THE EFFECTS OF GENISTEIN, A TYROSINE KINASE INHIBITOR ON ACUTE AND CHRONIC INFLAMMATION IN DIABETIC MICE

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ABSTRACT

The effects of tyrosine kinases on acute and chronic inflammation during diabetes are not fully determined. Therefore, the present study focuses on the effects of genistein, a tyrosine kinase inhibitor, on acute and chronic inflammation in diabetic mice. The mice either received normal saline (control, 0.1 ml, i.p., n=144) or streptozotocin (diabetic, STZ, 200 mg kg^{-1}, i.p., n=144). A week after injection of saline or STZ acute and chronic inflammation was induced by injecting carrageenan and implanting 2 cotton pellets. Before injection carrageenan or 5 day after implantation, 9 mice from each group (control or diabetic) received genistein (10 mg kg^{-1}, i.p.), indomethacin (2 mg kg^{-1}, i.p.) or L-NAME (0.1 mg kg^{-1}, i.p.). Paw edema and the weight of cotton pellets were significantly higher in diabetic mice. Pretreatment with either indomethacin or L-NAME significantly reduced the acute and chronic inflammation in the diabetic group. Genistein reduced chronic inflammation significantly. These results suggest that activation of tyrosine kinases as well as prostaglandins and nitric oxide pathways are involved in the increased chronic inflammatory responses observed in the diabetic animals.

Keywords: Diabetes, Genistein, Inflammation, Mice, Streptozotocin, Tyrosine kinase

INTRODUCTION

Tyrosine kinases phosphorylate proteins on the tyrosine residue which has an important role in the regulation of cell differentiation, and proliferation and also signaling processes in immune system cells. Activation of tyrosine kinases mediates the expression of the inducible isoform of cyclooxygenase (caused by endotoxin in murine macrophages, 1). The formation of arachidonic acid metabolites which have pro-inflammatory effects is enhanced by induction of cyclooxygenase (2). The important role of nitric oxide (formed due to the increased activity of inducible nitric oxide synthase) has also been shown in inflammatory diseases (3).

Cellular and vascular reactions in the inflammatory lesion are altered but are unrelated to the blood glucose levels in diabetic animals. For instance, in humans, a significant increase in cytokine production has been shown at the onset of diabetes (4, 5). Proinflammatory cytokines such as interleukin-1 and interleukin-6 have stimulatory effects on the synthesis of acute phase proteins (6, 7). This is of particular interest because inflammation plays an important role in the pathogenesis of diabetes and its complications. It has also been established that high glucose concentration significantly increases the expression of adhesion molecules (such as ICAM-1, E-selectin and P-selectin) on human umbilical vein endothelial cells (8). In many inflammatory diseases such as septic shock (9), and psoriasis (10) the change in protein tyrosine kinase activity has been shown. However, the involvement of tyrosine kinase either directly or through the prostaglandins and nitric oxide synthase in response to inflammation during diabetes is not completely understood. Therefore, the aim of this study is to investigate the effects of genistein, a protein tyrosine kinase inhibitor, on acute and chronic inflammation in diabetic mice (induced by streptozotocin). The effects of genistein on inflammatory responses were compared to those of indomethacin and N^{G}-nitro-L-arginine methyl ester hydrochloride (L-NAME).

MATERIALS AND METHODS

Animals

Groups of male albino mice weighing 25-30 g (Animal house of Qhaem Hospital, Mashhad, Iran) were used. All animals were fed a standard diet ad libitum. Housing conditions and experimental procedures were in accordance with the Animals (scientific procedures) Act of 1986 and conform to the National Institutes of Health guidelines for the use of experimental animals in Britain.

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Drugs

The following drugs were used; streptozotocin (Pharmacia & Upjohn Company, Kalamazoo, USA), ketamine (Rotexmedica, Germany), genistein, carrageenan, N^G-nitro-L-arginine methyl ester hydrochloride, dimethyl sulphoxide (DMSO) and xylazine were obtained from Sigma Laboratories. All solutions were freshly prepared in normal saline. Except genistein which dissolved in DMSO, in a final dose of 0.01/100 g body weight, other drugs were dissolved in distilled water and then diluted with normal saline.

