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NSC23766, a Known Inhibitor of Tiam1-Rac1 Signaling Module, Prevents the Onset of Type 1 Diabetes in the NOD Mouse Model

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Abstract

Background/Aims: Type 1 diabetes (T1D) is characterized by absolute insulin deficiency due to destruction of pancreatic β-cells by cytokines (e.g., interleukin-1β; IL-1β) released by invading immune cells. The mechanisms by which these cytokines induce β-cell dysfunction remain poorly understood. Recent evidence suggests that excessive generation of reactive oxygen species (ROS) by the phagocyte-like NADPH oxidase2 (Nox2), along with significantly low levels of antioxidants in β-cells, drive them toward oxidative damage. Rac1, a small G-protein, is one of the members of Nox2 holoenzyme. We recently reported that NSC23766, a known inhibitor of Rac1, significantly attenuated cytokine-induced Nox2 activation and ROS generation in pancreatic islet β-cells *in vitro*. Herein, we determined the effects of NSC23766 (2.5 mg/kg/day, i.p/daily) on the development of diabetes in the NOD mouse, a model for T1D.

Methods: Two groups of experimental animals (Balb/c and NOD mice) received NSC23766, while the two control groups received equal volume of saline. Body weights and blood glucose were measured every week for 34 weeks. Rac1 activation in pancreatic islets was measured by GLISA activation assay. Rac1 and CHOP expression was determined by Western Blotting.

Results: Our findings indicate that administration of NSC23766 significantly prevented the development of spontaneous diabetes in the NOD mice. Furthermore, NSC23766 markedly suppressed Rac1 expression and activity and the endoplasmic reticulum stress (CHOP expression) in NOD islets.

Conclusions: Our findings provide the first evidence implicating the role of Tiam1-Rac1-Nox2 signaling pathway in the onset of spontaneous diabetes in the NOD mouse model.

Keywords

Nox2; Rac1; NOD mice; Pancreatic islet; ER stress; Diabetes

Disclosure Statement

The authors report no conflicts of interest.

Introduction

Type-1 diabetes (T1D] is characterized by an absolute deficiency of insulin arising from progressive autoimmune destruction of pancreatic β-cells [1, 2]. It is well established that during the progression of the disease, pro-inflammatory cytokines (IL-1β, TNF-α, and IFN-γ), are released into pancreatic islets by infiltrated, activated T cells and macrophages [3–8]. However, the exact cellular and molecular mechanisms by which cytokines induce β-cell demise remain partially understood [4–7]. Even though cytokines have been shown to modulate several signaling pathways, apoptosis is considered as the primary mode of cell death in human and mouse models [8].

A growing body of evidence supports the hypothesis that oxidative stress from reactive oxygen species (ROS), and nitric oxide (NO] contributes to the damage in mitochondrial membrane, eventually causing defects in the membrane potential. In contrast with most other mammalian cell types, β-cells comprise relatively lower levels of redox-regulating enzymes, making them more vulnerable to oxidative damage [9, 10]. Recently, members of the NADPH oxidase (Nox) family (e.g., Noxl and Nox2) have emerged as one of the sources of pathological oxidative stress in pancreatic islets under the duress of exposure to pro-inflammatory cytokines [11–14]. Under basal conditions, the phagocyte-like NADPH oxidase (Nox2) holoenzyme is inactive as its individual subunits are distributed in the soluble and membranous compartments. The membrane-bound catalytic core consists of flavocytochrome b_{558} components p22^{phox} and gp91^{phox} and small G-protein Rap1. The regulatory core consisting of p47^{phox}, p67^{phox}, and p40^{phox} subunits and the small G-protein Rac1 reside in the cytosol. Upon stimulation, the cytosolic components are trans located to the membrane for holoenzyme assembly and activation of the enzyme [15, 16]. Our recent findings have also demonstrated that functional activation of Rac1 (i.e., GTP-Rac1) is vital for the NADPH holoenzyme assembly [12, 13].

