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Research Article

Increase in neuronal nitric oxide synthase content of the gastroduodenal tract of diabetic rats

E. Adeghate [a,*], B. al-Ramadi [b], A. M. Saleh [c], C. Vijayarasathy [c], A. S. Ponery [a], K. Arafat [d], F. C. Howarth [d], T. El-Sharkawy [d]

[a] Department of Human Anatomy, Faculty of Medicine & Health Sciences, United Arab Emirates University, P.O. Box 17666, Al Ain (United Arab Emirates), Fax: +971-3-7672033, e-mail: eadeghate@uaeu.ac.ae
[b] Department of Medical Microbiology,
[c] Department of Biochemistry and
[d] Department of Physiology, Faculty of Medicine & Health Sciences, United Arab Emirates University, P.O. Box 17666, Al Ain (United Arab Emirates)

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Abstract. This study examined the changes occurring in the pattern of distribution and expression of neuronal nitric oxide synthase (nNOS)-positive nerves in the gastroduodenal tract of streptozotocin-induced diabetic rats. The ganglion cells of the myenteric plexus of the gastric antrum of normal rats contain nNOS. We also observed nNOS-positive neurons and fibres in the myenteric plexus of the duodenum of normal rats. After the onset of diabetes, the number and intensity of staining of nNOS-positive nerve profiles in the gastric antrum and duodenum did not change significantly. However, Western blotting showed a significant increase in the expression of nNOS after the onset of diabetes. In conclusion, diabetes of 4 and 32 weeks duration induced an increase in the tissue content of nNOS in the gastroduodenum of rat. The increase in the level of nNOS in the gastroduodenum of diabetic rats may explain why impaired gastric emptying is common in patients with diabetes.

Key words. Diabetes mellitus; gastroduodenum; immunohistochemistry; Western blot; neural nitric oxide synthase; rat.

Nitric oxide (NO) regulates vascular tone, mediates haemodynamic states, influences coagulation and immune function and protects gastrointestinal mucosa [1, 2]. NO is also an important inhibitory transmitter in the gut [3, 4]. NO has been demonstrated in the myenteric plexus of the gastrointestinal tract of amphibian [5] and several mammalian species including the rat [6, 7], ferret [8] and guinea pig [9]. Recent studies have shown the ability of NO to regulate small-intestinal motility in humans [10]. NO-containing neurons have been classified as inhibitory neurons, which mediate the receptive and accommodative relaxation, and control the openings of sphincters. They are thus important in the regulation of normal gut motility [11]. Despite the large number of studies performed on the distribution and function of NO synthase (NOS) in the gastrointestinal tract, no data are available on the gastroduodenal tissue level of this important inhibitory neurotransmitter in diabetic rats. Since streptozotocin (STZ) has been shown to induce oxidative stress in rat, the aim of this study was to examine whether STZ-induced diabetes will cause changes in the pattern of distribution and content of neuronal NOS (nNOS) in the rat gastrointestinal tract.

* Corresponding author.

Materials and methods

Experimental animals and induction of diabetes mellitus

Twelve-week-old male Wistar rats, weighing approximately 250 g, were used in this study. Rats were obtained from the United Arab Emirates University breeding colony, and the Animal Ethics Committee's guidelines for the care and use of laboratory animals were followed. The rats were divided into two groups: STZ-induced diabetics and age-matched controls. Diabetes was induced by a single intraperitoneal injection of STZ (Sigma, Poole, UK) at 60 mg kg^{-1} prepared in 5 mM citrate buffer, pH 4.50 [12]. The animals were kept in plastic cages and maintained on standard laboratory animal diet and water ad libitum. A One-Touch II Glucometer (LifeScan, Johnson and Johnson, Milpitas, Calif.) was used to measure blood glucose for each individual animal. The animals were considered diabetic if the random blood glucose levels were equal to or more than 300 mg dl^{-1}. After 4 and 32 weeks from the date of induction of diabetes, all the animals from both groups were sacrificed under chloral hydrate (7%) general anaesthesia (6 ml kg^{-1} body weight, injected intraperitoneally). The gastroduodenal segments of normal (n = 6) and diabetic (n = 6) rats were removed and fixed overnight in freshly prepared Zamboni's fixative [13] of the following composition: paraformaldehyde (80 g/696 ml) and aqueous picric acid (300 ml) in 0.2 M phosphate-buffered saline (1000 ml).

