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The Role of Nitric Oxide in Airway Responsiveness in Diabetic-Antigen Sensitized Guinea Pigs *in Vitro*

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ABSTRACT

Clinical asthma and airway responsiveness appear to be less severe when diabetes is superimposed. The aim of the present study was to determine the possible role of Nitric Oxide (NO) in the airway reactivity under diabetic and diabetic-allergic conditions.

Twenty-five male guinea-pigs were divided into five groups of five each as follows: diabetic, antigen sensitized, diabetic- antigen sensitized, insulin-treated diabetic- antigen ovalbumin sensitized and control animals. Tracheal rings of all groups were mounted in an organ bath system for isometric contraction measurements. Tissues were pre-incubated with either of the following chemicals: L-NAME, L-arginine or methylene blue. Cumulative concentration response curve was made with histamine.

Decrease in the airway reactivity in diabetic and diabetic- antigen sensitized animals were shown compared to the antigen sensitized animals. pEC_{50} values of histamine in the presence of L-Arg showed increase in diabetic and diabetic- antigen sensitized animals compared to the controls. In the presence of methylene blue, these values showed an increase in diabetic and diabetic- antigen sensitized animals compared to the controls. However, incubation with L-NAME did not change the airway responsiveness to histamine in diabetic and diabetic-antigen sensitized animals compared to the controls.

Experimental diabetes causes were found to decrease the responsiveness of tracheal rings in the presence or absence of allergy.

Findings of this research work showed that NO had no role in hypo-responsiveness of airway in diabetic and diabetic- antigen sensitized animals.

Key words: Airway Reactivity; Allergy; Diabetes; Guinea pig; Nitric Oxide

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INTRODCUTION

Modulating effects of hormones and other endocrine factors in allergic inflammation have been shown in some researches. Attenuating effects of glucocorticoids or adrenergic agents are some obvious examples. Experimental finding indicates a relative lack of insulin in an organism causes an overall reduction in inflammatory reaction.[1] The mechanisms responsible for the reduced allergic reaction in diabetic patients are still unknown. Several studies however, indicate that the incidence of asthma in diabetic patients is less than that in the residual population. In addition, Douek et al (1999) showed that clinical asthma appears to be less severe when diabetes is superimposed. Moreover, the onset of the diabetic state is accompanied by diminution in symptoms of previously existing bronchial asthma. It is noteworthy that allergic disorders, including asthma, atopic dermatitis and eczema, have an uncommon occurrence in diabetic patients.[2-9]. The mechanism involved in this protection is not completely clear. In this line, It is well established that nitric oxide (NO) is involved in the regulation of airway tone as well as in asthmatic inflammation.[7-9] NO is synthesized from the semi-essential amino acid L-arginine by the enzyme NO synthase (NOS), of which different isoforms have been identified in the respiratory system. Recent experimental evidence has suggested different roles of NO in the regulation of mammalian airway function.[10] It is now well established that NO in low concentration has attenuating effects on the airway reactivity but the high concentration because of peroxynitrite has opposite effects. On the other hand, some evidence has implicated dysfunctional NOS and decreased NO availability in endothelium of diabetic humans and animals.[11] By consideration of NO effects on the respiratory system the aim of the present study was to investigate the role of NO in the airway reactivity in diabetic and diabetic-antigen sensitized guinea pigs.

MATERIALS AND METHODS

Experimental Groups

Twenty five male Dunkin-Hartley guinea pigs (400-500 g) were acclimatized in the animal house (12-hour light/dark periods, temperature of 22-25ºC) for 10 days and received a regular chow diet. After acclimation period guinea pigs were randomly categorized into five groups of five animals each:

I). diabetic; streptozotocin (STZ)-treated
II). antigen sensitized
III). antigen sensitized-diabetic
IV). antigen sensitized-diabetic plus insulin injection
V). nondiabetic, unsensitized controls

Induction of Diabetes and Allergy

Diabetes was induced with a single intra-peritoneal injection of 180 mg/kg streptozotocin (STZ; Zanosar, Upjohn, Kalamazoo, MI, USA). Antigen sensitizatin were performed by two intra-peritoneal injections of 1 ml/kg 5% (w/v) ovalbumin (grade III; Sigma, St Louis, MO, USA). Control animals were administered with the solvent of STZ and ovalbumin (normal saline).[12] In the antigen sensitized diabetic group, animals were sensitized four weeks after STZ injection on two consecutive days. In addition, a subset of the antigen-sensitized diabetic guinea pigs received long-acting (NPH) insulin (Isophane®, Lansulin Exir, Iran) in a dose of 2U/3days subcutaneously for 4 weeks, beginning from the first sensitization day. The presence of diabetes was verified by measuring the blood glucose levels > 130 mg/dl, using blood glucose monitor (Glucocare®, 77 Elektronika Kft. Haungray) in samples obtained from the vein of the ear. A value of 130 mg glucose/dl blood or more indicated a successful induction of diabetes.

