimental animals (2–3 μl). An Accu-Chek active blood glucose meter was used to monitor the blood glucose levels.

ELISA

Serum CXCL4 level measured successfully in all groups of mice and samples assessed by enzyme-linked immunosorbent assay (ELISA) using the (Mouse Platelet Factor 4 ELISA Kit, BT LAB Cat. No E0686Mo). Following the manufacturer's directions, at 450 nm absorbance was measured, a standard curve had been set on each microplate by diluting a known concentration standard. Using a logistic curve-fitting technique, the mean absorbance for the wells was used to calculate the chemokine concentration for each sample. The linear section of the standard curve contained all of the absorbance values. The data were represented as ng/ml.

Histopathology

Kidney tissue was fixed overnight in a 10% formaldehyde phosphate buffer before being dried and paraffin embedded. Hematoxylin and eosin were used to stain four micrometer sections. A modified scoring system was used to quantify kidney injury in a blinded manner.[24] Including mixed inflammatory cells, necrosis (reversible injury), apoptosis, fibrosis, vascular congestion and edema, and degeneration (reversible injury) Infiltration is evaluated on a scale of 0–4, zero represents (absence) and four represents (extensive). The mean value was calculated after assessing five random locations in each tissue sample. The sum of all six criteria determines the histopathology score.

Statistics

The data were provided as mean values with standard error (SE). Nonparametric tests were used to do statistical analyses (Mann–Whitney). n is the total number of mice in each group, and $P < 0.05$ was considered significant. Statistical analysis was performed by use of SPSS (IBM Corp., Armonk, N.Y., USA).

RESULTS

Streptozotocin-iInduced DM in Mice

Injection of (45 mg/kg body wt.) of STZ to mice significantly increased the fasting blood sugar of mice compared to sham group, $P < 0.05$. However, the group that treated with Rac1 inhibitor markedly reduced the high blood sugar induced by streptozotocin. Pre-treatment of mice with 5 mg/kg of the Rac1 inhibitor (NSC23766), reduced the fasting blood sugar from 556.20 ± 26.65 to 375.20 ± 20.7 with $P < 0.05$ [Figure 1]. Hence, attenuating Rac1 activation by NSC23766 prevents high blood glucose which might induce platelet activation.

Rac1 Regulates Platelet Secretion of CXCL4 in DM

DM increased plasma levels of CXCL4 from 6.40 ± 0.4 ng/ml in sham mice up to 13.60 ± 1.32 ng/ml, corresponding to a 2.12-fold increase [Figure 2]. The results showed induction of DM by injecting (45 mg/kg body wt.) SZT in plasma [Figure 2] suggesting that DM induces the platelet chemokine secretion CXCL4 in Diabetic mice. Notably, (NSC23766) a Rac1 inhibitor, significantly reduced DM-induced platelet aggregation and

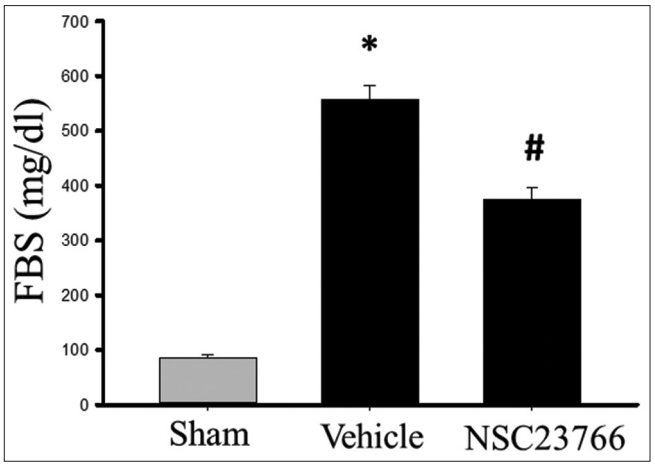

Figure 1: Blood Glucose concentration. Fasting blood glucose levels were measured on day of harvest after the mice were induced with STZ (Vehicle) for 5 days and pretreated with Rac1 inhibitor (NSC23766). Data represent mean ± SE (sham = 5, vehicle = 5). *$P < 0.05$ versus sham

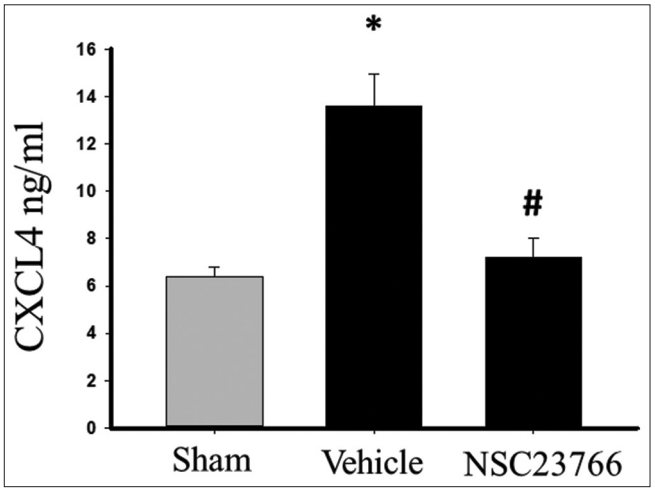

Figure 2: Activated platelets secrete chemokines in diabetic mice. ELISA was use d to quantify the levels of CXCL 4 in the diabetic mice plasma. Data represent mean ± SE (sham = 5, vehicle = 5). *$P < 0.05$ versus sham

