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ANALYSIS OF IMMEDIATE *EX VIVO* RELEASE OF NITRIC OXIDE FROM HUMAN COLONIC MUCOSA IN GASTROINTESTINALLY MEDIATED ALLERGY, INFLAMMATORY BOWEL DISEASE AND CONTROLS

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Nitric oxide (NO) is a local mediator in inflammation and allergy. The aim of this study was to investigate whether live incubated colorectal mucosal tissue shows a direct NO response *ex vivo* to nonspecific and specific immunological stimuli and whether there are disease-specific differences between allergic and chronic inflammatory bowel disease (IBD). We took biopsies (n=188) from 17 patients with confirmed gastrointestinally mediated food allergy, six patients with inflammatory bowel disease, and six control patients. To detect NO we employed an NO probe (WPI GmbH, Berlin, Germany) that upon stimulation with nonspecific toxins (ethanol, acetic acid, lipopolysaccharides), histamine (10^{-8} - 10^{-4} M), and immune-specific stimuli (anti-IgE, anti-IgG, known food allergens) directly determined NO production during mucosal oxygenation. Non-immune stimulation of the colorectal mucosa with calcium ionophore (A23187), acetic acid, and ethanol induced a significant NO release in all groups and all biopsies. Whereas, immune-specific stimulation with allergens or anti-human IgE or -IgG antibodies did not produce significant release of NO in controls or IBD. Incubation with anti-human IgE antibodies or allergens produced a ninefold increase in histamine release in gastrointestinally mediated allergy ($p<0.001$), but anti-human IgE antibodies induced NO release in only 18% of the allergy patients. Histamine release in response to allergens or anti-human IgE antibodies did not correlate with NO release ($r^2=0.11$, $p=0.28$). These data show that nonspecific calcium-dependent and toxic mechanisms induce NO release in response to a nonspecific inflammatory signal. In contrast, mechanisms underlying immune-specific stimuli do not induce NO production immediately.

Key words: nitric oxide, inflammatory bowel disease, allergy, colorectal mucosa, immunoglobulin E, tumor necrosis factor- α

INTRODUCTION

In 1980 Furchgott and Zawadzki discovered that nitric oxide (NO) is an "endothelium-derived relaxing factor" (1). Many cells with significant immunological and inflammatory roles synthesize NO, including fibroblasts, endothelial and epithelial cells and chondrocytes (1-6), monocytes and macrophages (7-9), antigen-presenting cells (10), natural killer cells (11), eosinophils (12), and mast cells (13-16). In the NO synthase family, NO synthase-1 (neuronal NOS) and NO synthase-3 (endothelial NOS) are always expressed whereas NO synthase-2 (NOS-2, or inducible iNOS) contributes significantly to the production of NO during immunological and inflammatory processes (3-6). NOS-2 is induced by bacterial lipopolysaccharide (LPS) or the classic proinflammatory cytokines (IL-1, TNF- α , IFN- γ) (17, 18) and regulated by a calcium-/calmodulin-dependent protein kinase (19). Several hours are required for mRNA and protein synthesis between cell activation and NOS-2-induced production of NO (18, 20). A similar effect has recently been shown by colonic tissue samples in rats (21).

In chronic inflammatory bowel disease (IBD) and gastrointestinally mediated allergy (GMA), increased amounts of histamine are secreted in the intestines; here, mast cell accumulation has also been reported and the cells show signs of activation. Therefore, in this project we investigated: 1) whether NO production can be detected *ex vivo* in human tissue biopsies from patients with these diseases; 2) whether it is possible to nonspecifically or specifically stimulate mast cells and other tissue cells to directly release NO; and 3) whether a possible rapidly occurring NO release might even be diagnostically useful to identify causally effective allergens in intestinal biopsy tissue.

MATERIALS AND METHODS

Patients selection

All patients gave written informed consent for the biopsy study. The project was approved by the local ethics committee (No. 330) and performed in accordance with the Helsinki declaration.

Exclusion criteria included medication (systemically or locally) with corticosteroids, 5-aminosalicylic acid, immunosuppressants, β -receptor antagonists or the presence of a neoplasia.

Patients from three different groups were examined.

Gastrointestinally mediated allergy: 17 patients with GMA (n=102 biopsies) in whom the final diagnosis was confirmed by clinical parameters (history, skin tests, food antigen specific IgE in serum/intestinal lavage fluid), histology (eosinophilic and mast cell infiltration), and prior double-blind, placebo-controlled oral food provocation testing as the gold standard (22).

Inflammatory bowel disease: 6 patients with chronic IBD (n=46 biopsies) in whom diagnoses of Crohn's disease or ulcerative colitis had previously already been confirmed clinically, endoscopically, and histologically and the patients were not taking any medication at the time of colonoscopy. The biopsies were taken from noninflamed areas.

Control patients: biopsies from six healthy control subjects (n=40 biopsies) that were taken at colonoscopic screening examinations.

Biopsy procedure

After sufficient peroral cleansing of the bowel (KLEAN-PREP, Norgine GmbH, Marburg, Germany) the human tissue biopsies were taken during colonoscopic examination under normal coagulation conditions. In order to avoid any artificial irritation of the bowel, the biopsy forceps were rinsed in saline after coming into contact with formaldehyde solution. Mucosal specimens were taken from the following sites in the lower gastrointestinal tract: terminal ileum, ascending colon, transverse colon, descending colon, sigma, and rectum.

Mucosal oxygenation

To protect the mucosal specimens from ischemic tissue damage, they were stored in a portable mucosal oxygenator directly after they were taken by endoscopy (Fa. Intestino-Diagnostics GmbH, Erlangen, Germany) (22, 23). Biopsy tissues were incubated live and maintained in physiological culture medium at 37°C, pH 7.0, and pO_2 80 mmHg, and flushed with atmospheric oxygen. On average 6 biopsies per patient (range 4-10 biopsies) were taken from throughout the entire lower gastrointestinal tract and divided up into two test tubes containing incubation medium for the transport and until biopsies were processed for the stimulation.

