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INCREASED NITRIC OXIDE SYNTHASE ACTIVITY in GASTRIC FUNDAL MUSCLES from RATS with DIABETES

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Abstract: *This study was conducted in diabetic rats after 8 weeks of streptozotocin-induced diabetes. The rats were sacrificed and gastric fundus portion was cut away. Longitudinal gastric fundal muscle strips were prepared by cutting parallel to the greater curvature to examine the relaxant responses to electrical field stimulation (EFS), and the citrulline assay and Western blot analysis were the techniques used to investigate the amount of nitric oxide production and nitric oxide synthase (NOS) proteins.The results showed that the nitric oxide production and pharmacological action of nitric oxide were increased despite neuronal NOS (nNOS) and endothelial NOS (eNOS) proteins in diabetic gastric fundal muscles being significantly decrease.*
These findings suggested that there is an increased NOS active in streptozotocin diabetic rat gastric fundal muscles.

Keywords: *nitric oxide synthase, neuronal NOS (nNOS), endothelial NOS (eNOS), gastric fundal smooth Cmuscle, diabetic rats.*

I. Introduction

Nitric oxide (NO) is considered to be the predominant non-adrenergic non-cholinergic (NANC) transmitter and mediates electrical field stimulation-induced relaxation of the rat gastric fundus [1, 2]. A preliminary experiment using rat gastric fundal strips from streptozotocin-induced diabetic rats precontracted with carbachol producing tissue tone above basal line to observe the obvious relaxation, revealed that the relaxant responses to electrical field stimulation were enhanced rather than impaired when compared to controls. However, the impairment of nitrergic relaxant response was found in diabetic rat tissues precontracted with 5-hydroxytryptamine [1]. The different results may be due to the contractile agonist used. There are a number of reports showing that the choice of contractile agonist can influence relaxant responses to both agonists and nerve stimulation.

The rat gastric fundal contractions induced by 5-hydroxytryptamine and prostaglandin F_{2a} were more sensitive than contractions to carbachol, the relaxant effect of the K^+ channel activator pinacidil, nitroglycerin and isoprenaline [3]. Relaxant response of the rat gastric fundus evoked by photoactivation of a nitric oxide-containing compound and nitrergic nerve stimulation was found to be less in high K^+-contracted tissues than in 5-hydroxytryptamine-contracted tissues [4].

Impairment of non-adrenergic non-cholinergic inhibitory transmission has been observed in a number of gastrointestinal tissues taken from streptozotocin-induced diabetic rats. The gastric fundus [1, 5] and duodenum [5] are examples. This impairment is thought to involve the nitrergic mechanisms [1, 4, 7].

NO is synthesized from the terminal guanidine nitrogen atom of L-arginine by a nitric oxide synthase (NOS) [8]. The neuronal NOS (nNOS) and inducible NOS (iNOS) were localized in the gastrointestinal tract [8]. The impairment of nitrergic transmission occurred in various tissues taken from streptozotocin-induced diabetic animals or incubated in elevated glucose, due to either a reduction in smooth muscle responsiveness to NO [9] or to the impairment in the synthesis and/or release of NO [1, 4]. Impairment of postjunctional smooth muscle reactivity to NO had been found in the rat anococcygeus muscle from streptozotocin treated animals [10]. The action of NO was impaired in penile tissues from 9 week and 14 week-streptozotocin diabetic rats despite an increase in NOS activity [11].

The NO production in gastric fundus from 4- week streptozotocin diabetic rats was enhanced as Ca^{2+}-independent NOS activity in this tissue was markedly increase [12]. NO release is increased in diabetes, which was likely to be due to and increase in the activity of NOS enzyme [13]. Conflicting results of NO production and/or activity, and NO derivatives have been shown in conditions of hyperglycaemia. This may be due to a number of reasons but the different agonist used to contract the tissues is the most likely to be involved. This study therefore was conducted to investigate the relaxant responses, the amount of nitric oxide production and the nitric oxide synthase proteins in the diabetic rat gastric fundus muscles precontracted with carbachol.

II. Materials and Methods

A. Induction of diabetes

Male Wistar rats weighing 200-250 g were the animals used in this study. The rats were housed in air conditioned rooms and allowed access to food and water ad libitum at least for 5 days, before the commencement of experiments. To induce diabetes, rats received a single intra-peritoneal injection of streptozotocin (65 mg/kg), freshly dissolved in 20 mM citrate buffer (pH 4.5). Control rats were injected with equal volume of vehicle alone. To prevent the initial hypoglycaemia phase, the streptozotocin-treated rats received 2% sucrose in their drinking water for the first 48 h following streptozotocin injection. Diabetes was verified by non-fasting blood glucose levels at, or above 200 mg/dl [14]. The nonfasting blood glucose level was measured in a drop of blood obtained from tail vein by puncturing with a sterile needle using test strips and Blood glucometer)Accu-chek Advantage, Roche Diagnostics, USA.