chemokines secretion in platelets [Figure 2], showing that NSC23766 which is an effective inhibitor of Rac1 activation, also inhibit the increased level of platelet chemokines. Pre-treatment with NSC23766 attenuate serum levels of CXCL4 in diabetic mice from 13.60 ± 1.32 ng/ml to 7.20 ± 0.8 ng/ml, equating to a drop of more than 80% [Figure 2].

Histopathology Alterations of Kidney in Diabetic Mice

Histopathological change of kidney regarding normal control showed normal kidney histology architecture, while treated group (induced diabetes) showed mild vacuolar degenerations, scattered chronic inflammatory cells infiltration. While after giving treatment, there will be reduction in the inflammatory cells and reduction in degeneration [Figure 3b]. The induction of DM by injection of streptozotocin, significantly induced

kidney injury in diabetic mice compared to the sham group (1.13 ± 0.08) with $P < 0.05$ [Figure 3a]. However, administration of NSC23766 to the mice reduced the histological score to 0.26 ± 0.21 with $P < 0.05$ [Figure 3a].

Histopathology Alterations of Liver in Diabetic Mice

Histopathological change of liver normal control showed normal liver histology architecture, while treated group (induced diabetes) showed vascular congestion, degenerative hepatocyte (reversible injury), and scattered chronic inflammatory cell infiltration (lymphocyte). While in the pre-treated group with NSC23766, there was only mild vascular congestion [Figure 4b]. Our findings revealed significant liver damage in the diabetes induced animals. Streptozotocin injections used to induce diabetes in mice dramatically increased liver damage compared to the control group 1.4 ± 0.08 with $P < 0.05$ [Figure 4]. NSC23766, on the other hand, reduced the histological score in mice to 0.73 ± 0.06, with $P < 0.05$ [Figure 4a].

DISCUSSION

The current findings suggest that Rac1-mediated platelet activation and CXCL4 secretion from Platelets play an important role in DM. These data imply that platelets play a critical role in diabetes, and that reducing Rac1 signaling and/or CXCL4 function could be effective DM therapy options and its complication associated with platelet activation. Platelets are important for wound healing and thrombosis, but they also contribute to the host's response to bacterial invasion by performing a variety of pro-inflammatory actions.[25] Platelets, for example, govern a variety of features of responses of leukocytes to severe infections, according to research.[13,14,19] Platelets have been demonstrated to play an important role in the development of DM in the previous investigations; however, in this study, we found that the injection of mice with streptozotocin (45 mg/kg body wt.) significantly induce the DM compare to sham group P value <0.05. Similar to our study, the previous studies showed the elevation of glucose after induction of streptozotocin.[23,26-28] However, the role of Rac1in regulation of platelet chemokines in the DM not

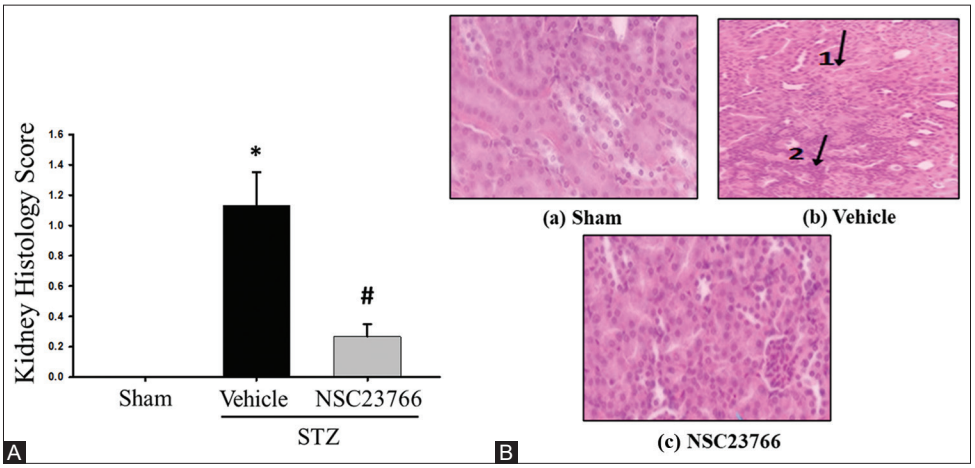

Figure 3: Rac1 regulates kidney damage in DM. (A) Histopathology score in the kidney. (B) Rac1 regulates kidney damage in DM. Representative (H&E ×100) sections of the kidney are shown. (a) Normal histology of the kidney of normal control group. (b) Scattered chronic inflammatory cells as shown in (arrow 1), and vacuolar degeneration (arrow 2). (c) Treatment group showed reduction in inflammatory cells and vacuolar degeneration