Several recent studies have demonstrated localization and functional activation of the phagocyte-like NADPH in clonal β-cells, normal rat islets, and human islets under the duress of various stimuli, including elevated levels of glucose, saturated fatty acids, and pro-inflammatory cytokines [12, 17–22]. Furthermore, pharmacological inhibition of Nox2 by diphenyleneiodonium chloride (DPI) or anti-sense oligonucleotides for p47^{phox} markedly attenuate glucose-induced ROS production and oxidative stress, suggesting critical involvement of Nox2 in the metabolic dysfunction induced by long-term exposure to elevated glucose [23]. These data implicated a significant contributory role for NADPH oxidase in the onset of metabolic dysfunction of the β-cell under conditions of oxidative stress [24, 25]. Along these lines, *in vitro* studies (pharmacological and siRNA) from our laboratory have demonstrated key roles for pro-inflammatory cytokines in the induction of Tiam1-Rac1-Nox2 pathway leading to mitochondrial dysfunction and caspase activation in pancreatic β-cells [12, 26]. As a logical extension to these *in vitro* findings, we have undertaken the current investigation to validate our hypothesis that Tiam1-Rac1-Nox2 pathway contributes to cytokine-mediated islet dysfunction in the NOD mouse, a well-established model for T1D. Our findings demonstrate that NSC23766, a known inhibitor of Tiam1-Rac1 signaling pathway inhibits spontaneous diabetes in this mouse model.

Materials and Methods

Materials

NSC23766 was from Calbiochem (Minneapolis, MN). Rac1 activation G-LISA assay kit was from Cytoskeleton Inc. (Denver, CO). CHOP antiserum was from Cell Signaling Technology (Danvers, MA). Mouse monoclonal antibody directed against Rac1 was purchased from BD Bioscience (San Jose, CA). Beta-actin antibody was from Sigma Chemical Company (St. Louis. MO). Blood glucometer and glucose strips were from Abbott Diabetes Care (Alameda, CA).

Experimental Animals

All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committees at Wayne State University and the John D. Dingell VA Medical Center. Balb/c control and NOD mice were purchased from Jackson laboratories (Bar Harbor, Maine) The animals were purchased at 7 weeks of age and were divided into four groups (n = 8/group). At 8 weeks of age two groups of experimental animals (Balb/c and NOD) received NSC23766 (2.5mg/kg/day, i.p./daily) and other two groups, which served as control Balb/c and NOD mice and received equal volume of saline. The body weights and blood glucose were monitored every week for 34 weeks.

Blood glucose monitoring

Blood glucose was monitored weekly by tail vein nick using Freestyle Lite blood glucose meter (Abbott Diabetes Care, Alameda, CA). Mice presenting with a blood glucose concentration of 250 mg/dl or higher, for two consecutive weeks were considered to be diabetic.

Islet isolation

Islets from all the experimental groups were isolated according to collagenase digestion method as we described previously [17, 19].

Rac1 activation assay

Rac1 activation from islets was quantified using a G-LISA assay kit as described in [27, 28].

Western blotting

Islet cell lysate proteins (20–30 μg/lane) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then probed using Rac1 and CHOP antibodies and further incubated with corresponding secondary antibodies. The same blots were stripped and used to probe for β-actin to ensure equal loading and transfer of proteins.

Statistical analysis of experimental data

The statistical significance of the differences between the experimental conditions was determined by ANOVA. P values < 0.05 were considered significant.

Results

Previous investigations from multiple laboratories including our own have implicated Tiam1-Rac1 signaling module in the activation of Nox2 and associated increase in ROS generation under conditions of metabolic stress [20, 28, 29]. Specifically, studies from our laboratory have reported significant attenuation of cytokine-induced metabolic dysregulation of β-cell following inhibition of Tiam1-Rac1 signaling pathway by NSC23766 [12]. Therefore, we undertook the current investigation to assess the role(s) of Tiam1-Rac1 pathway in the onset of spontaneous diabetes in NOD mouse, a model for T1D.

NSC23766 treatment elicited no significant effects on the growth (body weights) of either Balb/c or NOD mice

Data depicted in Fig. 1 (Panel A) demonstrate no significant effects of NSC23766 on the body weights of either the control Balb/c mice or the NOD mice. Their body weights at the beginning of the study (8 wks.) represented l8.75 ± 0.l3 grams and l9.68 ± 0.l9 grams for Balb/c mice and NOD mice, respectively. At the end of the study (32 wks.) the corresponding body weights of control group were 25.85 ± 0.95 grams (vehicle-treated) and 22.66 ± 0.52 grams (NSC23766-treated). The body weights of NOD mice represented 24.l3 ± 2.09 grams (vehicle-treated) vs. 24.86 ± l.32 grams (NSC23766-treated). Together, these data suggest no clear effects of NSC23766 on body weights of the animal throughout the course of the study.