For mediator stimulation tests (NO, histamine) one biopsy was transferred from the transport vial to the mucosal oxygenation culture with the installed NO probe. Only in cases without detectable NO production within 10 minutes, further stimulation experiments were possible at the same biopsy. Up to 4 stimulation series were done in some series with one vital biopsy.

Thus, with increasing experience about positive stimuli, we first applied unknown stimuli, and in the case of negative NO response within 10 minutes, further tests were possible and the positive stimuli were tested at the end to assure reactive vital tissue cells. The additional NO production compared with the baseline level without stimulus is given as the increase of NO over baseline and is expressed as Δ NO production.

Nitric oxide probe

NO was measured by using a thin, rod-shaped NO probe (ISO-NOP 2.0 mm, World Precision Instruments, Berlin, Germany) that could be inserted into the culture test tubes. Compared to other methods of determining NO by measuring its stable endproducts (nitrite and nitrate), this NO probe detected the actual NO and could record the NO levels with the aid of a

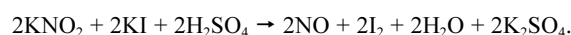
computer in real time (Software Duo 18, World Precision Instruments, Berlin (24)). The NO is detected by a highly selective, gas-permeable teflon membrane. The measurement is based on an electrochemical response. NO diffuses through the gas-permeable membrane and is oxidized on an electrode inside the probe. In this way a redox-flow is created that is proportional to the amount of NO in the probe, according to the following equation:



The probe reacts very sensitively to temperature and touch. Therefore, it was already installed an hour prior to stimulation in the experimental set up (compare Fig. 3) so that it could adapt to 37°C.

The setup was calibrated before each measurement (24). During the actual measurement the probe is firmly installed in a tube. In liquid medium the detection limit is 1 nM NO. For calibration the probe was installed in strong saline solution (1 molar, 37°C) in order to determine whether the curve was stable, thus indicating that the membrane is intact. Afterwards, it was placed in a tube filled with 10 ml calibration solution #1 (made up of: 0.1 MH_2SO_4 + 0.1 M KI, 37°C) which contained a small magnet and an oxygen tube to aerate the solution with ambient air.

The solution is aerated with ambient air and then stirred with the magnetic stirrer to mimic conditions identical to those that will exist for the measurements during mucosal oxygenation. As soon as the NO curve stabilizes again, 50 μ l, 100 μ l, and 200 μ l calibration solution #2 (made up of 50 μ M KNO_3) are pipetted into the solution. NO is then released according to the following equation:



After dilution, we calculated amounts of 249 nM, 493 nM, and 966 nM NO, respectively, for the additional amounts of calibration solution #2 pipetted into the solution. This curve is generated graphically on the computer with the current in pA (Fig. 1). The values measured are plotted against the known amounts of NO added during calibration and yield a straight calibration line. With respect to this line, any increases in values measured later can be converted to nM by the computer (Fig. 1). While the live biopsy tissues were being stimulated under mucosal oxygenation to detect NO, we also determined levels of histamine that were secreted in these samples (22).

Measuring fresh weight

Fresh weight was measured by using a highly sensitive analytical balance (Sartorius, Göttingen, Germany).

Incubation medium

As culture medium during mucosal oxygenation and while measuring NO, we used modified Hanks' solution (pH 6, volume 4 ml, without antibiotics or antimycotics), 2.5% Hepes buffer (Sigma, Munich, Germany), 1% fetal calf serum (Sigma, Munich), and 0.3% human albumin fraction V (Sigma, Munich) (22, 23). To prevent foam from developing as a result of continually aerating the tissue cultures with oxygen we added 40 μ l Simeticon (Endo-Paractol, Temmler Pharma, Marburg/Lahn, Germany) to each culture tube. The total volume of culture was 4 ml.

Stimulation of human tissue biopsies

To detect intact and viable cells in the tissue biopsies that could be stimulated, we used calcium ionophore (A23187, Sigma, Munich) as a positive control. Calcium ionophore

increases intracellular calcium, which induces NO release (19). In previous experiments we found a dose-dependent release of NO that was most significant at a concentration of 10^{-4} mol/l calcium ionophore. Furthermore, for a 4-h period of mucosal oxygenation, lactate dehydrogenase release, which would have been a sign of cell damage in the biopsies, was negligible.

The biopsy specimens were nonspecifically stimulated with substances known to be noxious, ethanol 96%, acetic acid 0.5, 1, 10%, endotoxin (LPS from *E. coli*, Sigma, Munich) and tested over a concentration range from 1, 10 and 100 ng/ml LPS.

As immunospecific stimuli we employed goat anti-human IgE and goat anti-human IgG (Sigma, Munich) at concentrations of 0.1, 1, 10, 100, 500 and 1000 μ g/ml and allergens already known from oral provocation testing (rye, fish, chicken egg, hazel nut, Allergopharma, Reinbeck, Germany) at concentrations of 0.1, 1 and 10 μ g/ml protein/ml (22, 23).

Histamine dihydrochloride (Sigma, Munich, Germany) was added exogenously to the cultures and tested at concentrations of 10^{-8} M to 10^{-4} M.

Histamine detection

At each of the time points 0, 15, 30, 60, 120, and 240 min during mucosal oxygenation, we extracted 400 μ l from the culture medium to determine the amounts of histamine in the cultures, as reported in previous publications (22, 23). At 60 min the extracted volume of culture medium was added again with fresh solution containing the stimulant to make 4 ml. The dilution factor for calculation of the actual mediator concentration was included in the computer software. The histamine content was measured in the incubation supernatant by enzyme immunosorbent assay (ELISA, Beckmann/Coulter, Krefeld, Germany).