Isolation of Trachea

Animals in the diabetic and antigen sensitized groups were anesthetized with Ketamin (Alfasan, The Netherlands) 50 mg/kg plus Xylazine (Alfasan, The Netherlands) 5 mg /kg IM, and their trachea removed. Guinea pigs in the diabetic-antigen sensitized and diabetic-antigen sensitized plus insulin injection groups were anesthetized in the same way after 8 weeks and their trachea removed. In addition five guinea pigs were assigned to the control group in each time of trachea isolation (4 and 8 weeks).

Measurement of Airway Contraction in vitro

The tracheas were prepared free of serosal connective tissue, cut into four triple rings and mounted in 20-ml organ bath (semi-Automatic organ bath LSI Lecta, Spain) containing Krebs-Henseleit solution

(NaCl 117.5 mM, KCl 5.60 mM, $MgSO_4$ 1.18 mM, $CaCl_2$ 2.50 mM, NaH_2PO_4 1.28 mM, $NaHCO_3$ 25.0 mM, D-glucose 5.50 mM). The buffer was aerated with carbogen (5% CO_2 and 95% O2) at a constant pH (7.4) and temperature (37ºC). Each side of the trachea rings was tied to a stainless steel hook and connected to a force transducer for measurement of isometric tension (powerlab/4sp AD Instrument Company, Australia). Tracheal preparations were allowed to equilibrate for 45 min under an initial tension of 1 g, with wash-outs every 15 min. After equilibration, the tracheal preparations were incubated for 30 min with vehicle (Krebs-Henseleit), 1mM of the NOS substrate L-arginine (1543 Sigma, St Louis, MO, USA), 120µM of the subtype nonselective NOS inhibitor L-NAME (N5751 Sigma, St Louis, MO, USA) or 10µM of the guanylyl cyclase inhibitor methylene blue (FN1002543 630 Merck, Darmstadt, Germany) for 30 min. Subsequently, cumulative dose response curves to histamine (4370 Merck, Darmstadt, Germany) were constructed ranging from 10^{-7} to 10^{-3} mmol/L.

Statistical Analysis

Airway reactivity to histamine was evaluated as pEC_{50} ($-\log_{10}$ EC_{50}) values. Data were expressed as mean ± SEM. Comparison of cumulative concentration-dependent curves for contraction was performed by nonlinear regression analysis based on a four logistic equation. The pEC_{50} values of the fitted curves were compared by 1 way ANOVA which were constrained at the bottom (0) and top (100). $P<0.05$ was considered to be significant.

RESULTS

Treatment with STZ induced diabetes in guinea pigs as shown by the increased glucose levels in blood compared to control animals (Figure 1). Antigen sensitization did not affect basal or STZ-induced blood glucose levels, whereas cotreatment with insulin normalized the STZ-induced increase in blood glucose (Figure 1)

Dose response to histamine showed a significant increase after antigen sensitization compared to the control (3.4 times). In diabetic guinea pigs, response to histamine was significantly decreased compared to the antigen-sensitized (21.8 times) and control animals (5.6 times). Dose response to histamine showed significant decrease in diabetic antigen-sensitized guinea pigs compared to the antigen sensitized (13.1 times) and control (3.3 times) groups. Furthermore, significant increase in dose response to histamine was shown for insulin-treated diabetic antigen-sensitized guinea pigs, compared to the diabetic (19.4 times), diabetic antigen-sensitized (11.7 times) and control groups (3.4 times) (Figure 2).