B. Gastric fundal muscle preparation

After eight weeks, the rats were sacrificed by carbon dioxide asphyxiation. The gastric fundus portion was cut away. Longitudinal gastric muscle strips measuring 2 mm in width and 15 mm in length were prepared by cutting parallel to the greater curvature.

C. Relaxant responses to EFS

The gastric muscle strips were mounted between paired platinum ring electrodes and suspended with one end attached to the base of a stimulating electrode and the other end attached by a thread to a force displacement transducer. The muscle strips were mounted in 30 ml organ baths contained Kreb's solution of the following compositions (in mM): NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, K_2HPO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 11.1. The solution was maintain at 37 ^{0}C and gassed with 95%O_2-5%CO_2. The strips were allowed to equilibrate for at least 1 h before commencing experiments.

To examine the EFS evoked NANC relaxation on the diabetic rat gastric fundal muscle, studies were carried out using a frequency response curve (1-16 Hz, 40V, 0.5 ms for 10 s every 5 min), a parameter was used by Williams and Parsons (1995). The experiments were performed by incubating the strips in the presence of guanethidine sulphate (10^{-6}M), an adrenergic neuronal blocked agent to reduce catecholamine release, and indomethacin (10^{-6}M), a cyclo-oxygenase inhibitor to inhibit prostaglandin synthesis, to isolate the NANC transmitter component. EFS induced NANC relaxation was performed by stimulating the carbachol (10^{-6}M) precontracted tissues via paired platinum ring electrodes using a Grass S11 stimulator in the absence and in the presence of N^G-nitro-L-arginine (L-NOARG; 10^{-6}M).

The tissue responses were recorded isometrically using a force transducer (Dynamometer UF1) amplified by a preamplifier (Lectromed 3552) and recorded on a Lectromed 5041 recorder.

D. Citrulline assay

NO production was measured as the amount of L-[H^3] citrulline produced. The technique used by Takahashi *et al*. (2000) was applied in the present study. Six muscle strips from each group of animals were pool after incubated in Kreb solution at 37 ^{0}C for 30 min loading with 3μCi/ml L-[H^3] arginine in the absence or presence of carbachol (10^{-6}M). The tissues were homogenized and centrifuged. The supernatant was applied to a Dowex AG 50 WX-8 resin column to separate L-[H^3] citrulline from L-[H^3] arginine. L-[H^3] citrulline production was measured using scintillation counter. The data were expressed as dpm/g tissue wet weight.

E. Western blot analysis

To detect the NOS proteins (nNOS, eNOS and iNOS) in diabeteic rat gastric fundal muscle, the Western blot analysis was applied. The gastric fundus were isolated, rapidly cleaned with ice-cold Krebs buffer and snap frozen with liquid nitrogen and then stored at –80°C. Tissues were solubilised in a lysis buffer containing 10% glycerol, 2% SDS and 76.5 mM Tris-HCl pH 6.8 and heated at 95°C for 5 min. The lysate was clarified by centrifugation (Hettich Zentrifugen EBA 12) at 10,000 rpm, for 3 min at room temperature. The protein concentration was estimated using bovine albumin as a standard. Samples were mixed with 2% bromophenol blue and 5% β-mercaptoethanol, heated at 95 °C for 5 min and then loaded into a SDS-polyacrylamide gel. For determination of molecular mass, a molecular weight standard (New England Biolabs) was used. After completion of the electrophoresis, proteins were electroblotted 3 h onto a PVDF membrane (BDH, UK). The membrane was blocked for 2 h with blocking buffer (5% non-fat dry milk, 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20) and then stained with primary monoclonal antibody obtained from Affinity (0.25 mg/ml stock in 50% glycerol, 20 mM sodium phosphate pH 7.5, 150 mM NaCl, 1.5 ml NaN_3, 1 mg/ml BSA) and anti mouse IgG: horseradish peroxidase as secondary antibody diluted 1:2,500 and 1:5,000 in 5% non-fat dry milk, respectively. Enhanced chemiluminescent staining using ECL reagent (Amersham Pharmacia Biotech) developed following extensive washing of the reaction. NOS protein was expressed as % relative intensity.