Griess reagent

As a complementary, second means of determining NO, we employed the Griess reagent system (Promega, Madison, WI, USA) (25). Here, NO is measured *via* one of its stable endproducts, NO_2^- (nitrite). The underlying principle is a chemical reaction in

which sulfanilamide and N-1-naphthylethylenediamine dihydrochloride in an acidic environment react to form an azole compound that, according to the manufacturer's instructions, can be photometrically measured at wavelengths of 520-550 nm (detection limit $\geq 2.5 \mu\text{M}$ (25-27)). A reference curve is created for the corresponding incubation medium of 0-1.56, 3.13, 6.25, 12.5, 25, 50 and 100 μM nitrite standard for each measurement during mucosal oxygenation. To each of the experimental probes undergoing mucosal oxygenation and each of the reference curves, 50 μ l of sulfanilamide solution is added under light-protected conditions at room temperature and incubated for 5-10 min. Then, 50 μ l of N-1-naphthylethylenediamine dihydrochloride is added and at the end of the incubation period at 30 min, absorption is measured at 520-550 nm (most staining pink/magenta was already observed after 5-10 min). The deviation of the measured values from the accompanying standard curve was $<3\%$.

Statistical methods

Descriptive statistics of the resulting data were evaluated by using Microsoft Excel XP (mean $\pm$ standard deviation). The statistical significance, data comparability, and linear regression were computed for these data using the statistical software program Graph Pad Prism 3.0 and rendered graphically.

As two independent samples with two dichotomous or two-categorical features were statistically investigated in the test situations of the present study, we could work with a four-field distribution. Fisher's exact test was used as a special form of the chi-squared test. A significance level of $\alpha=0.05$ was selected and we considered p values of <0.05 to be statistically significant.

RESULTS

Calibrating and adapting nitric oxide measurements to the tissue culture during mucosal oxygenation

As recommended by the manufacturer, the NO measurements were calibrated every day before conducting the experiments

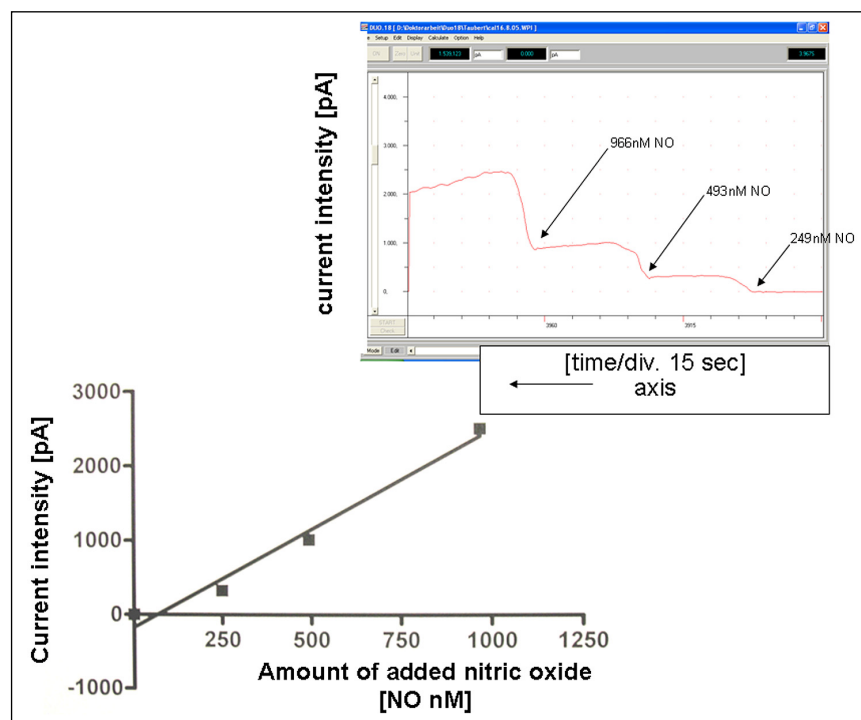


Fig. 1. Calibration and standardization curve for NO measurement adapted for the use of live colorectal tissue samples during mucosal oxygenation. The left pointing arrow under the x axis in the upper figure at the right top shows the direction of the real-time monitoring of the redox current, which is given by the computer software during on line registration in opposite direction from right to the left side (24). During on line registration of the NO induced redox current (pA) the picture shows at the right side the earlier time points when the stimulation has been started. By use of the calibration standards (figure at the left bottom) the changes of the registered redox current (pA) are calculated by the computer software to nM NO.

