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Inducible nitric oxide synthase activity in colon biopsies from inflammatory areas: correlation with inflammation intensity in patients with ulcerative colitis but not with Crohn's disease

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Summary. Nitric oxide synthase (NOS) activities are responsible for the enzymatic conversion of L-arginine into NO and L-citrulline. Relatively low amounts of NO are produced in intestinal epithelial cells or are released from nerve endings. The effects of NO production are related to the maintenance of epithelial integrity and permeability. A pathological role of an increased NO production has been suggested to play a role in models of experimental colitis. In humans, NOS activity in colon mucosa from patients with ulcerative colitis is clearly increased when compared with the activity of the control group. In contrast, an increase of NOS activity in the colon mucosa from patients with Crohn's disease remains controversial. In the present work, we have measured NOS activity in colon biopsies originating from the control group ($n = 16$), from patients with ulcerative colitis ($n = 23$) and Crohn's disease ($n = 17$) using the radiochemical method of the conversion of L-[guanido-^{14}C] arginine into radioactive L-citrulline. In the control group, NOS activity was mainly of the inducible type (88% of total NOS activity) since it was characterised by its insensibility to the absence of calcium in the assay medium. In colon biopsies originating from patients with ulcerative colitis, inducible NOS activity was increased 3 fold ($p < 0.005$) and in patients with Crohn's disease, inducible NOS activity was increased 5 fold ($p < 0.005$). Correlations between NOS activity in colon biopsies and the intensity parameters of the disease i.e. Truelove index, endoscopic score and histological parameters were evidenced in patients with ulcerative colitis. In contrast, in patients with Crohn's disease, the high inducible NOS activity was not correlated with any intensity parameters of the disease. From these data, we concluded that although inducible NOS activity was increased several fold in colon biopsies originating from patients with both ulcerative colitis and Crohn's disease, a correlation between this activity and the severity of bowel inflammation was not found in either cases.

Keywords: Amino acids – Nitric oxide synthase – Colon biopsies – Ulcerative colitis – Crohn's disease

Introduction

Nitric oxide synthase (NOS) activities are responsible for the enzymatic transformation of L-arginine into nitric oxide (NO) and L-citrulline. Constitutive NOS are characterised by the production of low amounts of NO, and the dependency upon the presence of calcium for catalysis (Knowles and Moncada, 1994). Inducible NOS, an isoform of the enzyme, was mainly studied in macrophages (Hauschildt et al., 1990; Hevel et al., 1991). This isoform activity is not dependent upon the presence of calcium in the assay medium, and the expression of the enzyme appears to be increased by stimuli such as interferon-γ, lipopolysaccharide and tumor necrosis factor α (Lowenstein et al., 1993). Both constitutive and inducible NOS are characterised by a very high affinity for their L-arginine substrate (i.e. micromolar K_M).

In intestinal tract, NO can be produced in relatively low amounts from epithelial cells (M'Rabet-Touil et al., 1993) or from non-adrenergic non-cholinergic nerves (Bult et al., 1990). Effects of NO on the maintenance of intestinal epithelium integrity (MacKendrick et al., 1993; Miller et al., 1993) and permeability (Kubes, 1992; Kubes, 1993) have been reported. Furthermore, NO appears to modulate colonic secretion (Wilson et al., 1996; Stack et al., 1996) and intestinal motility (Colignano et al., 1992). Increased NO production has been described following lipopolysaccharide treatment and seems to be due to an increased inducible NOS expression in enterocytes (Tepperman et al., 1993) and colonocytes (Bouthton-Smith et al., 1994). A pathological role of NO has been suggested in animal models with experimental colitis (Ribbons et al., 1995; Seo et al., 1995; Mourelle et al., 1996). However, toxic NO effect would partly be explained by its capacity to react with reactive oxygen species to form strong oxidant like peroxynitrite (Beckman and Koppenol, 1996).

Although of unknown etiology, intestinal inflammatory diseases such as Crohn's disease or ulcerative colitis are characterised by uncontrolled inflammatory reactions which evolve through iterative upsurges. This inflammatory reaction leads to lesions in tissues and functional disturbances including both secretory and motility features. At the microscopic level, there is an infiltration of the injured tissues by numerous inflammatory cells: macrophages, lymphocytes, polynuclear neutrophiles. The biological mediators released by these activated cells could constitute the origin of the histological and functional anomalies observed during the upsurges. Several inflammatory mediators have been proposed including eicosanoids, platelet activating factor, proteolytic enzymes and oxygen radicals. NO by itself or through reactions with other oxygen species could play a role in the genesis of colon mucosa lesions. Indeed, an excess of NO could be cytotoxic to colonic

cells (Blachier et al., 1996) or could be implicated in functional alterations like digestive hypomotility (i.e. colectasy), mucosa vasodilatation, increase in intestinal permeability or digestive hypersecretion. However, it has been proposed that NO could play a protective role during active inflammation (Dijkstra et al., 1998; Perner and Rask-Madsen, 1999).