F. Drug

Carbachol, quanethidine sulphate, indomethacin, streptozotocin, N^G-L-arginine, bromophenol blue and β-mercaptoethanol were pershased from Sigma UK.

G. Analysis of the results

The relaxant responses were expressed as percentage relaxation, L-[H^3] citrulline as dpm/g tissue wet weight. NOS protein was expressed as % relative intensity. Data were shown as mean $\pm$ SEM, n indicates the number of animals. Differences between means were determined by using Student's *t* - test for unpaired data. Probability levels less than 0.05 ($P<0.05$) were taken to indicate statistical significance.

III. Results

A. Relaxant responses to EFS

The electrically-evoked NANC relaxations in the presence of L-NOARG (10^{-6}M) of the gastric fundal strips were abolished at 1 Hz and 2 Hz, significantly reduced ($P<o.o5$) at 4 Hz, and markedly reduced at 8 Hz but unaltered at 16 Hz of stimulation (Fig. 1b) when compared to the relaxant responses in the absence of L-NOARG (10^{-6}M) (Fig.1a)**.**

Percentage relaxation to EFS was significantly ($P<0.05$) greater in the gastric fundal muscle strips obtained from STZ-treated animals than age-matched control rats. L-NOARG (10^{-6}M) significantly inhibited the relaxant responses to EFS in the tissues from STZ-treated and age-matched control rats over the whole frequency range (1Hz to 16 Hz) although the degree of inhibition was more marked at the lower frequencies (1Hz to 4Hz). In the presence of L-NOARG, the relaxant responses over the whole frequency range were almost identical in STZ-treated and control tissues (Fig.2).

B. Citrulline assay

L-[H^3] citrulline formations were compared between the gastric fundal strips taken from control and STZ-treated rats under the basal condition (non-treated) and treated with carbachol (10^{-6}M). Basal L-[H^3] citrulline formation obtained from STZ-treated tissues was slightly less than from controls but was slightly greater in STZ carbachol-treated tissues when compared to controls (Fig.3).

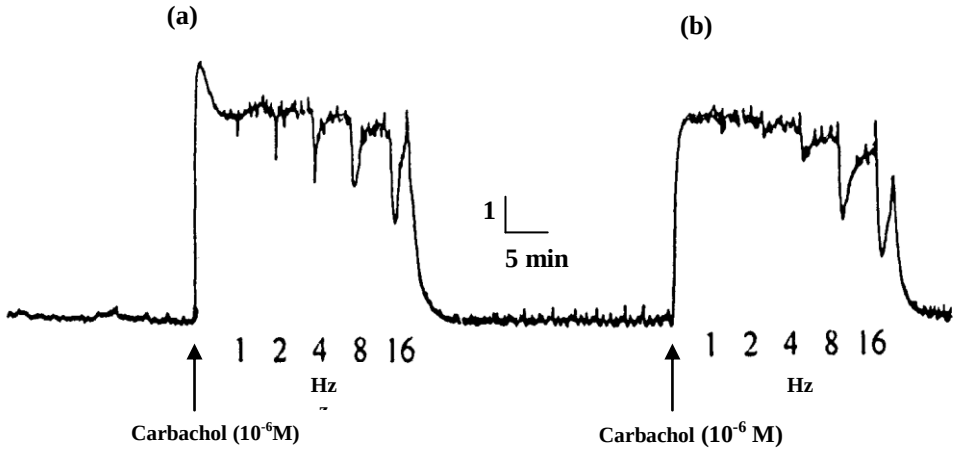

Figure 1 Original trace illustrating the relaxant responses to EFS (frequency response curve; 40V, 0.5 ms for 10s, every 5 min) on gastric fundal strips in the absence (a) and in the presence (b) of L-NOARG (10^{-6}M).