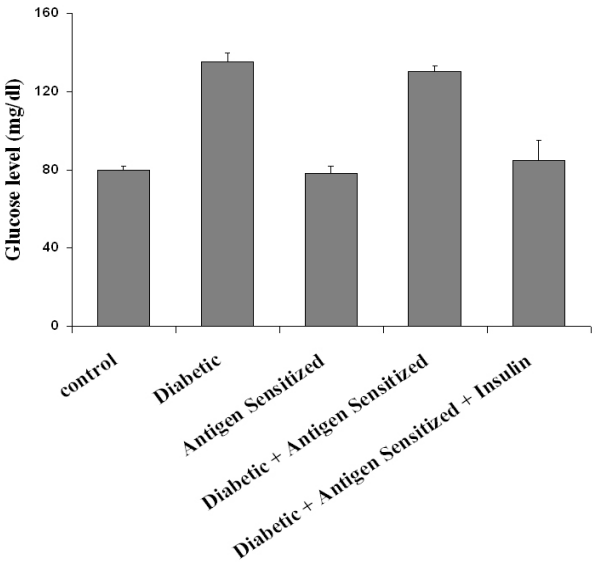

Figure 1. Blood glucose level (mg/dl) in all experimental groups: In all experimental groups, glucose level in blood (mg/dl) was measured using blood glucose monitor. A value of 130 mg glucose/dl blood or more assigned as a confirmation of diabetes.

We showed that airway reactivity to histamine in the presence of L-NAME in diabetic guinea pigs was significantly decreased compared to both antigen-sensitized (6.3 times) and control groups (4.5 times). On the other hand, airway reactivity to histamine in the presence of L-NAME in diabetic-antigen sensitized guinea pigs showed significant decrease compared to both antigen-sensitized (5.4 times) and control groups (3.9 times). Response to histamine in the presence of L-NAME, in insulin-treated diabetic-antigen sensitized guinea pigs showed significant decrease compared to antigen-sensitized (6.4 times) and control groups (4.6 times) (Figure 2).

Dose response to histamine of diabetic guinea pigs incubated with L-Arg was significantly increased compared to the control (6 times). Dose response to histamine of diabetic-antigen sensitized guinea pigs in incubation with L-Arg showed significant increase when compared to the control (7.7 times) (Table 1).

Methylene blue significantly increased response to histamine in diabetic guinea pigs compared to control (12 times). Methylene blue significantly increased dose response to histamine in diabetic-antigen sensitized guinea pigs compared to control (15.4 times) (Table 1).

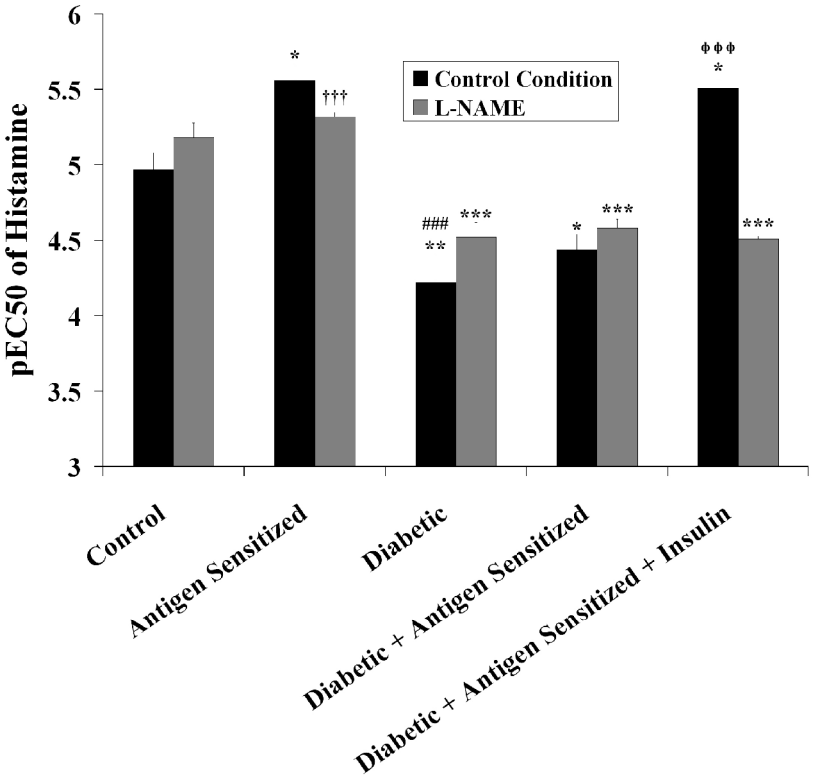

Figure 2. Airway reactivity of guinea pigs to histamine in the control condition(■) and the presence of L-NAME(□). In all experimental groups, Prepared trachea rings were tied to the hook of the stainless steel and connected to a force transducer for measurement of isometric tension. Trachea rings were incubated with only Krebs-Henseleit (control condition) or L-NAME for 30 min and in each case dose response curve was obtained with cumulative increases in histamine from 10^{-7} – 10^{-3} mmol/L. Data shown for isometric tension test are the means ± s.e.mean of five experiments. * p<0.05; ** p<0.01; *** p<0.001; compared to control. ### p<0.001; compared to antigen sensitized guinea pigs. ϕϕϕ p<0.001; compared to diabetic and diabetic-antigen sensitized guinea pigs; ††† p<0.001 compared to diabetic, diabetic-antigen sensitized and insulin-treated diabetic-antigen sensitized guinea pigs.