Immunohistochemistry

The Zamboni-fixed tissue samples were later dehydrated and embedded in paraffin wax at 55 °C according to a previously described method [14]. Six-micrometer-thick sections were cut on a microtome (Shandon AS325, Pittsburgh, Pa.), and placed in a water bath at 48 °C. Thereafter, sections were transferred onto prewashed microscopic slides, which were dried in an oven at 55 °C for 30 min to enhance attachment of sections. The sections were then de-paraffinized in xylene and processed for immunohistochemistry using a previously described method of Adeghate et al. [14]. Briefly, after 30 min incubation in the blocking reagent, the appropriate dilution of primary antibodies (mouse monoclonal anti-nNOS from Transduction Laboratories, Kentucky) and negative control reagents were applied. The sections were incubated in primary antibodies for 24 h at 4 °C. The slides were then washed and incubated for 30 min with prediluted biotinylated anti-mouse IgG. After washing in Tris-buffered saline (TBS), the sections were incubated with streptavidin-peroxidase conjugate for 45 min followed by washing with TBS. The peroxidase activity was visualised by incubating the specimens for 3 min in TBS solution containing 3,3-diaminobenzidine tetrahydrochloride and 0.03% hydrogen peroxide. The slides were later washed, counter-stained with haematoxylin, and dehydrated before mounting in Cytoseal 60 (Stephens Scientific, Riverdale, Calif.).

The antiserum to nNOS was used at 1:1000 dilution. The specificity of the antibody was confirmed by processing tissue samples in the absence of anti-nNOS serum. No specific immunostaining was observed when anti-nNOS antibody was omitted.

Western blot analysis

Gastric and duodenal tissues, obtained from rats 4 and 32 weeks after the onset of diabetes, were washed twice with cold phosphate-buffered saline (PBS) and directly frozen in liquid nitrogen.

Frozen tissues (1 – 2 g) were broken under liquid nitrogen with a ceramic mortar and pestle. Two volumes of extraction buffer (100 mM HEPES, pH 7.5, 10% sucrose, 10 mM DTT, 0.1% CHAPS, 150 mM NaCl) and protease inhibitors (1 mM PMSF and 1 mg/ml of leupeptin, aprotonin and pepstatin A) were added to the powder of broken cells. The suspension was cleared by centrifugation at 14,000 g for 30 min. The supernatant was collected and stored at – 80 °C until further use. Protein concentrations of cellular lysates were determined by the Bradford method using the colorimetric protein assay kit (BioRad). Sixty micrograms protein from the tissue extracts was separated by 8% SDS-PAGE and transferred to PVDF membrane (Millipore) by standard methods [15, 16]. The blots were probed at 1:1000 dilutions of mouse monoclonal anti-nNOS (Transduction Laboratories, Lexington, Ky.) in PBS containing 0.1% Tween-20 and 2% nonfat dry milk for 2 h at room temperature. The blots were then incubated with HRP-conjugated antibody against mouse IgG (1:2000 dilution; Sigma) and the immunoreactive bands were visualised and analysed using a Super Signal West Pico chemiluminescent kit following the manufacturer's protocol (Pierce Chemical Company). To confirm equal loading of proteins, the blots were also immunoprobed with a polyclonal antibody against the cytoskeletal protein α-tubulin (Santa Cruz Biotechnology; 1:2500 dilution). Gastric and duodenal tissues obtained from age-matched controls were processed according to the above procedure.

Determination of secreted NO in culture supernatants

Stomach and duodenal segments, obtained from normal and diabetic rats (4 weeks after onset of diabetes), were minced into small pieces (1 – 3 mm^3) and washed in 0.02 M PBS (pH 7.4). Approximately 3 – 4 mg of gastric and duodenal tissue fragments from normal and diabetic rats were placed in 2-ml glass vials containing 1 ml of PBS and preincubated for 30 min in a water bath at 37 °C to wash away the contents of damaged cells due to cutting of the tissues. Immediately after the preincubation step, the PBS was drained and the specimens were subsequently

incubated at 37 °C for 1 h with acetylcholine (10^{-4} M). Tissues incubated with PBS alone were used as controls. After incubation, the supernatants were collected, spun free of any debris, and assayed for nitrite (NO_2^-) content. Accumulation of NO_2^- was used to determine production of NO according to the Griess method [17]. Briefly, 100 µl of culture supernatant were mixed with an equal volume of Griess reagent, incubated at room temperature for 15 min and absorbance read at 562 nm in an automated microplate reader. A solution of $NaNO_2$, of a known concentration, was used as standard.