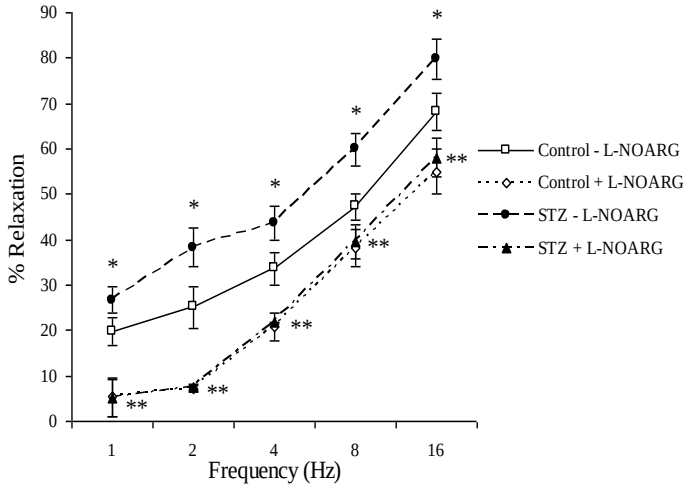

Figure 2 Percentage relaxation to EFS in the absence and in the presence of L-NOARG (10^{-6}M) (frequency response curve; 40V, 0.5 ms for 10 s every 5 min) of gastric fundal muscle strips from controls and STZ-induced diabetic rats. Values represent the mean ± SEM for 8 animals. *P<0.05 is significantly different from controls and ** P<0.05 is significantly different in the presence of L-NOARG from in the absence of L-NOARG (Student's *t* test for unpaired observations).

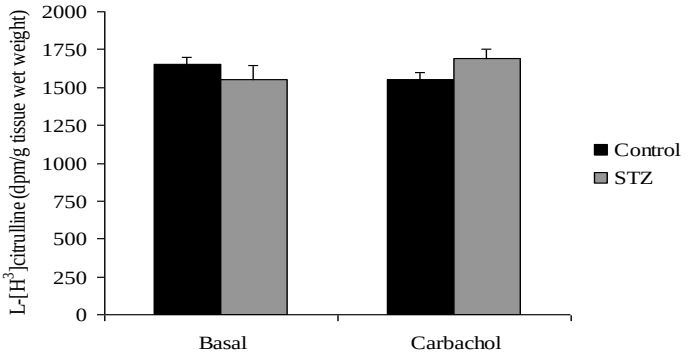

Figure 3 L-[H^3] citrulline formation obtained from control and STZ-treated gastric fundal tissues in basal and treated with carbachol. Values represent the mean ± SEM for 6 animals. *P<0.05 is significantly different from controls (Student's *t* test for unpaired observations).

C. Western blot analysis

Using monoclonal antibody, proteins tentatively identified as nNOS and eNOS but not iNOS has been found as a single band of about 160 kDa for nNOS and 140 kDa for eNOS both in gastric fundus from STZ-

treated and age-matched control tissues. The band of nNOS protein was clearly observed in the gastric fundal tissues from control rats but was reduced in the STZ-treated tissues. The density of eNOS bands were less when compared to nNOS and was again lower in STZ-treated tissues.

Interestingly, the evidence of iNOS protein was not found in the present study. Quantification shown the nNOS and eNOS protein expression in STZ-treated tissues were significantly ($P<0.05$ in nNOS and $P<0.001$ in eNOS) reduced compared to controls (Fig. 4a, Fig. 4b).

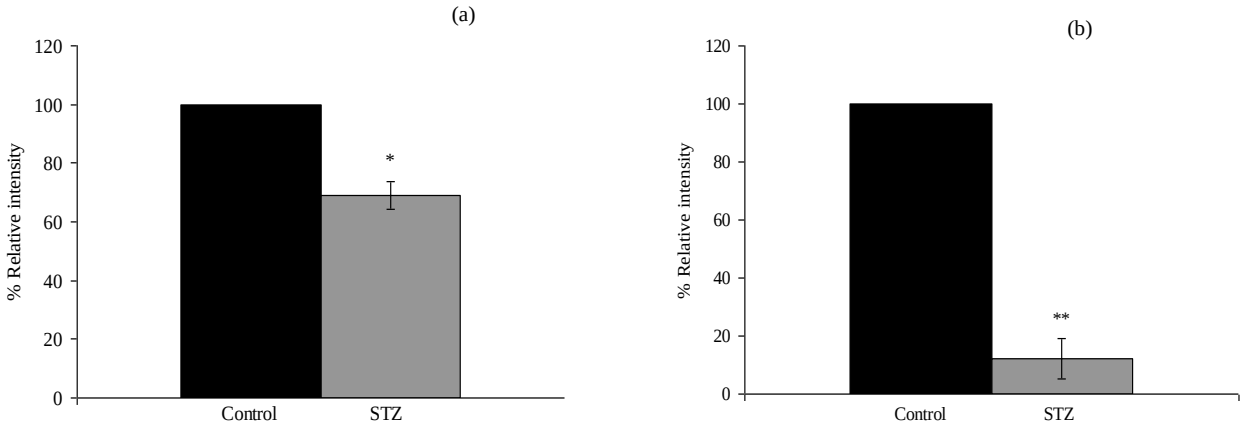

Figure 4 Relative intensity of nNOS (a) and eNOS (b) protein band quantified by scanning densitometry in gastric fundus tissues from controls and STZ-induced diabetic rats. Data were expressed as a percentage of the maximum value obtained from controls. Values represent the mean ± SEM for 8 animals.
* $P<0.05$ and ** $P<0.001$ are significantly different from controls. (Student's *t* test for unpaired observations).