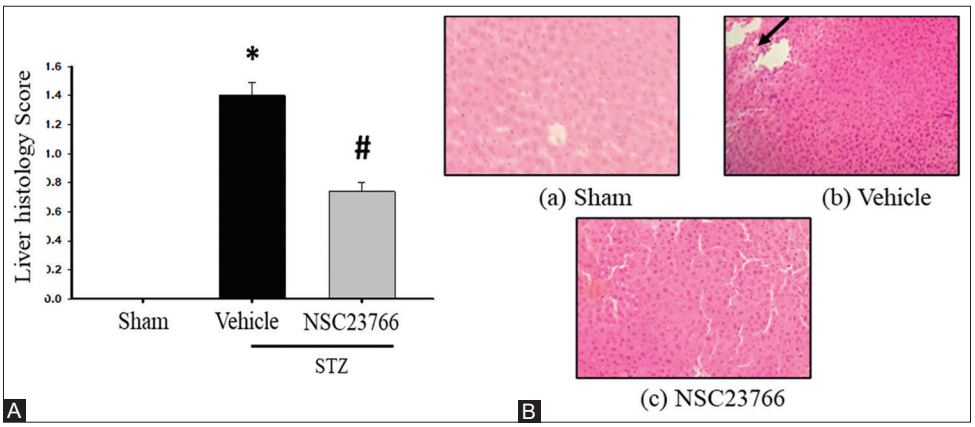

Figure 4: Rac1 regulates liver damage in DM. (A) Rac1 regulates liver damage in DM. Histology score in the liver. (B) Rac1 regulates liver damage in DM. Representative (H&E ×100) sections of the liver are shown. (a) Normal histology of the liver of normal control group. (b) vascular congestion, and degenerative hepatocyte as shown in the (arrow on the vehicle), and scattered chronic inflammatory cell infiltration. (c) Treatment group showed only mild vascular congestion

been studied earlier. Induction of streptozotocin-induced DM, significantly increased platelet chemokine secretion. However, the induction of DM by streptozotocin was abolished by the pre-treatment with Rac1 inhibitor (5 mg/kg), by more than 80% reduction [Figure 2]. This emphasizes the role of platelets and Rac1 in the DM molecular process. Moreover, histological study showed the kidney destruction in diabetic mice which induced by injection of streptozotocin. Pre-treatment with Rac1 inhibitor (5 mg/kg) significantly reduced the kidney damage induced by DM. The role of platelets in renal damage was demonstrated in the previous studies.[29-32] Talat *et al*. showed that platelet count significantly increased in the diabetic patients.[33] The glomerulus and tubules basement membrane thickens and this leads to recruitment of inflammatory cells. Gene expression and protein production of extracellular matrix components such as collagen IV, laminin, and fibronectin increase when extracellular matrix components accumulate. The weight of the kidneys was reported to increase as a result of these changes.[34] Our result showed pathological changes in the liver produced in diabetic mice induced by (45 mg/kg body wt.) streptozotocin. A recent study has shown liver injury induced by STZ.[35] Furthermore, another study revealed that the occurrence and progression of diabetic liver injury are influenced by oxidative stress.[36] In this study, we documented a significant reduction of mixed inflammatory cells and apoptosis of pre-treatment hepatocytes with Rac1 inhibitor in mice. The Rac1 inhibitor (5 mg/kg) administration significantly reduced necrosis, fibrosis, vascular congestion and edema, an extreme change shown between diabetic mice and pre-treated Rac1 inhibition. Furthermore, the latest study showed that NSC23766 treatment significantly alleviated the hepatic RI in mice as well as reducing ROS production and oxidative stress.[37] Pro-inflammatory cytokines were observed to be acutely induced in the liver and kidneys of rats exposed to GNPs in several studies.[38-40]

In platelets, CXCL4 is one of the most prevalent chemokines.[13,14] Numerious studies have found that patients with metabolic syndrome had higher levels of (IL-6), (TNF), CXCL16, and (CRP) than healthy people. Furthermore, metabolic syndrome has been linked to an increase in the number of immune cells such as leukocytes, monocytes, and platelets.[41-43] Interestingly, the level of CXCL4 in diabetic mice's serum was higher than in sham animals, according to this study findings [Figure 2].

Rac1 has been implicated in the regulation of platelet chemokine production in the previous investigations.[13,14,19] Furthermore, it is well known that high blood glucose levels assist in the development of ROS on the endothelium lining of arteries.[44,45] ROS, a signaling molecule produced by NADPH, is vital in the advancement of inflammation and vascular damage in diabetes.[46] Rac1, a small G protein, is a key signaling molecule that connects intracellular signaling pathways to NADPH oxidase activity.[47]

CONCLUSION

In the present study, we showed that Rac1 has a function in platelet activation and that the chemokine CXCL4 is overexpressed in diabetic mice. In addition, morphological change associated with DM in mice as a consequences of platelet activation. Rac1 inhibition may thus be a therapeutic drug to regulate DM through attenuation of chemokine, which is a signaling molecule in aggravating inflammation, as well as chemokine production liver and kidney damage by activated platelets in DM.

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