Results

Immunohistochemistry of the gastric antrum

Figure 1a, b shows nNOS-positive ganglion cells among nNOS-negative ganglion cells of the myenteric plexus of the gastric antrum of age-matched control (fig. 1a) and diabetic (4 weeks after onset) rats (fig. 1b). The distribution pattern of nNOS in the ganglion cells of the myenteric plexus of the gastric antrum of STZ-induced diabetic rats was similar to that observed in age-matched controls. In addition, nNOS-immunoreactive nerves were also observed in the circular muscle layer of the lamina muscularis propria of the gastric antrum of both control and diabetic rats. Figure 1c, d shows nNOS-immunoreactive ganglion cells in the myenteric plexus of the gastric antrum of normal control and long-term (32 weeks after onset) diabetic rats. nNOS was still observable in the myenteric plexus of the gastric tissue fragments of rats taken 32 weeks after the onset of STZ-induced diabetes.

Immunohistochemistry of the duodenum

Figure 2 shows nNOS-positive neurons in the myenteric plexus of the duodenum of normal, age-matched control and diabetic rats. nNOS-immunopositive ganglion cells were observed in the myenteric plexus of control (fig. 2a) and short-term diabetic (4 weeks after onset) rats (fig. 2b). The nNOS-positive neurons of the myenteric plexus of the duodenum appeared to be smaller than the nNOS-positive ganglion cells of the duodenum of age-matched control rats. In addition, nNOS-positive nerves were also identified in the circular layer of the lamina muscularis propria of the duodenum. The pattern of distribution of nNOS in ganglion cells in the myenteric plexus of the duodenum of STZ-induced diabetic (4 weeks after onset) rats was similar to that observed in age-matched control rats. The nerves innervating the smooth muscle cells of the circular layer of the lamina muscularis propria of diabetic rats contained nNOS, as observed in the lamina muscularis propria of normal rat. Figure 2c, d shows nNOS-positive neurons in the myenteric plexus of the duodenum of normal, age-matched control and long-term (32 weeks after onset) dia-