Induction of diabetes

Diabetes was induced by injection of streptozotocin (STZ, 200 mg kg^{-1}, dissolved in 0.2 ml of normal saline, i.p.) as previously described (11 and 12). Control mice received similar volumes of normal saline by the same route. Mice with serum glucose levels above 400 mg dl^{-1} were considered diabetic.

Carrageenan-induced paw edema in mice (acute inflammation)

Inflammation was induced in the hind paw of mice by injection of 0.05 ml of 1% carrageenan S.c. in the sub plantar region. Mice were randomly divided into different groups (Table 1A). The hind paw volume was measured before and after the challenge plethysmometrically at 60, 150, 300 and 1440 min. Results are expressed as the increase in paw volume (ml) which was calculated by subtraction of the basal volume.

Cotton pellet granuloma in mice (chronic inflammation)

Chronic inflammation was produced by cotton pellet induced granuloma in mouse by the method which was previously described (13). Mice were randomly divided into different groups (Table 1B). Briefly, under anesthesia (ketamine, 65 mg kg^{-1} and xylazine, 6.5 mg kg^{-1}, i.p.) two sterilized cotton pellets (density cotton) weighing 30 mg were implanted subcutaneously in the groin region of mice, one on each side (n=144). These cotton pellets were sterilized in an air oven at 121°C for 20 minute before the use. The animals were sacrificed on the 8th day. The granulation tissues with cotton pellets were dried at 60˚C overnight and then dry weight was recorded. The weight of the cotton pellets before implantation was subtracted from the weight of the dried, dissected pellets.

Statistical analysis of data

Results are expressed throughout as means± S.E.M. and were analyzed by one way ANOVA followed by a Tukey-Kramer multiple comparison test (for comparison of the volume of paw and weight of the cotton pellets in different groups). A P value of less than 0.05 was considered to be significant.

RESULTS

Injection of streptozotocin to the mice clearly caused an increase in the plasma glucose level as well as polydipsy and polyuria.

Diabetes and acute inflammation

One week after intraperitoneal administration of either normal saline or streptozotocin (200 mg kg^{-1}, i.p.) to the mice, carrageenan was injected. The hind paw edema induced by carrageenan injection was significantly higher in the diabetic mice at all time intervals (Table 2). However, pretreatment of diabetic mice with either indomethacin (2 mg kg^{-1}, i.p.) or L-NAME (0.1 mg kg^{-1}, i.p.) prior to the carrageenan injection, but not genistein (10 mg kg^{-1}, i.p.), significantly reduced the level of paw edema (Table 2).

Diabetes and chronic inflammation

The weight of cotton pellets granuloma, a marker of chronic inflammation, was significantly higher in the diabetic mice in comparison to the control (control: 172 ± 17 mg, diabetes: 268 ± 11 mg, P<0.001, Fig. 1). However, pretreatment of mice with genistein (10 mg kg^{-1}, i.p.) showed a non-significant anti-inflammatory effect in the control but a significant effect in the diabetic mice (genistein + diabetes: 180±9 mg, P < 0.0001 vs. diabetes, Fig. 1) groups. The anti-inflammatory effect of genistein in diabetic mice was comparable to those of L-NAME (0.1 mg kg^{-1}, i.p., Fig. 1) and less than indomethacin (2 mg kg^{-1}, i.p.).
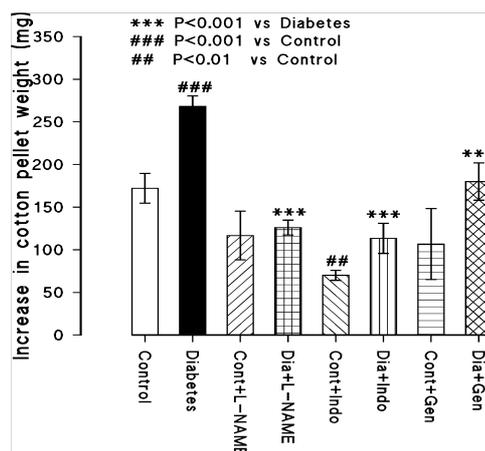