NSC23766 treatment significantly attenuated the onset of spontaneous diabetes in NOD mice

Data shown in Fig. 1 (Panel B) demonstrate a marked reduction in the development of hyperglycemia in NOD mice treated with NSC23766. At the beginning of the study (8 wks.), the blood glucose values corresponded to l27 ± l.50 mg/dl and l09 ± ll.4 4 mg/dl for Balb/c and NOD mice, respectively. At 32 wks., the blood glucose values remained unchanged in Balb/c mice treated with either vehicle (96 ± 6 mg/dl) or NSC23766 (91 ± 5 mg/dl). As expected, blood glucose levels were significantly higher in NOD mice at 32 wks. (370 ± 50 mg/dl). NSC23766 treatment of NOD mice significantly reduced their blood glucose levels (187 ± 46 mg/dl). Together, data in Fig. 1 (Panels B) indicate a marked reduction in the onset of spontaneous hyperglycemia in NOD mice following treatment with NSC23766, a known inhibitor of Tiam1-Rac1 signaling pathway.

Administration of NSC23766 markedly attenuates increased expression of Rac1 and CHOP, a marker for ER-stress, in islets from NOD mice

In the next of studies, we quantified expression of Rac1 and CHOP, an ER-stress marker, in islets derived from Balb/c mice and NOD mice treated without or with NSC23766 (32 wks.). Data shown in Fig. 2 indicate a significant increase in the expression of Rac1 in islets derived from NOD mice (Panel A and B), which as suppressed significantly in islets from NOD mice treated with NSC23766. Further, we noticed a marked increase in the expression of CHOP in NOD mouse islets (Fig. 2; Panel B), which was inhibited completely in islets from NOD mice treated with NSC23766. Together, these data indicate that activation of Nox2 may be upstream to the induction of CHOP expression (and ER stress).

Treatment of NOD mice with NSC23766 significantly reduces Rac1 activation

In the last set of studies, we quantified Rac1 activation (G-LISA; Methods for additional details) in islets derived from Balb/c or NOD mice treated with vehicle or NSC23766. Data in Fig. 3 indicate a paradoxical increase (~2 fold) in Rac1 activation in Balb/c mice treated with NSC23766. We also noticed nearly ~4.5 fold stimulation in Rac1 activation in islets from NOD mice, which was inhibited significantly following treatment of NOD mice with NSC23766. Together, these data of significant suppression of Rac1 activation in NOD mice by NSC23766 suggest a critical regulatory role for Tiam1 in the induction of Rac1 activation (Fig. 3) and induction of hyperglycemia in the NOD mouse model.

Discussion

Emerging evidence from multiple laboratories including our own implicates regulatory roles for Nox2 in the metabolic dysfunction under the duress of glucolipotoxicity and cytokines. The overall goal of this study was to further understand the role of Tiam1-Rac1-Nox2 in the induction of islet dysfunction in the NOD mouse model. Our studies yielded some novel clues suggesting that: (i) expression of Rac1 and CHOP are increased in the NOD mouse model (at 32 wks.) and NSC23766 significantly attenuated expression of both Rac1 and CHOP; (ii) Rac1 is also activated in NOD mouse islets which was significantly inhibited by NSC23766; and (iii) NSC23766 administration to NOD mouse significantly prevented the spontaneous development of hyperglycemia in this model. Based on these findings we suggest novel roles for Tiam1-Rac1-Nox2 in the induction of T1DM in the NOD mouse model.

Several recent studies have explored potential utility of inhibitors of specific guanine nucleotide exchange factors (GEFs) for Rac1 as modulators of its activation under the duress of glucolipotoxicity, pro-inflammatory cytokine exposure and diabetes [12, 18, 19, 30]. It is well established that activation of Rac1 is mediated by a variety of GEFs including Tiam1, Vav2 and Trio [16, 27, 31, 32]. In 2004, Gao and associates discovered NSC23766, a small molecule compound, that specifically inhibited Tiam1 or Trio-induced activation of Rac1 [33]. Activation of other small G-proteins (Cdc42 and Rho) remained unaffected by NSC23766. Furthermore, NSC23766 significantly attenuated Rac1-mediated proliferation, anchorage-independent growth and invasion phenotypes in human prostate cancer PC-3 cells [33]. Since then, more than 200 studies (a recent Medline search] employed NSC23766 as inhibitor of Rac1 activation.