Table 1. pEC50 values in all experimental groups

Groups of Guinea pigs	Krebs-Henseleit (control condition)	L-NAME	L-Arg	methylene blue
	pEC_{50}	pEC_{50}	pEC_{50}	pEC_{50}
Control	4.97 ± 0.11	5.18 ± 0.1	4.98 ± 0.23	5.25 ± 0.07
Antigen Sensitized	5.56 ± 0.1	5.32 ± 0.03	5.25 ± 0.1	5.29 ± 0.12
Diabetic	4.22 ± 0.18 [a, c]	4.52 ± 0.1 [e, h]	5.00 ± 0.14 [a, e]	5.30 ± 0.1 [c, h]
Diabetic- Antigen Sensitized	4.44 ± 0.1 [b, d]	4.58 ± 0.06 [f, i]	5.33 ± 0.05 [b, f]	5.63 ± 0.03 [d, i]
Insulin treated Diabetic- Antigen Sensitized	5.51 ± 0.06	4.51 ± 0.02 [g, j]	5.55 ± 0.13 [g]	5.54 ± 0.23 [j]

In all experimental groups, prepared trachea rings were tied to the hock of the stainless steel and connected to a force transducer for measurement of isometric tension. Trachea rings were incubated with Krebs Henseleit (control condition), L-NAME, L-Arg or Methylene blue for 30 min and in each case dose response curve was obtained with cumulative increases in histamine from 10^{-7} – 10^{-3} mmol/L. Airway reactivity to histamine was evaluated as pEC_{50} ($-\log_{10} EC_{50}$) values. Data shown for isometric tension test are the means ± s.e.mean of five experiments. The same letters were used to show significance difference between two groups. a, p<0.01; b, p<0.001; c, p<0.001; d, p<0.001; e, p<0.05; f, p<0.001; g, p<0.001; h, p<0.001; i, p<0.001; j, p<0.01.

Dose response to histamine in the presence of L-NAME showed a significant decrease in diabetic, diabetic-antigen sensitized and insulin treated diabetic-antigen sensitized guinea pigs when compared to the L-Arg incubated diabetic (3 times), diabetic-antigen sensitized (5.6 times) and insulin treated diabetic-antigen sensitized guinea pigs (10.9 times) (Table 1).
Dose response to histamine in the presence of methylene blue made significant increase in the airway reactivity of diabetic, diabetic-antigen sensitized and insulin treated diabetic-antigen sensitized guinea pigs compared to the effect of L-NAME in diabetic (6 times), diabetic-antigen sensitized (11.2 times) and insulin treated diabetic-antigen sensitized guinea pigs (10.7 times) (Table 1).

DISCUSSION

We found that dose response to histamine showed significant decrease in both diabetic and diabetic-antigen sensitized guinea pigs when compared to the control animals. In this respect, our findings on the airway reactivity in diabetic and diabetic-antigen sensitized guinea pigs are in line with previous reports.[12,13] In this respect, Szilvássy et al. (2001) believed that the attenuated bronchomotor response in insulin deficient diabetic rats is related to a decrease in release of sensory neuropeptides like substance P, Somatostatin and CGRP. They showed that these contractile agents play an important role in neurogenic bronchoconstriction. In this regard, Cavalher-Machado et al. (2004) reported a reduced contraction to ovalbumine in isolated bronchial segments from diabetic rats. They found the number of degranulated mast cells and histamine release reduced by a 50% in diabetic ones. Furthermore, they showed a complete recovery of the impaired responses under the influence of insulin. Cavalher-Machado et al. (2004) believed that insulin might modulate the controlling of mast cell degranulation. In addition, it has been shown that the amount of stored histamine in diabetic mast cell granules was not different from that found in healthy ones.[14]