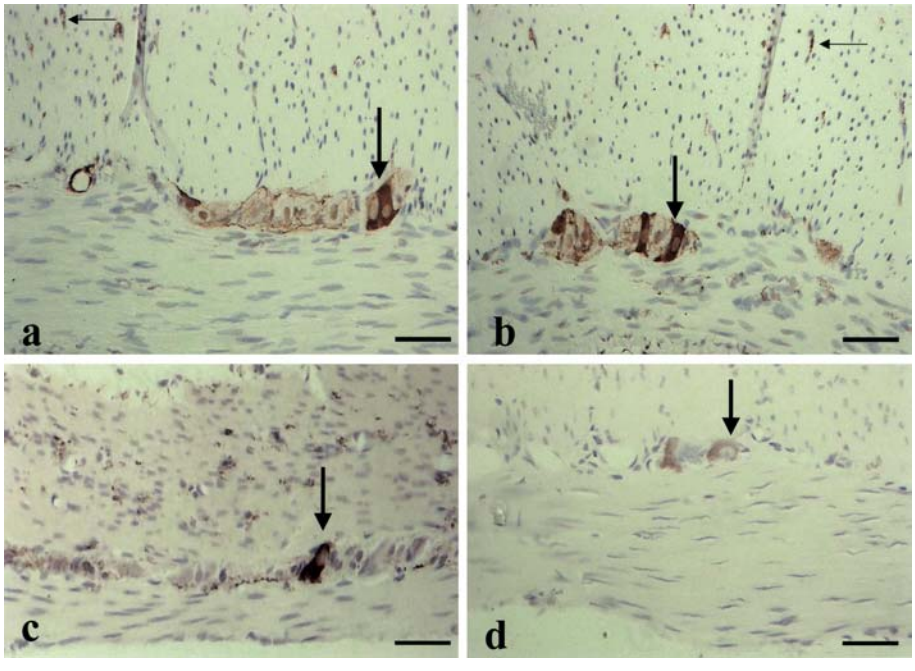

Figure 1. nNOS-positive ganglion cells (thick arrow) in the myenteric plexus of rat gastric antrum 4 (*b*) and 32 (*d*) weeks after the onset of diabetes and in age-matched control rats (*a* and *c*, respectively). The pattern of distribution of nNOS-positive nerves in the gastric antrum of STZ-induced diabetic rats is similar to that observed in age-matched control rats. nNOS-positive nerve fibres (thin arrow) were also observed in the circular layer of the lamina muscularis externa of the gastric antrum of both normal and diabetic rats. However, the density of nNOS-positive nerves appeared to be more sparse in long-term (32 weeks) compared to short-term (4 weeks) diabetic rats. This micrograph is typical of six such animals. Scale bars, 50 µm.

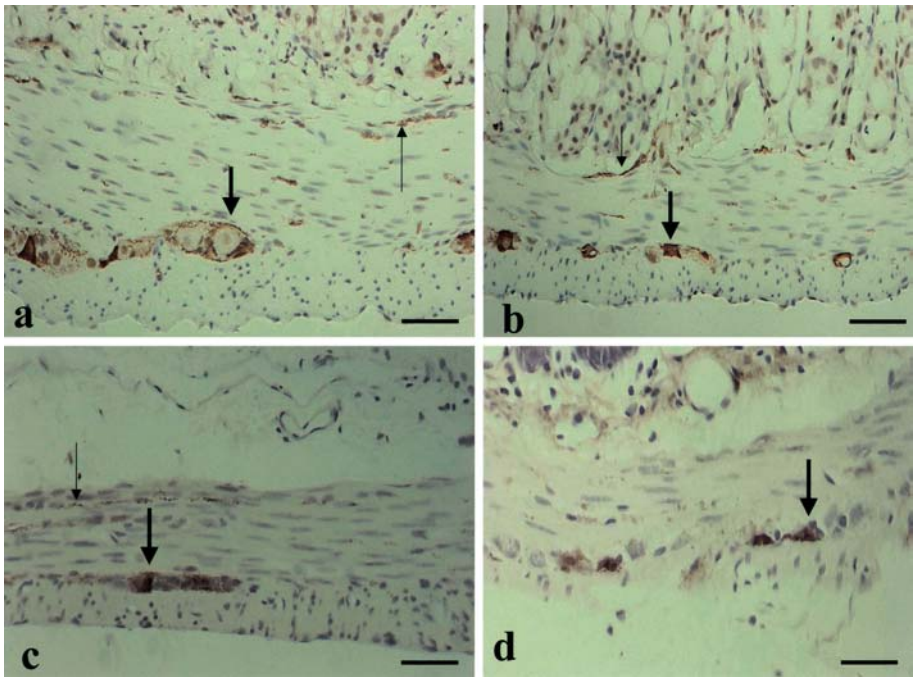

Figure 2. nNOS-positive neurons (thick arrows) in the myenteric plexus of the duodenum obtained 4 (*b*) and 32 (*d*) weeks after the onset of diabetes and in age-matched control rats (*a* and *c* respectively). The circular muscular layer of the lamina muscularis externa contains nerve fibres (thin arrow). The size of nNOS-positive ganglion cells in the duodenum of STZ-induced diabetic rats appears to be smaller compared to that of age-matched control rats 4 weeks after onset of diabetes. This micrograph is typical of six such animals. Scale bars, 50 µm.

betic rats. The pattern of distribution of nNOS-immunoreactive neurons was similar. However, there appeared to be fewer nNOS-immunoreactive nerves in the circular layer of the muscularis propria in rats with long-term diabetes compared to those with short-term diabetes.

Western blot

To determine the potential changes that may occur in nNOS level after the onset of diabetes, we assessed the expression of nNOS by Western blot in 60 µg of total protein from the gastric antrum and duodenal tissues of normal and STZ-diabetic rats using anti-nNOS antibody. There was a pronounced increase in the tissue level of nNOS in the stomach (fig. 3) and duodenum (fig. 4) of rats 4 and 32 weeks after the onset of diabetes. The level of nNOS in the gastric and duodenum tissues of diabetic rats was three- to six fold that measured in the gastric and duodenum tissues of age-matched controls.

Production of NO by gastroduodenal tissue

The potential release of NO by gastroduodenal segments from normal and diabetic (4 weeks after onset of diabetes) rats was studied by incubating the segments with or without acetylcholine in PBS for 1 h and measuring the amount of NO released into the medium. NO production was quantified by determining the extent of NO_2^- accumulation over the 1 h incubation period.