Figure1. Mean ± S.E.M of the weight of cotton pellets (mg), in different groups of mice (n= 9 in each group). One way ANOVA followed by a Tukey-Kramer multiple comparison test was applied to compare the data which showed a significant difference (P<0.001) between diabetic mice who received genistein and L-NAME in compare to diabetes alone (*** P<0.001 vs. diabetes).

Table 1 (A). Different experimental groups in acute inflammation and B. in chronic inflammation.

Animal groups	N	Treatment
Control	9	received saline, 0.2 ml, i.p.
Diabetes	9	received 200 mg kg $^{-1}$ of STZ, i.p.
Control + genistein	9	received saline, 0.2 ml, i.p. + 10 mg kg $^{-1}$ of genistein, administered 2h prior to the carrageenan injection, i.p.
Diabetes + genistein	9	received 200 mg kg $^{-1}$ of STZ, i.p. + 10 mg kg $^{-1}$ of genistein, administered 2h prior to the carrageenan injection, i.p.
Control + indomethacin	9	received saline, 0.1 ml, i.p. and indomethacin 2 mg kg $^{-1}$, administered 30 min prior to the carrageenan injection, i.p.
Diabetes + indomethacin	9	received 200 mg kg $^{-1}$ of STZ, i.p. + indomethacin 2 mg kg $^{-1}$, administered 30 min prior to the carrageenan injection, i.p.
Control + L-NAME	9	received saline, 0.2 ml, i.p. and L-NAME 0.1 mg kg $^{-1}$, administered 30 min prior to the carrageenan injection, i.p.
Diabetes + L-NAME	9	received 200 mg kg $^{-1}$ of STZ, i.p. + L-NAME 0.1 mg kg $^{-1}$, administered 30 min prior to the carrageenan injection, i.p.

Table 1 (B). Different experimental groups in chronic inflammation.

Animal groups	N	Treatment
Control	9	received saline, 0.2 ml, i.p.
Diabetes	9	received 200 mg kg $^{-1}$ of STZ, i.p.
Control + genistein	9	received saline, 0.2 ml, i.p. + 10 mg kg $^{-1}$ of genistein, i.p. injected at day 5 of implantation
Diabetes + genistein	9	received 200 mg kg $^{-1}$ of STZ, i.p. + 10 mg kg $^{-1}$ of genistein, i.p. injected at day 5 of implantation
Control + indomethacin	9	received saline, 0.2 ml, i.p and indomethacin 2 mg kg $^{-1}$, i.p. at day 5 of implantation
Diabetes + indomethacin	9	received 200 mg kg $^{-1}$ of STZ, i.p. + indomethacin 2 mg kg $^{-1}$, i.p. at day 5 of implantation
Control + L-NAME	9	received saline, 0.2 ml, i.p and L-NAME 0.1 mg kg $^{-1}$, i.p. at day 5 of implantation
Diabetes + L-NAME	9	received 200 mg kg $^{-1}$ of STZ, i.p. + L-NAME 0.1 mg kg $^{-1}$, i.p. at day 5 of implantation

Table 2. Mean ± S.E.M of amounts of increase in the hind paw volume after carrageenan injection in different groups of mice.

Time (min)	Control	Diabetes	Cont + L-NAME	Dia + L-NAME	Cont + Indo	Dia + Indo	Cont + Gen	Dia + Gen
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
60	7.8±0.8	12±1.2**	8±2.8	10±1.4	8.3±1.5	8.1±1.2$^{\#}$	6.3±1.3	10.6 ±1.6
150	8.1±1.3	15.6±1.6***	5.7±1.9	9.3±1.83$^{\#}$	4.1±1. 1*	9.9±1.5$^{\#}$	9.7±2.4	17±1.9
300	13 1±1.7	21±0.9***	11±1.1	12±1.3$^{\#}$	8±1.9*	12±0.8$^{\#}$	13±1.8	23.6±2
1440	19.3±1.2	26±2**	16±1.7	14±0.9$^{\#}$	12±2*	14±1.1$^{\#}$	14.8±1.4	27±2

$* P < 0.05, ** P < 0.01, *** P < 0.001$ vs. Control, and $\# P < 0.05$ vs. Diabetes.