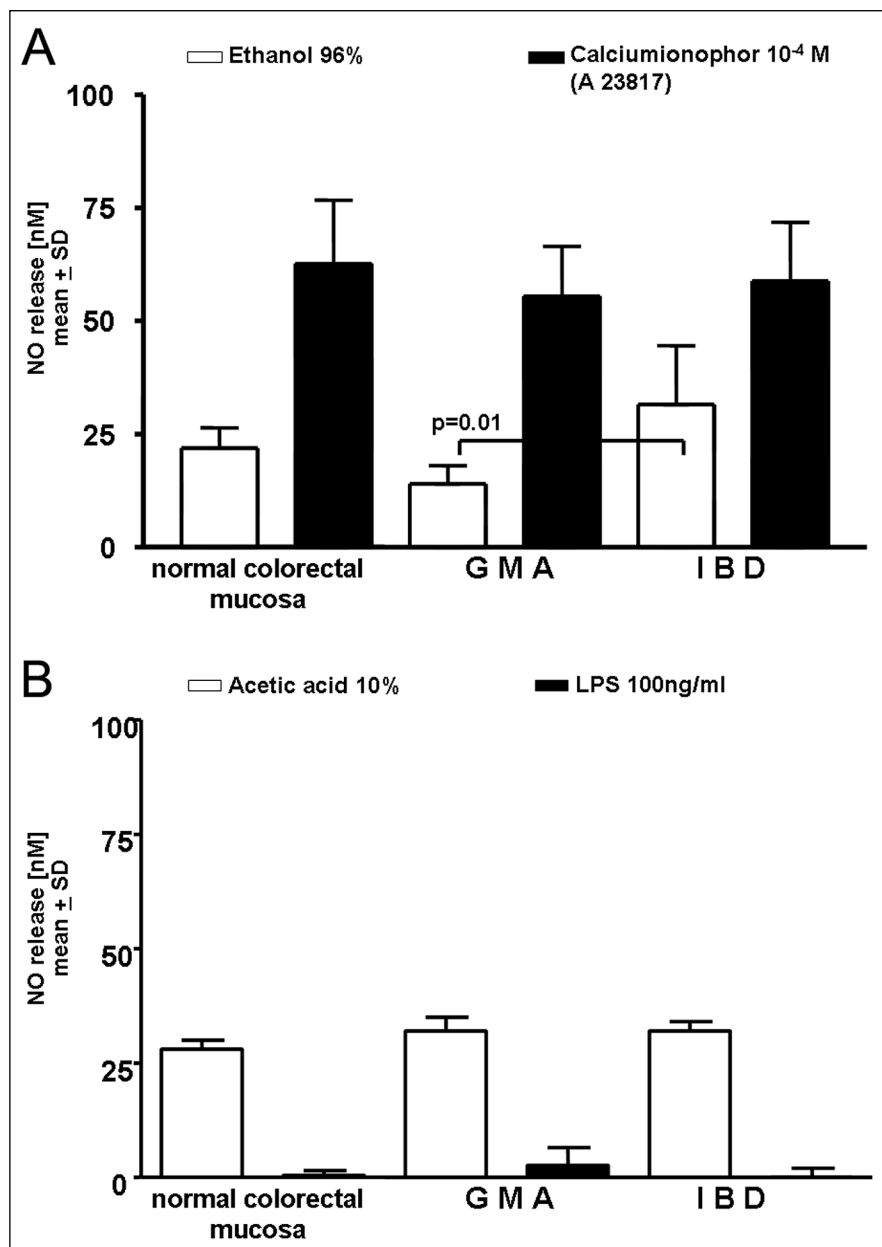


Fig. 2A-B. NO release from human colorectal mucosal tissue during mucosal oxygenation in response to (2A) calcium-dependent tissue cell stimulation by calcium ionophore A23187 and toxic injury by ethanol 96%, and (2B) toxic injury by acetic acid (10%) and endotoxin (LPS from *E. coli*; 100 ng/ml). Modified Hanks solution was used for mucosal oxygenation of vital colorectal samples 37°C, pH 7.0, and pO_2 80 mmHg (22, 23). Biopsies were incubated with different concentrations of stimulants and the immediate NO response was detected via a highly selective, gas-permeable teflon membrane of the NO electrode, measuring the redox current intensity online. After stabilization and calibration of the NO electrode the increase of NO production from baseline was calculated by computer software and the Δ NO release is shown in Figs. 2A and 2B. GMA - gastrointestinally mediated allergy; IBD - inflammatory bowel disease

(Fig. 1). The mean slope of the calibration curve at various test days was calculated and followed a mathematical equation of 2.62 ± 0.27 . The mean sensitivity was 2.62 ± 0.35 pA/nM. The probe oscillated around the zero value at ± 1.5 -2.9 nM on average between different test days, but these different baseline levels were without rapid dynamics, as shown for positive stimuli in figure 4 and 5, and were not recorded as NO production.

During mucosal oxygenation we demonstrated that the substances such as nitrite, nitrate, oxygen, culture components, etc. that were used did not have any adverse effect on the NO measurements (24, 26), i.e., no NO was detected when the tissue specimens were not being stimulated. However, living biopsies without stimulus did not produce significant NO amounts over baseline within 12 h. Over the course of 12 h, NO production was in the oxygenated biopsy tissue cultures ($n=12$ biopsies; time points for NO production 0 min $\Delta 15 \pm 20$ nM; after 12 h $\Delta 10 \pm 20$ nM), whereas in the nonoxygenated biopsies ($n=10$) already after 1 h $\Delta 90 \pm 20$ nM NO, and after 12 h $\Delta 100 \pm 50$ nM NO was being produced. Thus, we concluded that there are no other relevant redox currents in oxygenated biopsy cultures with

noninflamed tissue, but we did not prove it exactly. Theoretically other radical-producing mechanisms or electron reactions may occur within inflamed tissue, but to exclude this possibility we investigated only inflamed tissue.

Nitric oxide measurements using Griess reagent

NO was also determined with the NO probe in 14 biopsies from three control subjects using Griess reagent. Here, NO is measured via one of the stable end products (NO_2 -nitrite) (25, 27). The NO values only deviated from the standard curve of the Griess reagent by <3% of the reference curve. The test run using Griess reagent was thus correct and sensitive (measurement range 0, 2.5 and 100 μ M). The results achieved by using Griess reagent were qualitatively similar to those from the NO probe; however, the values measured by using the Griess method were higher because the amounts of NO are cumulatively determined from the total amount of nitrite that has developed over time. Upon stimulation with calcium ionophore or 96% ethanol, respectively, the corresponding nitrite production was 2.9 ± 0.6