The results of this analysis are shown in figures 5, 6. As can be seen, acetylcholine induced an increase in NO production in both gastric (fig. 5) and duodenal (fig. 6) tissues of normal rats. However, the level of NO induction was smaller in gastric tissue and did not reach significance compared to basal levels. The results obtained from diabetic tissues are significant in two important respects. First, both gastric and duodenal tissues of diabetic rats exhibited significantly ($p < 0.001$ Student's t-test) reduced basal levels of NO secretion compared to normal controls. Second, even when stimulated with acetylcholine, the level of NO produced remained significantly ($p < 0.001$ Student's t-test) lower than in age-matched control rats (figs. 5, 6). Similar to the situation in normal rats, gastric tissue segments from diabetic rats exhibited a lower capacity to secrete NO compared to duodenal tissue. Taken together, these results demonstrate that gastroduodenal tissues from diabetic rats are impaired in their ability to secrete both basal and acetylcholine-inducible levels of NO compared to normal rats.

Discussion

The present study shows that NOS, the enzyme that converts L-arginine to NO, is well distributed in the upper gastrointestinal tract of both normal and diabetic rats.

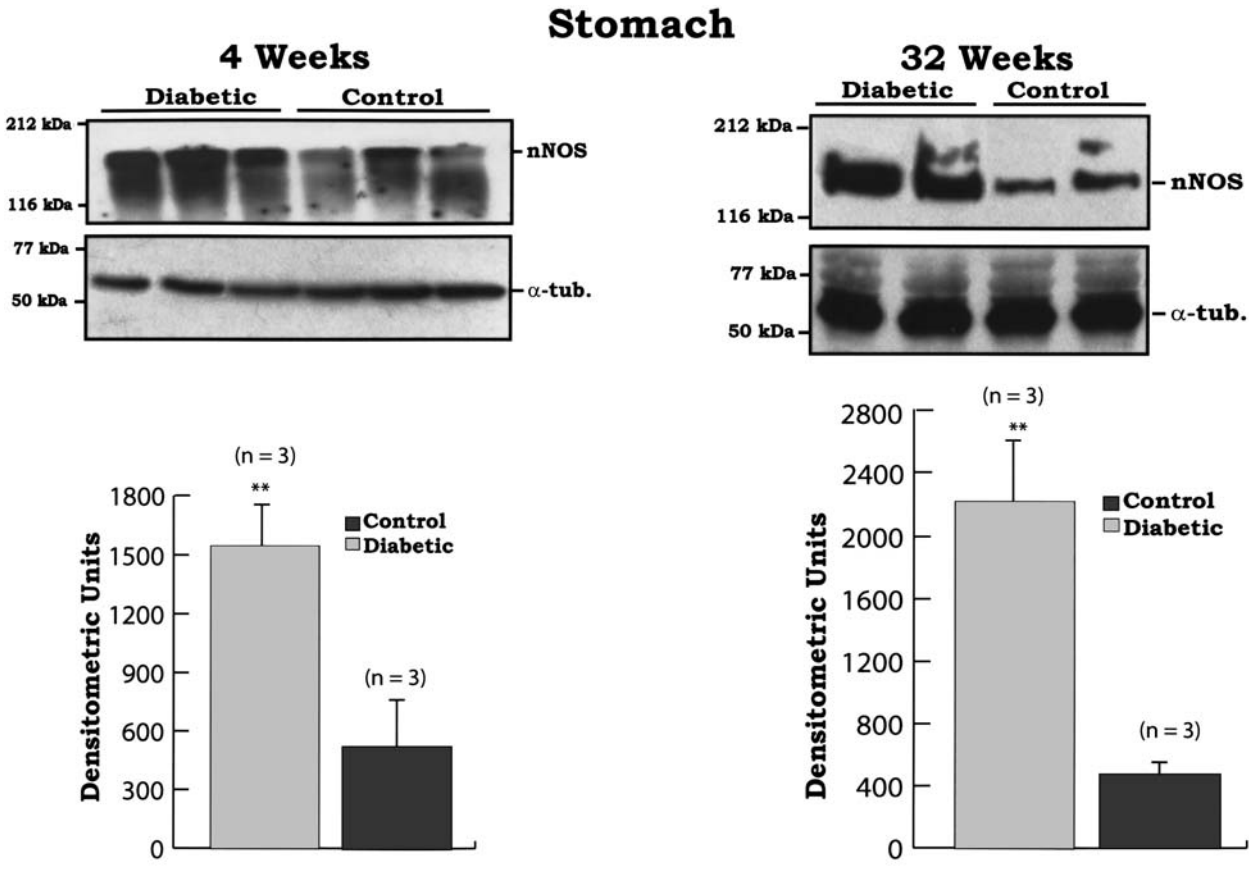

Figure 3. Western blot analysis of nNOS protein expression in the gastric antrum of normal and STZ-diabetic rats with a short (4 weeks) and long (32 weeks) duration of diabetes. In both groups of rats, nNOS protein was increased significantly (**$p < 0.001$) in the gastric antrum of diabetic rats compared to age-matched controls. The expression of α-tubulin was included as a control of equal loading of protein in the different samples. The histogram represents a densitometric scanning profile of the nNOS-immunoreactive bands obtained from two to three different animals of each group.