In humans, several reports recently described increases in citrulline production (Middleton et al., 1993), in luminal NO (Lundberg et al., 1994), in NOS activity (Boughton-Smith et al., 1993; Rachmilewitz et al., 1995), in NOS immunoreactivity (Singer et al., 1996; Leonard et al., 1998) and in NOS mRNA (Mc Laughlan et al., 1997; Zhang et al., 1998) in colon of patients suffering from ulcerative colitis and/or Crohn's disease. The increase of NOS activity in Crohn's disease appeared however to be controversial as recently reviewed (Guslandi, 1998). The aim of the present work was to measure both constitutive and inducible NOS activities in colon biopsies originating from a large number of patients with ulcerative colitis or Crohn's disease and to search for a possible correlation between NOS activities and severity of these inflammatory bowel diseases.

Material and methods

Subjects

Mucosal colon biopsies were routinely taken during coloscopy. Biopsies were taken from 40 patients suffering from inflammatory intestinal cryptogenetic diseases. 30 biopsies were taken from 23 patients suffering from ulcerative colitis (10 men and 13 women with an average age of 39 $\pm$ 2 years) and 19 biopsies were taken from 17 patients suffering from Crohn's disease (9 men and 8 women, with an average age of 35 $\pm$ 3 years). In the ulcerative colitis group, the severity of the illness, according to the Truelove score (i.e. a clinical score (Truelove and Witts, 1955; Edwards and Truelove, 1963)), was low in 9 cases, moderate in 13 cases and high in 3 cases. In 2 cases, biopsies were reitereted in patients in remission. In patients with Crohn' disease, 6 episodes were characterised by a Best score (i.e. a clinical score (Best et al., 1979)) less than 150, 11 by a Best score between 150 and 300 and 6 by a Best score higher than 300; several episodes were characterised in some patients. Crohn's disease and ulcerative colitis were diagnosed after clinical, endoscopic (i.e. modified Baron score for ulcerative colitis (Baron et al., 1964)), radiological and in some cases, histological examinations (i.e. altered epithelium differentiation, neutrophiles infiltration and cryptic abscess for ulcerative colitis (Truelove and Witts, 1955)). Corticosteroid treatment was already initiated at the time of coloscopy in 16 cases (10/30 for ulcerative colitis and 6/19 for Crohn's disease). The parameters of illness intensity were evaluated within a delay of 48 hours following the biopsies.

In all patients, the intensity of the inflammatory upsurge was evaluated on the basis of general clinical parameters (Truelove index for ulcerative colitis and Best score for Crohn's disease) and systemic biological parameters (sedimentation rate, orosomucoide, c-reactive peptide, total leucocytes and polynuclear neutrophils, blood platelets). In patients suffering from ulcerative colitis, the intensity of mucosal erosion was evaluated using local parameters: endoscopic (modified Baron score) and histological. Healthy mucosal samples (control group) were obtained from 16 control subjects (with an average age of 56 $\pm$ 4 years) with no digestive pathologies as evaluated from coloscopies made in a context of functional colopathy, and no parietal lesions.

Biopsies

Biopsies were taken from colonic segments with macroscopic lesions in active disease patients, and healing areas in patients in remission. In control subjects, biopsies were taken in the recto-sigmoid region. Biopsies were immediately frozen in liquid nitrogen and maintained at −80°C until measurement of NO-synthase activity.

NO-synthase activity determination

L-[guanido-^{14}C] arginine was purchased from New England Nuclear. 6-(R,S)-5, 6, 7, 8-tetrahydro-L-biopterin (BH_4) was obtained from Serva.

After sonication (3 × 5 sec at 4°C), homogenates of biopsies containing 0.2–0.6 mg proteins were incubated for 60 min at 37°C in 100 μL of a Tris/HCl buffer (100 mmol/L pH 7.2) containing 50 μmol/L-[guanido-^{14}C] arginine, 1 mmol/L dithiothreitol, 1 mmol/L NADPH, 10 μmol/L flavine adenine dinucleotide, 10 μmol/L flavine mononucleotide, 10 μmol/L 6-(R-S)-5, 6, 7, 8-tetrahydrobiopterine and 5 mmol/L-valine used as an arginase activity inhibitor (Hrabak et al., 1996).

The measurement of inducible NOS activity was performed using 5 mM EGTA in the incubation medium. Total NOS activities (i.e. constitutive and inducible activities) were measured in the presence of 1 mM $CaCl_2$ in the incubation medium. The constitutive activity was then calculated from the difference.