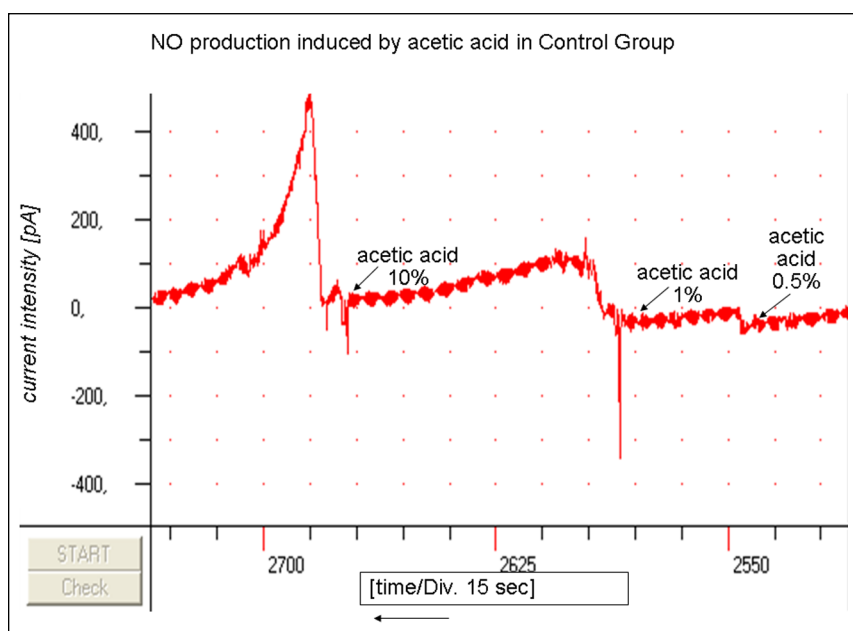


Fig. 3. NO production from normal colorectal tissue as demonstrated by detecting the redox current intensity in response to acetic acid during mucosal oxygenation. The left pointing arrow under the x axis in the upper figure at the right top shows the direction of the real-time monitoring of the redox current, which is given by the computer software during on line registration in opposite direction from right (earlier time points) to the left side (24).

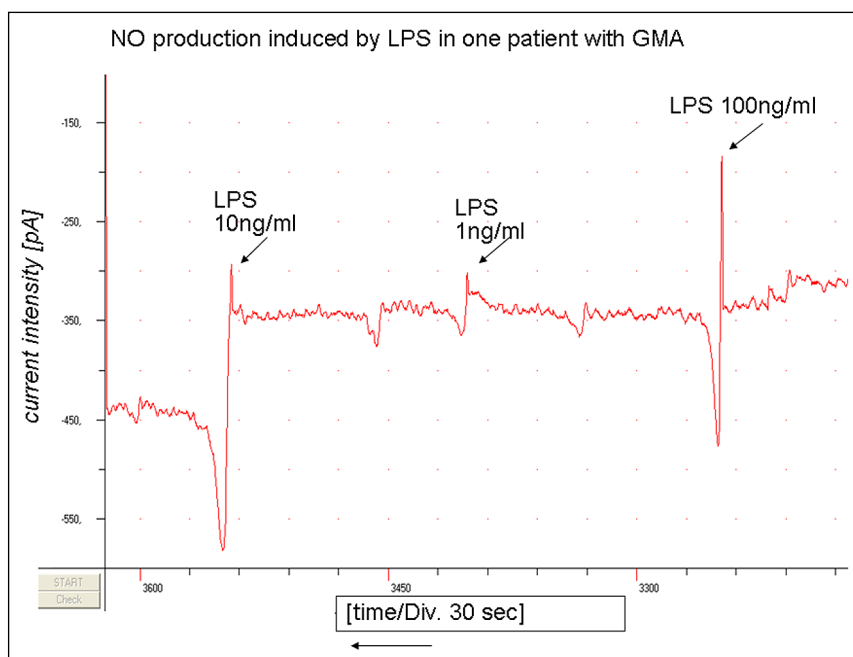


Fig. 4. Kinetics of dose-dependent NO production from live colorectal tissue as demonstrated by detecting the redox current intensity in one patient with GMA in response to different LPS concentrations. Only 1 GMA patient out of 4 (25%) showed release of NO in response to LPS. The left pointing arrow under the x axis in the upper figure at the right top shows the direction of the real-time monitoring of the redox current, which is given by the computer software during on line registration in opposite direction from right (earlier time points) to the left side (24).

μM ($n=5$) or $2.19 \pm 1.1 \mu\text{M}$ ($n=4$), respectively. If not stimulated, the NO, *i.e.*, nitrite production, was found to be $1.92 \pm 1.56 \mu\text{M}$ ($n=5$). Thus, calcium ionophore stimulation induced a greater increase of $\Delta 0.98 \mu\text{M}$ or 51% over baseline, while 96% ethanol induced only an increase of $\Delta 0.27 \mu\text{M}$ (14.1%), respectively.

From this analysis we concluded that Griess reagent (μM) is not as sensitive as the NO probe (nM) and differences between Griess reagent and NO probe are caused by the fact that Griess reagent measures the cumulative amount of NO *via* stable nitrite, while NO probe precisely detects the actual amount of NO (24, 27).

Fresh weight

The fresh weight of the 188 mucosal specimens examined ranged between 11.2 and 30.8 mg (mean 20.1 ± 10 mg). The differences in fresh weight of specimens from the three groups

examined were not statistically significant: GMA 18.8 ± 9 mg, IBD 20.4 ± 5 mg, controls 24.1 ± 7 mg fresh weight.

Nitric oxide production in the control group

Previous investigations at concentrations of 10^{-8} – 10^{-4}M calcium ionophore showed a dose-dependent stimulation of NO production in human colon biopsies. Here, a calcium ionophore concentration of 10^{-4} stimulated the highest NO production. The positive control with 10^{-4}M calcium ionophore produced an increase in NO to $\Delta 62.7 \pm 18$ nM in a total of eight measurements in all control patients ($n=6$ controls, 8 experiments, 100%). The 96% ethanol also induced a release of $\Delta 22 \pm 6$ nM NO in all biopsies from the 6 control patients and in 8 experiments (100%, Fig. 2A).