Our observation on the distribution of nNOS in the myenteric plexus of the gastroduodenum of normal rats corroborates those of previous reports [9]. Moreover, the observation of numerous nNOS-positive nerves in the circular layer of the muscularis externa also correlates well with previously reported data [18]. The rich innervation of the circular layer of the muscularis externa by nNOS-positive nerves may indicate a selective dependence of this muscle layer on NO transmission. The results of the present study indicate that the pattern of innervation of the gastric antrum and the duodenum is similar in normal rats and in rats with short-term (4 weeks) diabetes. The similarities in the pattern of innervation of the gastroduodenum of normal and diabetic rats suggest that diabetes mellitus of 4 weeks duration is probably not long enough to cause conspicuous morphological changes. However, the gastric and duodenal tissue fragments of rats with long-term (32 weeks) diabetes appeared to have fewer nNOS-positive neurons. This observation agrees with other reports, which have demonstrated a decrease in the number of NOS-positive nerves in the antrum of diabetic rats [19]. Moreover, our observation on the pattern of distribution of nNOS in the duodenum of rats with short-term diabetes corroborates that of Wrzos et al. [19]. Worth noting is that quantitation of cellular structures based on immunohistochemical staining may be grossly influenced by many technical factors such as the intensity of staining, duration of the development of the chromogen and the sensitivity of the antisera used.

The potential release of NO by gastroduodenal segments was examined by incubating the segments in PBS for 1 h and measuring the amount of NO released into the medium. Acetylcholine at 10^{-4} M evoked significant increases in NO release from the duodenal tissue fragments of rats (with 4-week-long diabetes) when compared to basal. The fact that the incubation period was only 1 h suggests that acetylcholine is a potent stimulator of constitutive NO. Despite the fact that the gastric and duodenal tissues of diabetic rats contained significantly more nNOS, the quantity of NO released from diabetic tissues was significantly smaller than that of normal rats. A possible reason for this is that Ca^{2+} signaling has been shown to be impaired in diabetes [20]. Since acetylcholine is known to act through Ca^{2+} during the stimulated release