In present study, we showed that in the presence of L-Arg, dose response to histamine increased in both diabetic and diabetic-antigen sensitized animals compared to control. On the other hand, airway reactivity to histamine in the presence of L-NAME in diabetic, diabetic-antigen sensitized guinea pigs showed no significant difference when compared with the control. In addition, dose response of the tracheal rings to histamine in the presence of L-Arg showed a significant increase in diabetic, diabetic-antigen sensitized and insulin treated diabetic-antigen sensitized guinea pigs when compared with the L-NAME incubated diabetic, diabetic-antigen sensitized and insulin treated diabetic-antigen sensitized ones. This condition may be due to high amount of NO formed by iNOS. The role of iNOS in inflammatory diseases like asthma has been documented by some investigators.[15,16] It is well established under inflammatory conditions, iNOS may be expressed in variety of pulmonary cells, including epithelial,[17] endothelial[18] and vascular smooth muscle cells,[19] fibroblasts,[20] and various inflammatory cells, such as alveolar macrophages,[21] eosinophils[22] and neutrophils.[23] Increased iNOS expression causes generation of high NO concentration. In this respect, previous findings showed that in asthmatic patients the higher levels of iNOS derived NO has deleterious effects and appears to be associated with airway inflammation by causing mucosal swelling, infiltration of inflammatory cells, epithelial damage and airway hyperreactivity (AHR).[24-27] In this line, it is well documented cNOS activity reduces after allergen exposure. Mechanism of reduced cNOS activity has implicated reduced protein expression of both nNOs and eNOS, which may occur after repeated allergen exposition.[28] On the other hand, Cerchiaro et al. (2001) showed an increase in expression and activity of iNOS in neutrophil obtained from alloxan-induced diabetic rats.[29] In this respect some evidence has implicated dysfunctional NOS and decreased NO availability in endothelium as a major pathogenic mechanism in diabetic vascular complications in humans and diabetic animals.[11] By considering our finding and above-mentioned findings, we can probably conclude increase iNOS expression occurred in diabetic and diabetic antigen-sensitized guinea pigs. In this respect, while L-NAME inhibited the pro-inflammatory effects of iNOS derived NO on the respiratory system the effect of L-Arg increased production of NO and its detrimental effects on the respiratory tract. Mechanism involved in deleterious effects of NO include inhibition of key enzyme of the mitochondrial respiratory chain and energy metabolism, mitochondrial Ca^{2+} efflux, activation of apoptotic enzymes, lipid peroxidation and DNA strand breakage with subsequent activation of

poly (ADP-ribose) synthetase.[30] Most of these mechanisms can be attributed to $OONO^-$ (peroxynitrite) generated by concomitant overproduction of NO and O_2^- under these conditions. Notably, $ONOO^-$ formed during oxidative stress may have a procontractlie action, which involves oxidative inactivation of the K_{Ca} channel as well as sarco/endoplasmatic reticulum Ca^{2+}-ATPase type 2.[31]

We found that methylene blue significantly increased response to histamine in diabetic and diabetic-antigen sensitized guinea pigs compared to control. By considering these findings, it can be concluded that guanylyl cyclase may be involved in the hyporeactivity of the airway in both diabetic and diabetic- antigen sensitized guinea pigs. This ascertain is in line with findings of Suzuki et al.(1986), Watanabe et al. (1990), Jones et al. (1994) and Ijima et al. (1995) on the soluble guanylyl cyclase (sGC). In fact, sGC is regarded as the key enzyme in mediating tracheal relaxation induced by NO and NO-related compounds through elevating the intracellular concentration of cyclic GMP.[32-35] However, there are also indications that NO-donors relax tracheal smooth muscle via a cyclic GMP-independent mechanism.[36-39] But our findings on the opposite effects of L-NAME and methylene blue in the responsiveness of tracheal rings may implicate a non-specific effect for methylene blue.

In conclusion, based on the results of this study, experimental diabetes was found to decrease the responsiveness of tracheal rings in the presence or absence of allergy. Unexpectedly, increase of NO production enhanced airway responsiveness in response to histamine in diabetic and diabetic- antigen sensitized animals; on the other hand, blockage of NO production showed an opposite effect. The findings of this research work showed that NO had no role in hypo-responsiveness of airway in diabetic and diabetic-antigen sensitized animals. The opposite effects of L-NAME and methylene blue in the responsiveness of tracheal rings may implicate a non-specific effect for methylene blue, i.e., it may exert effects other than inhibition of guanylyl cyclase.

However, further studies for finding the correlation between the NO levels and the expression of the NOS isoforms genes in diabetic and diabetic-antigen sensitized animals are necessary.

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