Dose-dependent NO responses to various concentrations of acetic acid are illustrated in Fig. 2B and 3 and were detected in

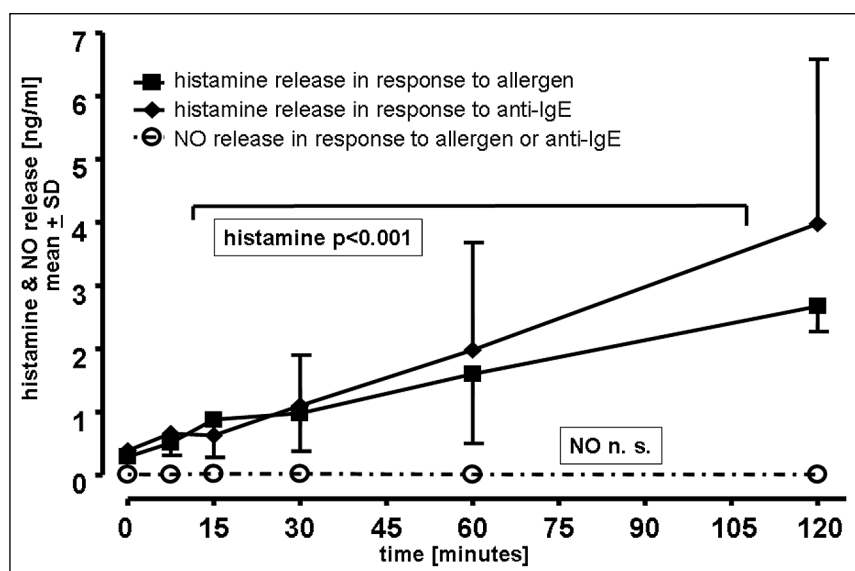


Fig. 5. Kinetics of anti-human IgE- and food allergen-induced histamine and NO release from human colorectal mucosa in patients with GMA.

all six patients, all biopsies in the control group (100%) and in the patient groups. The increase in NO production induced by 10% acetic acid in the controls was $\Delta 28 \pm 2$ nM.

In a further eight experiments each in eight biopsies from four patients, neither anti-human IgE nor anti-human IgG at concentrations ranging from 0.1, 1, 10, 100, 500 and 1000 $\mu\text{g/ml}$ induced the release of NO.

Likewise, stimulation experiments using exogenously added histamine at concentrations ranging from 10^{-8} to 10^{-4}M or LPS at 1, 10 and 100 ng/ml didn't induce any directly detectable NO production (0%) over a time period of 0 to 20 minutes in healthy colorectal mucosa.

Nitric oxide production in individuals with gastrointestinally mediated allergy

The 10^{-4}M calcium ionophore used as positive control induced a rapid increase in NO release in all 17 patients (100%; $n=34$ biopsies). Mean NO production in this group was $\Delta 55.45 \pm 11.1$ nM (Fig. 2). In five patients 96% ethanol was used for stimulation ($n=10$ biopsies) and a NO response of $\Delta 14.0 \pm 4.02$ nM was also observed in all biopsies and patients (100%; Fig. 2A). Again, the response to toxic ethanol stimulus was lower than for calcium ionophore.

The acetic acid (10%) induced an NO release of $\Delta 32 \pm 3$ nM ($n=6$ biopsies) in this patient group and in all biopsies (100%, Fig. 2B). We conducted 22 stimulation experiments in 22 biopsies from 11 patients by using anti-human IgE at concentrations ranging from 0.1, 1, 10, 100, 500 and 1000 $\mu\text{g/ml}$. In only four biopsies from two of the 11 tested patients (18%) there was an NO increase of 9.5 ± 4 nM. No NO production could be identified in the rest of the patients.

Closer analysis of the two NO-producing patients showed multiple, IgE-mediated allergies and an eosinophilic gastritis in one. In the other patient NSAID intolerance and a type IV nickel hypersensitivity were present. Upon multiple stimulation trials of the tissue biopsies by using anti-human IgE at concentrations of 500 and 1000 $\mu\text{g/ml}$, respectively, NO production of $\Delta 7.4 \pm 3.3$ nM and $\Delta 14.8 \pm 4$ nM was observed in both patients.

In another 18 tissue biopsies from individuals with allergies that were incubated with anti-human IgG at concentrations of 0.1-1, 10, 100, 500 and 1000 $\mu\text{g/ml}$, no NO release could be detected (0%).

In eight patients from the GMA group, we carried out 24 stimulations using different known causative food allergens

(corresponding to the results of double-blinded provocation tests (22, 23)). In contrast to the induced histamine secretion (see below), these allergens didn't induce any significant direct NO release in any of the allergy patients at any concentration in the range 0.1, 1 and 10 $\mu\text{g/ml}$ protein (0%).

Histamine at concentrations of 10^{-8}M to 10^{-4}M each was exogenously added to the tissue biopsies ($n=16$) from four patients and then incubated. A concentration-dependent NO response was detected in the 8 biopsies from two patients (50%) at relatively high histamine concentrations. At a histamine concentration of 10^{-4}M in culture medium, a moderate NO release of $\Delta 5.9 \pm 3$ nM was observed; at 10^{-3}M histamine this was $\Delta 29.5 \pm 20$ nM.

Upon LPS stimulation in four patients with GMA ($n=16$ biopsies), only four biopsies from one patient (25%) demonstrated a concentration-dependent NO response. The kinetics for this response is presented in Fig. 4, giving a mean NO production of $\Delta 3.5 \pm 3$ nM in the GMA group.

Nitric oxide production in individuals with inflammatory bowel disease

Calcium ionophore at 10^{-4}M produced a steady NO response of $\Delta 58.8 \pm 12$ nM in all six patients (100%, $n=15$ biopsies; Fig. 2). In 10 biopsies from five patients (100%), 96% ethanol also resulted in a steady NO increase of $\Delta 31.5 \pm 10$ nM. Here, the NO release was statistically significantly higher in IBD patients than in the allergy group ($p=0.01$), but not in the controls ($p=0.1$, Fig. 2A). Acetic acid 10% produced an increase in NO of $\Delta 32 \pm 2$ nM in a further six biopsies from all three patients with IBD who were tested (100%, Fig. 2B).

In six patients from the IBD group no NO release was detected in 10 additional stimulation experiments for anti-human IgE and anti-human IgG at concentration ranges from 0.1, 1, 10, 100, 500 and 1000 $\mu\text{g/ml}$. Stimulation with exogenously added histamine and with LPS ($n=6$ biopsies each) didn't induce an NO response either.