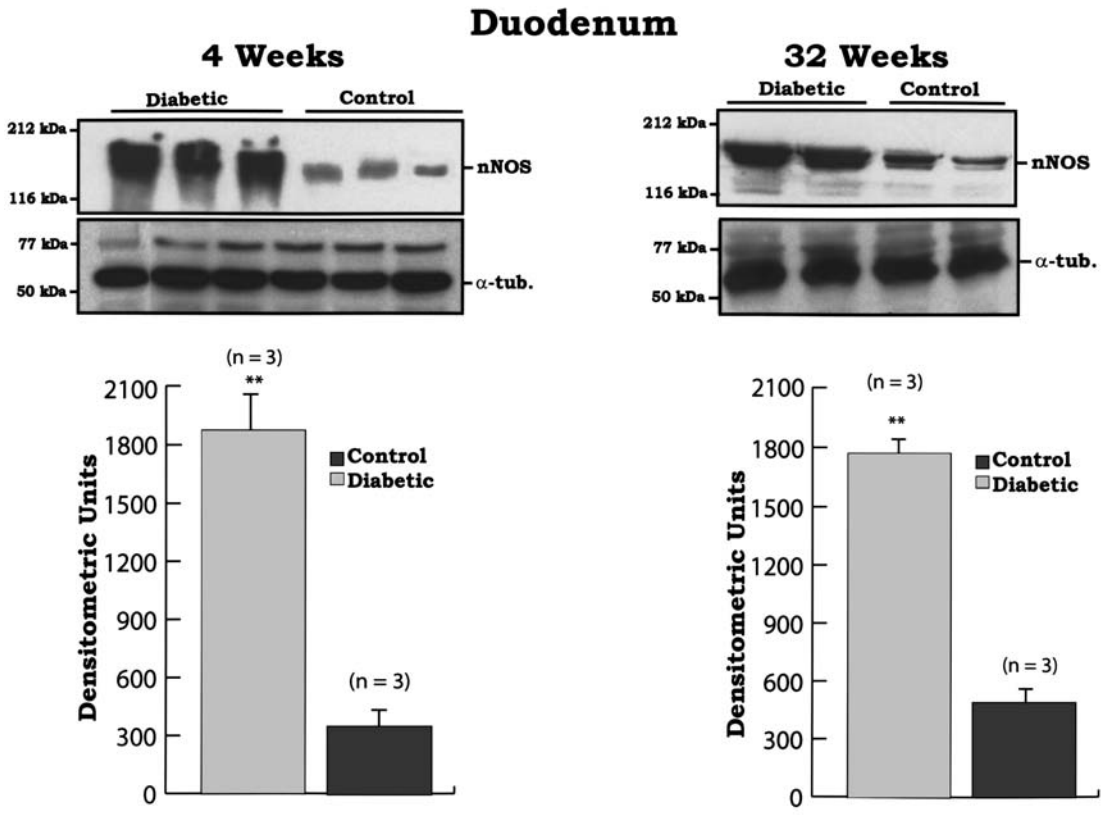

Figure 4. Western blot analysis of nNOS protein expression in the duodenum of normal and STZ-diabetic rats with a short (4 weeks) and long (32 weeks) duration of diabetes. nNOS protein was increased significantly (**$p < 0.001$) in the duodenum of both groups of diabetic rats compared to controls. The expression of α-tubulin was included as a control of equal protein loading in the different samples. The histogram represents a densitometric scanning profile of the nNOS-immunoreactive bands obtained from two to three different animals of each group.

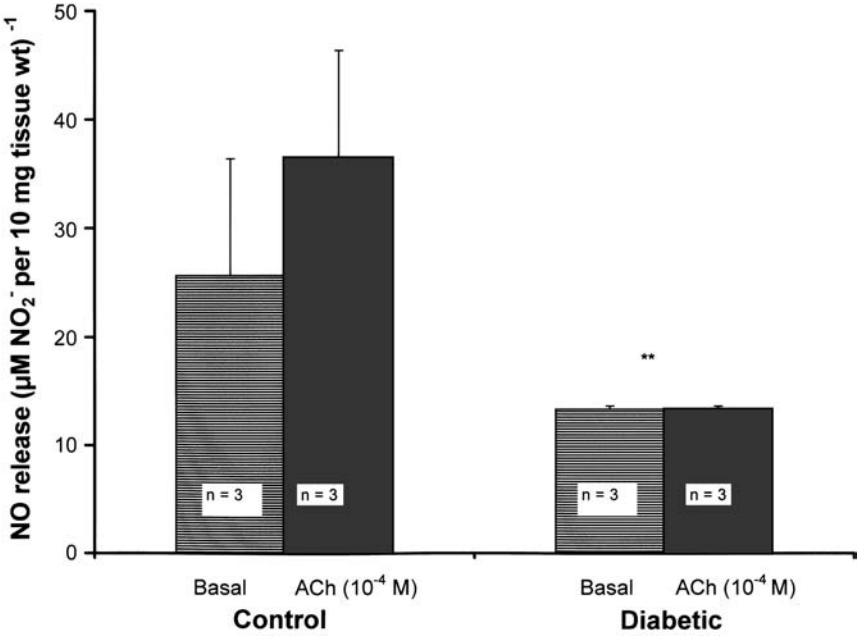

Figure 5. Release of NO from the gastric tissue fragments of rats 4 weeks after the onset of diabetes (n = 3). Basal NO release from age-matched control rats (n = 3) is shown for comparison. Note that acetylcholine (ACh) (10^{-4} M) induced a small but not significant increase in NO release from the gastric antrum of normal rats compared to basal. NO release was significantly (**$p < 0.001$) lower in diabetic rats (both basal and acetylcholine induced) when compared to that of age-matched controls. Data are the mean ± SD.