In the context of islet β-cell, we have utilized NSC23766 to further investigate regulatory roles of Tiam1-Rac1 signaling cascade in islet function. Compatible with original findings of Gao and coworkers [33], we observed that NSC23766 significantly inhibited only Rac1, but not Cdc42 or Rho activation [32]. Our *in vitro* [12] and *in vivo* (current study] observations clearly implicate regulatory roles for Rac1 in cytokine-induced islet dysfunction. We also identified Tiam1 as one of the GEFs that is involved in cytokine-induced Rac1 activation since NSC23766, a known inhibitor of Tiam1, inhibited cytokine-induced Rac1 activation. In the current study, we observed that administration of NSC23766 (2.5mg/kg/day, i.p./daily] prevents the onset of spontaneous diabetes in the NOD mouse model. We recently used this dosage in mouse models to demonstrate regulatory roles of Tiam1-Rac1-Nox2

pathway in diabetic retinopathy [28, 34]. Studies by Shibata et al. have employed NSC23766 at a dose of l0 mg/kg/day which is four times the dose we have used, and observed reduced Rac1 activity in the kidney without apparent evidence of organ toxicity in mice [35]. In summary, these findings in the NOD mouse model confirm our *in vitro* observations suggesting that cytokine-induced islet dysfunction may be mediated *via* the activation of Tiam1-Rac1-Nox2 signaling module.

Interestingly, we observed increased Rac1 activation in Balb/c mice treated with NSC23766. These data suggest potential involvement of other GEFs in the activation of Rac1 in this model system. Along these lines, recent data from our laboratory have demonstrated that two GEFS, namely Tiam1 and Vav2, are involved in the activation of Rac1 to facilitate glucose-stimulated insulin secretion [GSIS] since simultaneous inhibition of Tiam1-Rac1 [NSC23766] and Vav2-Rac1 [Ehop-0l6] pathways yielded much greater inhibition of GSIS, compared to inhibition of either of these pathways alone [27]. Therefore, the increased Rac1 activation in control Balb/c mice might be due to compensatory mechanism[s] by which Vav2 might be activating Rac1. Furthermore, based on our previous findings, it appears that post-translational geranylgeranylation and palmitoylation of Rac1 are also critical for regulatory effects of Rac1 since we reported significant inhibitory effects of inhibitors of geranylgeranylation (GGTI-2l47) and palmitoylation (2-bromopalmitate) attenuated cytokine-mediated effects on the islet β-cell [12, 26]. One potential caveat in these conclusions is that both GGTI-2l47 and 2-bromopalmitate would impede geranylgeranylation and palmitoylation of other candidate proteins. Therefore, additional studies are needed to further validate the above model.

Our data also suggest significant increase in CHOP expression (i.e., ER-stress] in NOD mouse islets, which was inhibited significantly in islets derived from NSC23766-treated NOD mice suggesting that Tiam1-Rac1 activation may be upstream to CHOP expression and induction of ER stress. Additional studies are needed to further validate this formulation. Our findings in NOD mice are compatible with observations of Tersey and associates demonstrating that ER stress precedes the onset of T1D in the NOD mouse. They were able to substantiate this hypothesis both in *in vitro* and *in vivo* model systems [36].

Lastly, it should be pointed out that while our previous and current findings are suggestive of key regulatory roles for Nox2 in cytokine-mediated islet dysfunction, it is likely that other Noxs could contribute to the pathogenesis of islet defects. Indeed, recent studies from Taylor-Fishwick's group have demonstrated key modulatory roles for Noxl in cytokine-mediated dysfunction of the islet β-cell [11]. M Ll7l, a selective inhibitor of Noxl significantly reduced cytokine-induced ROS generation and caspase activation in clonal β-cells and mouse islets. It would be worthwhile to determine the cytoprotective effects of NSC23766 and M Ll7l (in combination] to further assess the validity of the hypothesis that Nox (Noxl and 2] derived ROS generation and oxidative stress are causal for β-cell damage under the duress of cytokines. In conclusion, we provide the first pharmacological evidence to implicate Tiam1-Rac1-Nox2 signaling pathway in the onset of T1D in the NOD mouse model.