Simultaneous measurement of histamine and nitric oxide in gastrointestinally mediated allergy

Histamine and NO were measured simultaneously for 120 min in five patients from the allergy group ($n=13$ biopsies). Whereas histamine concentrations increased from 0.1 ± 0.07 to

Table 1. Qualitative summary of all stimulation experiments performed in patients with GMA (n=17 patients), IBD (n=6 patients), and normal healthy colorectal mucosa (control group, n=6 patients); +++ NO release in all experiments (100%); + NO release in $\geq 50\%$; (+) NO release in $\leq 25\%$; - no NO release. (LPS) lipopolysaccharide (*E. coli*); anti-human IgE goat anti-human IgE antibody; anti-human IgG goat anti-human IgG antibody.

	Control group	G M A	I B D
Number patients	n=6	n=17	n=6
Number biopsies	n=40	n=102	n=46
Biopsies/patient	6.6	6.0	7.6
Calcium-Ionophore (A 23817)	+++	+++	+++
Ethanol	+++	+++	+++
Acetic acid	+++	+++	+++
LPS	-	(+)	-
Histamine	-	+	-
Food allergens	-	-	-
Anti-human IgE	-	(+)	-
Anti-human IgG	-	-	-

0.39 $\pm$ 0.3 ng/ml within 120 min when the culture medium was not stimulated (28), a steep allergen-induced increase in histamine secretion from 0.29 $\pm$ 0.04 ng/ml to 2.67 $\pm$ 0.48 ng/ml was observed during mucosal oxygenation (9.2-fold increase, $p < 0.001$, Fig. 5). With anti-human IgE (500 μ g/ml) a statistically significant increase in histamine release from 0.39 $\pm$ 0.09 ng/ml to 3.89 $\pm$ 2.68 ng/ml was observed (9.9-fold increase, $p < 0.001$, Fig. 5).

Interestingly, these results for histamine release did not correlate with those from the NO measurements: in the same stimulation experiment no direct (<20 min) or delayed (20-120 min) NO release could be detected (no increases, Fig. 5). Nor was a significant correlation between histamine secretion and NO production observed ($r^2=0.11$, $p=0.28$).

Qualitative summary of nitric oxide production in human colorectal biopsies

Table 1 presents a qualitative summary of the experimental results for NO production in the live human colorectal biopsy tissue.

DISCUSSION

NO regulates vascular permeability and mucosal barriers in the colon and mediates, according to the organ, tissue, or species being examined, highly varying effects in immunological and nonimmunological cells (3-6, 29). Because of its short half-life NO can mediate these pro- and anti-inflammatory effects relatively rapidly for a few seconds up to minutes, depending on the individual microenvironment and concentration.

Independent of the source *in vivo*, it is highly likely that this molecule also plays an important local, tissue-specific immunomodulatory role, for example, by selectively suppressing mast cell activation (or more selectively) histamine secretion and thus reducing the damaging effects of these cells and excessive histamine secretion in the surrounding tissue (13, 30).

The presence of proinflammatory mast cell mediators in the intestines has been frequently reported for IBD and GMA (31, 32). However, except for indirect immunohistochemical findings of NO synthase, no studies have been conducted to determine whether NO can be directly detected as a mediator in live human colorectal tissue, whether NO release as a mast cell inhibitory signal is lacking in IBD, and which signals in human mucosa stimulate NO production.

Therefore, we applied a computer-assisted procedure to dynamically measure NO *via* an electrode during mucosal oxygenation. In this way human colorectal biopsy tissues can be maintained in live condition by using physiological incubation. Furthermore, this method has been used for diagnosing gut mucosal allergic reactions and the secretion of histamine, tryptase, or tumor necrosis factor (TNF)- α upon allergen-specific stimulation or by anti-human IgE antibodies can be followed over time (22, 23, 31). Over a 4 h period no nonspecific lactate dehydrogenase was released as a sign of increasing cell damage. By using this method, we can also diagnostically quantify allergen-induced histamine or mast cell secretion in live human colorectal biopsy tissue, which has previously been reported to strictly correlate with a change in transepithelial resistance (23). In this *ex vivo* system dynamic NO production could be determined more quickly and more clearly *via* the redox flow by the NO probe, than by determining nitrite using Griess reagent (27).

The limitations of the measurements using the probe are related to the fact that only short-term (immediate) NO release can be measured on the electrode *via* redox flow (time interval 90-180 s), whereas Griess reagent can determine the total amount of stable nitrite that has accumulated at a defined time point as an indirect parameter for NO production. As can be seen in Figs. 1 and 3, reproducible NO calibration curves could be achieved during mucosal oxygenation of human colorectal biopsy tissue that, after adding a suitable stimulus for NO release, produced a rapidly visible redox current or changes in NO, which for calcium ionophore and histamine were concentration dependent. Thus, according to the aforementioned aims of this study, colorectal mucosal biopsies were found to produce significant amounts of NO and may thus be used for NO investigation from human gut.

Calcium ionophore induced a pronounced, direct NO response in both healthy and diseased colorectal mucosa (Fig. 2A). This shows that during mucosal oxygenation the live incubated biopsy tissue that is composed of very heterogeneous types of cells can synthesize calcium-dependent mediators and dynamic NO secretion when intracellular calcium levels have been increased.

Under these conditions, calcium ionophore-induced NO production shows rapid kinetics and a maximum NO peak within 90 to 180 s. While NO release *via* calcium ionophore proved to be equally high in all the disease groups examined, the addition of nonspecific, toxic stimuli like 96% ethanol or acetic acid 10%, also directly induced NO production; however, it was about 50% lower in both cases (Fig. 2A, 2B).

A significant difference in NO production induced by 96% ethanol was observed between GMA and IBD. Because NO is directly measured *via* the NO electrode within a few minutes of starting the experiment, it seems obvious that the NO that is measured was synthesized by the constitutively expressed NO synthases (eNOS or nNOS): no time is required to induce them as they are always present.