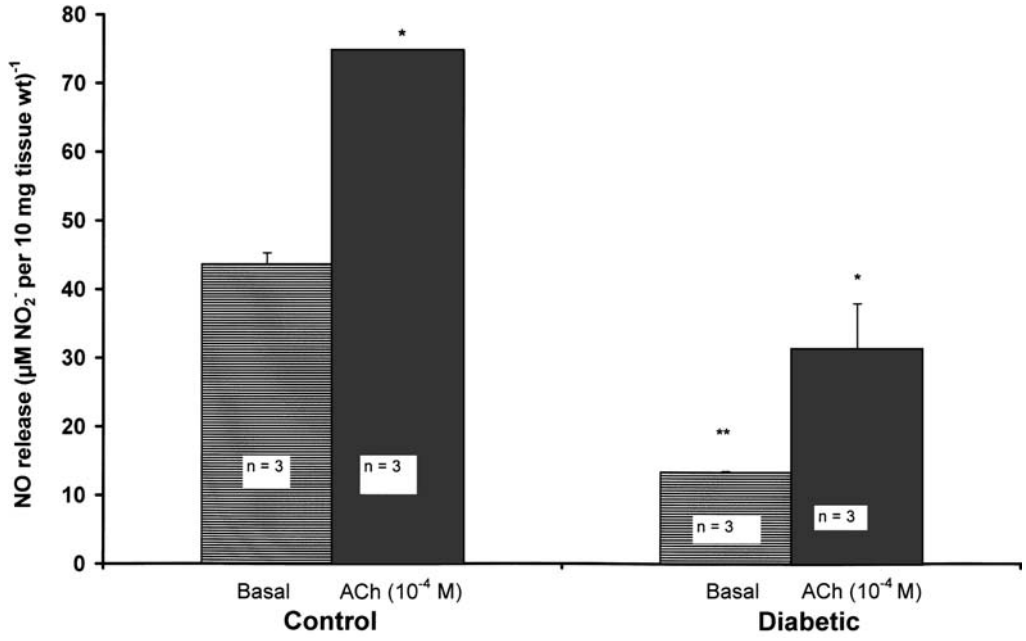

Figure 6. Histograms showing NO release from duodenal tissues of rats 4 weeks after the onset of diabetes (n = 3). NO release from age-matched control rats (n = 3) is shown for comparison. Acetylcholine (ACh) (10^{-4} M) evoked a large and significant (*$p < 0.05$) increase in NO release from the duodenal tissue fragments of normal and diabetic rats compared to basal. NO release was significantly (**$p < 0.001$) lower in diabetic rats (both basal and acetylcholine-induced) when compared to that of age-matched controls. Data are the mean ± SD.

of constitutive NO from endothelial and neural cells [21], the impaired Ca^{2+} signaling may contribute to the failure of acetylcholine to stimulate NO release from the gastroduodenum of diabetic rats. Why the response of the duodenal segment to acetylcholine challenge resulted in a significant increase in NO release compared to the stomach is not known.

The tissue level of nNOS increased significantly in the stomach and duodenum of rats suffering from a short (4 weeks) and long (32 weeks) duration of diabetes mellitus. The ratio of the densitometric values of nNOS in the gastroduodenum of normal and diabetic rats was similar in both groups of rats. Our observation is in contrast to that reported by Takahashi et al. [22] in the BB/W diabetic rat. They observed that nNOS was significantly reduced in insulin-treated BB/W diabetic rats. In another similar experiment, a partial depletion of nNOS was observed in the gastric fundus of non-obese diabetic mice [23]. The difference between the literature reports and ours may originate from the animal model of diabetes used in these studies. Moreover, the increase in the level of nNOS in the gastroduodenum may be attributed to the toxic effect of STZ, since NO production is increased in STZ-induced oxidative stress [24].

In conclusion, the gastroduodenal segment of STZ-diabetic rats contained a significantly higher tissue level of nNOS. This increase in tissue nNOS may play a role in the pathogenesis of abnormal gastroduodenal function observed in diabetic patients.

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