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Abbreviations

NOD mice	Non obese diabetic mice
T1D	Type 1 diabetes
Rac1	Ras-related C3 botulinum toxin substrate 1
Tiam1	T-lymphocyte invasive and metastasis proteinl
ROS	Reactive oxygen species
Nox2	NADPH oxidase2
CHOP	C/EBP homologous protein
IL-1β	Interleukin-1β
TNF – α	Tumor necrosis factor-α
IFN-γ	Interferon-γ
GEF	Guanine nucleotide exchange factor
ER Stress	Endoplasmic reticulum stress

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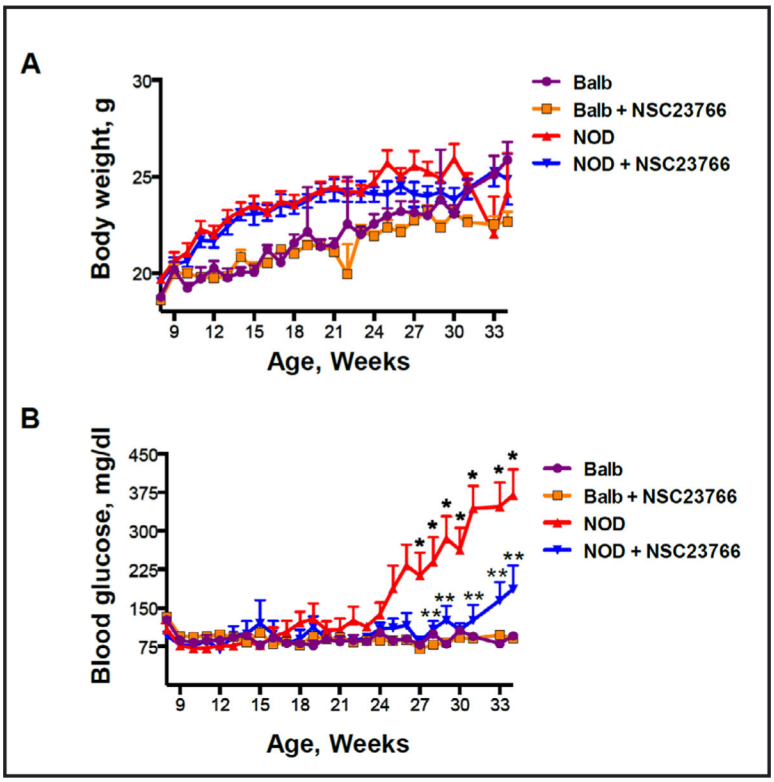

Fig. 1.

NSC23766 prevents the incidence of diabetes in NOD mice. (Panel A) NOD mice and Balb/c control mice were divided into two groups each. NSC23766 administration (2.5 mg/kg/day) was started at 8 weeks of age and the corresponding control group received saline. Body weights were measured every week. Data are mean from 10–14 mice/group. (Panel B) NOD mice and Balb/c control mice were divided into two groups each. NSC23766 administration (2.5 mg/kg/day) was started at 8 weeks of age and the corresponding control group received saline. Blood glucose was monitored every week. Data are mean from 10–14 mice/group. * represents $p < 0.001$ *vs* Balb/c and ** represents $p < 0.001$ *vs* NOD control mice.

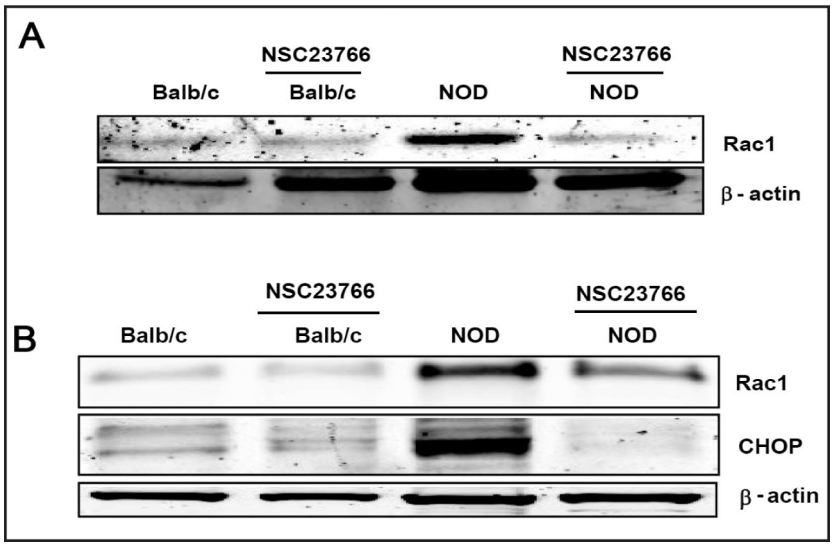

Fig. 2.