This explains why all patients show a nonspecific immune NO response to ethanol and acetic acid. The quantitatively significantly higher release of NO in response to ethanol in the IBD than in the allergy group can possibly be explained by the fact that the number and densities of immune cells and NO producers in the tissue are different in IBD (*e.g.*, macrophages,

lymphocytes) and food allergies (mast cells, eosinophils) and are differently preactivated (IL-23, IL-17, IL-12 *etc.* versus IL-13, IL-5, IL-4 *etc.*) and/or regulated, that is, cells expressing iNOS in the tissue may already be present in IBD (*e.g.*, macrophages) (4, 5, 33, 34). In addition, as has been recently pointed out by Konturek *et al.*, physical and psychological stress may have a further impact on *in vivo* function of immune effector cells like mast cells, which constitute an important effector cell population of the brain-gut axis and differences in afferent or efferent neuroimmunologic translation of stress to the gut may further explain different levels of *in vivo* iNOS activity (4, 5, 35).

Whereas a short-term NO response couldn't be induced in control subjects or IBD patients either through anti-human IgE or anti-human IgG antibodies, food allergens, or histamine and LPS, heterogeneous reactions to anti-human IgE antibodies (18%) and LPS (25%) were observed in the allergy group. That could speak for the presence of certain immunological or inflammatory subpopulations in GMA, in which different types of allergies, different allergens, different dominant types of immune cells, various phases of mediator secretion (early versus late phase reaction), various paths of mediator degradation have been described (12, 14-16, 30).

As seen by the detailed clinical analysis of the two GMA patients who responded to anti-human IgE antibodies with distinct NO production, these two patients suffered from numerous allergies and, accordingly, activated eosinophilic granulocytes were present. Thus, in addition to the possible presence/existence of iNOS expression in these patients already before taking the biopsy, NO production may have been stimulated by the low-affinity IgE eosinophilic receptors (12).

But apart from these two patients from the GMA group with complex polyvalent allergic diseases and eosinophil activation, all other NO stimulation results showed that an immediate NO release occurs only to nonspecific stimulation of mast cells and other tissue cells within human colorectal mucosa, but not to immune-specific stimuli like anti-human IgE, or -IgG and food allergens.

However, by using known food allergens and anti-human IgE antibodies, we observed an over ninefold increase in histamine secretion over a 120 min period in GMA tissues. This endogenous histamine release stood in complete contrast to NO production, however, because neither anti-human IgE antibodies nor the causally effective allergens, *via* the high-affinity mast cell IgE receptors, mediated immediate NO release in human colorectal mucosa, except in the two patients presented above. As histamine secretion correlates very closely with the release of mast cell tryptase in the biopsy tissue (32, 36), we presume that, although mast cells are activated *via* the high-affinity IgE-receptor I (Fc_ε RI) in the biopsy tissue, rapid production of NO didn't ensue in the human colorectal tissue at the anti-IgE concentrations tested. Hence, because allergens, as a specific signal for colorectal mucosal mast cells, could not induce a short-term NO response during mucosal oxygenation, our third aim of the study, to use NO as a rapid diagnostic parameter to identify causal allergens, must be rejected. Thus, NO release with its advantage to act as an immediate signal from vital tissue, cannot be used for intestinal allergy diagnostics. Therefore, gastrointestinal allergens must be diagnosed using other previously described mediators such as histamine, tryptase, TNF- α , or eosinophilic cationic protein and not *via* a NO sensitive electrode (22, 23, 31).

These results in humans are in a certain contrast to studies in murine mucosal mast cells in culture, where it was shown that upon allergenic or anti-IgE antibody stimulation the cells expressed the inducible NO synthase (iNOS) in the form of mRNA and protein and subsequently synthesized NO (14). In mouse models of asthma iNOS is also upregulated upon allergen stimulation with aerosol (37, 38).

There are several reasons why an allergen-induced NO production could not be reproduced in human colorectal mucosa in the present study. Firstly, time plays a role since in this study only immediate reactions within a few minutes (<10 min) after stimulating the tissue were considered, whereas in the aforementioned studies NO was measured in a period from 15 to 18 h after stimulation. Indeed, a certain amount of time is required to induce and synthesize iNOS (18, 20). Furthermore, the aforementioned models involve isolated animal mast cell lines and in our study human cells naturally embedded in tissues were examined. Thus, there may be decisive differences between animal and human mast cells as far as NO production is concerned. On the other hand, Berkman *et al.* (1997) showed in human lung epithelial cells that typical interleukins of the Th2 response like IL-4 and IL-13 inhibit *de novo* synthesis of iNOS, but IL-10 does not (39). Therefore, in addition to the source of NO synthesis (cNOS versus iNOS), species-specific, organ-specific, tissue-specific, as well as immunoregulatory, neurovegetative stress responses and local differences need to be considered in interpreting these NO results (9, 12, 30, 35, 39).

Histamine at high concentrations ($\geq 10^{-4}$ M) added exogenously to the biopsy tissue induced a concentration-dependent NO release in half of the allergy patients. In 1997 Mannaioni also observed that histamine increases NO release from mast cells (36). Since we know that NO can inhibit the release of histamine and leukotrienes from mast cells, this would indicate that, in the sense of negative feedback, NO has an inhibitory or regulatory effect on degranulating activated mast cells and thus could contribute to preventing excessive histamine secretion and to anaphylactic reactions (40).

In summary, in human colorectal tissue, release of NO shows a rapid response in response to nonspecific stimulation (calcium ionophore, ethanol, and acetic acid), and NO is produced through the activity of constitutively available NO synthases (isoenzymes). Upon stimulation with immune-specific stimuli or LPS (*via* Toll-like receptors) we could not detect a direct NO release in control subjects and IBD patients, while GMA showed somewhat mixed results in our model using dynamic NO measurement *via* a probe. Thus, further investigations need to be conducted over a longer incubation period to draw final conclusions about immune-specific stimuli.

Conflict of interests: None declared.

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