DISCUSSION

In the present study plasma glucose levels were significantly higher in diabetic mice which confirm results of the previous studies (14, 15). The relation of different mediators of inflammation such as C-reactive protein, tumor necrosis factor-α and interleukin-6 and metabolic syndrome such as diabetes previously has been shown (16). It has been shown that different type of diabetes has different effects on inflammatory responses; for instance in some studies diabetes were attenuated (17, 18) or unchanged (19), while in others diabetes increased the inflammatory responses (4, 5, 8). In the present work it is shown that the response to carrageenan (injected into the paw) and cotton pellet implantation were significantly increased in diabetic mice. Edema formation due to carrageenan in the rat paw is biphasic event (19, 20). The early phase (1-2 h) is attributed to the release of histamine, serotonin and the increase of prostaglandin (PG) synthesis in the surroundings of the damaged tissues while the late phase is mainly mediated by bradykinin, leukotrienes, polymorphonuclear cells and PGs which are produced in tissue macrophages. In diabetic mice administration of indomethacin significantly reduces the volume of paw edema at all time intervals, whereas pretreatment with L-NAME caused a non-significant reduction at 60 min (after carrageenan injection) and a significant reduction in other time intervals (Table 2). Previous studies have shown that nitric oxide synthase inhibitors reduce the development of carrageenan-induced inflammation which support a role for nitric oxide in the pathophysiology of this model of inflammation (22, 23, 24). The ineffectiveness of L-NAME in early phase of inflammation does not seem to be due to the dosage and route of administration since it has previously been shown that this amount of L-NAME could significantly reduce the inflammation in septic rats (9). One possible explanation is that the amount of other mediators rather than nitric oxide is important in diabetic mice in the early phase of acute inflammation (induced by carrageenan injection). However, further studies are required to find out the exact mechanism. In the present study, inhibition of tyrosine kinase by genistein could not reduce the volume of paw edema in the diabetic mice. Recently, it has been shown that inhibition of tyrosine kinase significantly reduces the acute and chronic inflammation in normal rats (25) as well as in septic rats (9). The reason for the discrepancy between present and the previous results (9) and others (25) is not clear. One possible explanation might be the difference between the amount of inflammatory mediators and also the type of inhibitor (tyrphostin AG-126 in Cuzzocrea et al's study, 25) which has been used. However, further investigations are needed to clarify these differences. Although, genistein failed to change the acute inflammation in diabetic mice but it can significantly reduce the chronic inflammation which is induced by the cotton pellet implantation. The inflammatory granuloma is a typical feature of the established chronic inflammatory reactions (26) and the dry weight of the pellet correlates well with the amount of granulomatous tissues (27). To compare the anti-inflammatory effects of genistein, indomethacin, a prostaglandin synthase inhibitor, and L-NAME, a nitric oxide synthase inhibitor, were used. As it was shown, the effect of genistein in reduction of inflammation was comparable to those of L-NAME but less than indomethacin.

Although plasma concentration of genistein in the present study was not measured, but in past study (9) using the same protocol, it was found that it can inhibit tyrosine kinase in vivo (9) and in vitro (28). However other mechanisms such as antioxidant effects might mediate the anti-inflammatory effects of genistein (29).

In conclusion, these observations provide evidence to our knowledge for the first time that tyrosine kinases play a significant role in chronic but not acute inflammation in diabetic animal. Further studies will be undertaken to investigate the effects of tyrosine kinase inhibitors on other kind of inflammation (such as formaldehyde induced paw edema) and in different animal models.

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