NSC23766 suppresses the expression of Rac1 and CHOP NOD mouse islets. NOD mice and Balb/c control mice were divided into two groups each. NSC23766 administration was started at weeks of age (2.5 kg/day) daily and 32 for first study (Panel A) and week 34 for second study (Panel B), islets were isolated by collagenase digestion method and expression of Rac1 and CHOP was determined by Western blotting. Protein loading in each lane was quantified by stripping the same blot and re probing with β-actin. A representative blot from two independent studies is shown here.

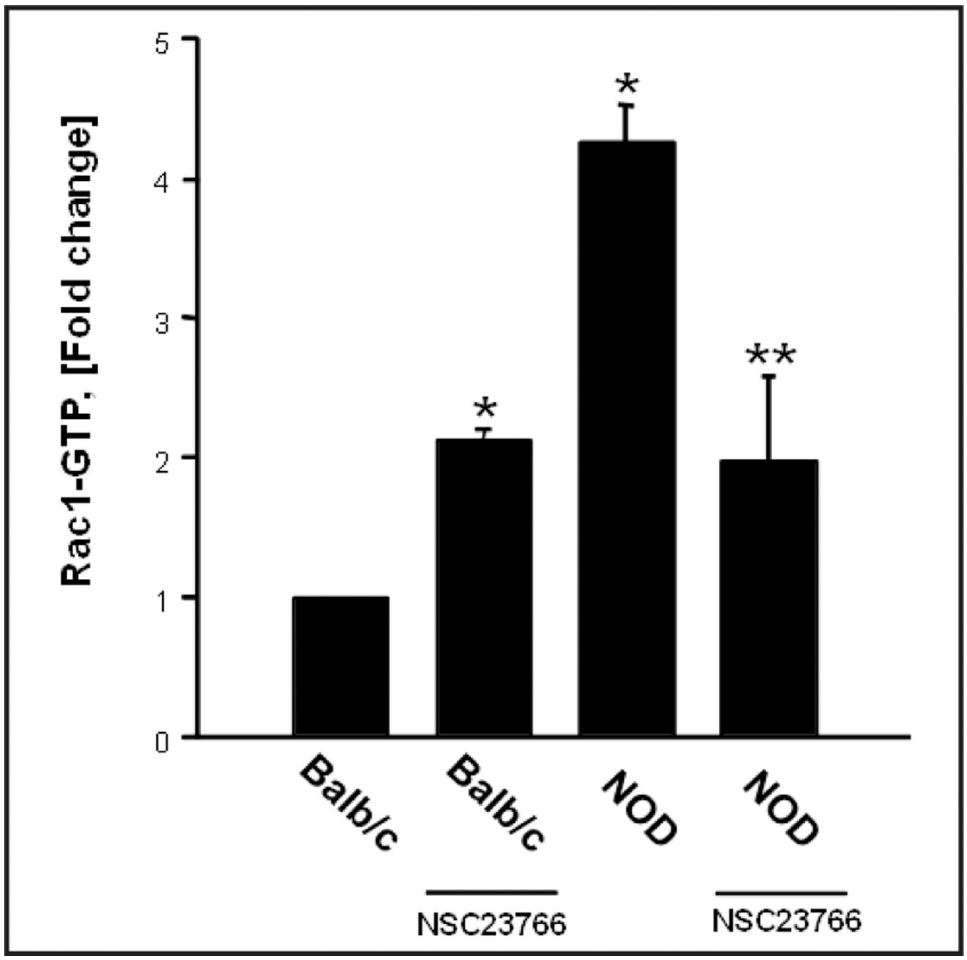

Fig. 3.

NSC23766 prevents Rac1 activation in NOD mouse islets. NOD mice and Balb/c control mice were divided into two groups each. NSC23766 administration was started at 8 weeks of age (2.5 mg/kg/day) daily and the corresponding control group received saline. After the end of week 34, islets were isolated by collagenase digestion method and activation of Rac1 was quantified by the G-LISA method. Data are expressed as fold change ± SEM. * represents $p < 0.05$ *vs.* Balb/c and ** represents $p < 0.05$ *vs.* NOD, N = 3.