IV. Dicussion

The electrically-evoked NANC relaxations of the gastric fundal strips were abolished at 1 Hz and 2 Hz, significantly reduced ($P<0.05$) at 4 Hz, and markedly reduced at 8 Hz in the presence of L-NOARG (10^{-6}M), a nitric oxide synthase inhibitor, indicating that at the lower frequencies, NO was the sole transmitter involved in the frequency dependent NANC relaxation.

In the absence of L-NOARG (10^{-6}M) the relaxant responses were greater in the gastric muscle strips taken from streptozotocin-induced diabetic rats and the relaxant responses were reduced by L-NOARG (10^{-6}M) to a greater extent than those strips taken from controls. This finding suggested that the strips taken from streptozotocin-induced diabetic rats produced or released more NO than the strips taken from controls.

Nitric oxide production (expressed as the conversion of [^{3}H] arginine to [^{3}H] citrulline) of the gastric fundal strips taken from STZ-treated rats under the basal condition (non-treated) was slightly less than controls under the basal condition (non-treated) but was slightly greater when treated with carbachol (10^{-6}M). This finding indicated that NO production was slightly increased in streptozotocin carbachol treated strips and this might result from an activation of muscarinic receptors. Activation of muscarinic receptors resulting in an invrease in NO production, NOS activity and NO/NO_2 release has been reported. Espanol and Sales [15] found that mAChR activation by carbachol increased NO formation in the rat ileum. Acetylcholine acting at muscarinic receptors contributes to stimulation of NO formation in the guinea-pig ileum longitudinal muscle [16]. Nerve-induced NO/NO_2 release in guinea-pig colon is in a substantial part, due to M_1 receptor activation as has been reported [17]. *Leiros et al* [18] found that carbachol significantly increased basal activation of NOS in rat sublingual and submandibular salivary glands and the maximum increase of NOS activities occurred at a carbachol concentration of 10^{-6}M.

The use of carbachol to treat the strips in the presence study might therefore activate an increase in the EFS-induced relaxant responses mediated by NO. It is clearly that Streptozotocin strips are more responsive to carbachol than control strips, and thus an increased activation of muscarinic acetylcholine receptors could lead to increase NO production.

Since the major amount of the constitutive NOS protein found in gastric fundal strips in the presence study is nNOS isoform, and increased in NO production in streptoZocin-treated animals are most likely resulted from an increase in nNOS activity.

Expression of the constitutive nitric oxide synthase (nNOS and eNOS) of the gastric fundus in the presence study was similar to the study carried out by Qu *et al* [19] who found that in the rat intestine nNOS is the predominant isoform, there is little eNOS but no iNOS. The decrease in NOS protein in streptozotocin-treated gastric fundus compared with controls in the present study was similar to the decreased NOS protein levels in the gastric fundus of diabetic rats found by *Takahashi et al* [20] and in the antrum myenteric plexus [20]. Decreased NOS protein in other tissues have also been reported, for example, penile [22, 23] and skeletal muscle [24, 25]. In contrast, increased NOS protein levels were found in some streptozotocin-diabetic rat tissues e.g. oesophagus [26], cerebral cortex [27], renal cortex [28], and rabbit gastric glands [29]. Furthermore, NOS expression in the duodenum, ileum and colon myenteric plexus of diabetic rats was not different from controls [21]. NOS protein expression has been studied in many tissues from streptozotocin-diabetic rats and different results have been obtained even when the same tissues were used. For example, Shin *et al* [30] used an RTPCR technique and found that the neuronal and endothelial nitric oxide synthase mRNA were increased in the renal outer medulla with no differences found in the renal cortex and renal internal medulla of streptozotocin-induced diabetic rats. In contrast, the Western blotting studies showed that the three isoform of NOS (nNOS, iNOS and eNOS) all increased in the renal cortex of streptozotocin-induced diabetic rats, whereas, they remained unchanged in the renal medulla [28]. The conflicting results in this case seem to be dependent of the methodology employed.

Despite the constitutive NOS protein being decreased, NO production in the gastric fundal muscles from streptozotocin-treated rats was not changed when compared to controls, suggesting that the NOS in streptozotocin fundal strips is more efficient in producing NO.

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