Radioactive L-citrulline was separated by HPLC using a C18 column (Kromasil, AIT, France) after O-phthaldialdehyde derivatization for 2 min. Buffers A and B were those described by Seiler and Knödgen (Seiler and Knödgen, 1985) with the following gradient: from 43.3 to 68.3% B for 20 minutes.

The production of L-citrulline was measured by reference to the specific activity of L-[guanido-^{14}C] arginine in the assay medium and expressed as pmol/mg proteins/h after determination of protein content by the Lowry procedure (Lowry et al., 1951).

Presentation of results and statistical methods

Results from NOS activities are expressed as the mean ± SEM, together with the number of patients. When several biopsies were taken from one patient, the data were pooled. The statistical significance of differences between NOS activities in patients vs the control group was assessed by the unpaired Student t test. Differences were calculated by the non-parametric Mann-Whitney test. Correlations were established by the Spearman non-parametric test.

Results

In control biopsies, total NOS activity represented 113 ± 28 pmol/mg proteins/h. This activity was mainly due to the inducible form of NOS since 88 ± 6% of the total activity was represented by this Ca^{2+}-independent activity (Fig. 1). In ulcerative colitis patients, the total NOS activity measured in colon biopsies increased more than 3 fold. This increase was mainly due to an increase in the inducible form of the enzyme activity. In patients with Crohn's disease, the total NOS activity was increased 5 times when compared with the data recorded in control patients. Once again, inducible NOS activity can account for this important increase since it represents 91 ± 5% of total NOS activity. Correlations between NOS activity in colon biopsies and the intensity parameters of the disease were evidenced in patients with ulcerative

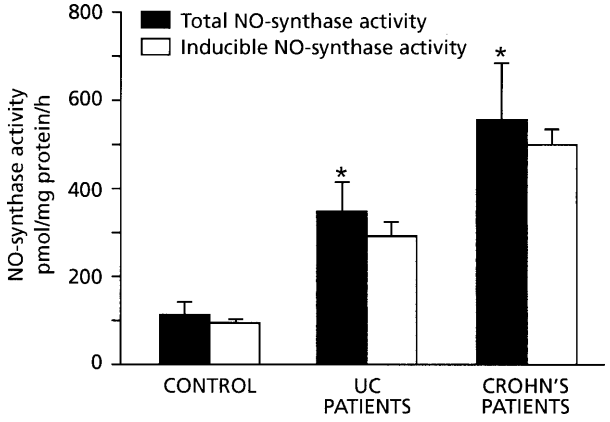

Fig. 1. Total (constitutive and inducible) and inducible NOS activities in colon biopsies originating from control subjects, ulcerative colitis patients and Crohn's patients. Total NOS activity was measured as the production of radioactive L-citrulline from 50 μmol/L-[guanido-^{14}C] arginine in the presence of cofactors and 1 mmol/L $CaCl_2$ as described in Material and methods. Inducible NOS activity was measured in the absence of $CaCl_2$ but in the presence of 5 mM EGTA used as a calcium chelator. Total NOS activity is given as mean value ± SEM, and is derived from respectively 16, 23 and 17 subjects in control, ulcerative colitis and Crohn's groups. *: $p < 0.005$ vs control

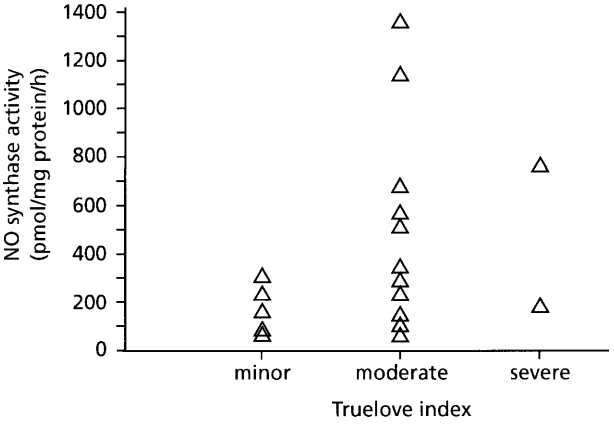

Fig. 2. NOS activity as a function of Truelove index in ulcerative colitis patients

colitis. Indeed, in this group, when the total NOS activities were compared with the Truelove indexes, the correlation coefficient was found to be equal to 0.5 with a p value equal to 0.02 (Fig. 2). When NOS activities were compared with the endoscopic scores, r was equal to 0.4 with a p value equal to 0.05 (Fig. 3). Finally, by comparison with histological parameters, r was found to be equal to 0.7 with a p value equal to 0.05 (Fig. 4).

In contrast, in patients with Crohn's disease, the high NOS activity was not correlated with any intensity parameters of the disease studied (data not shown).

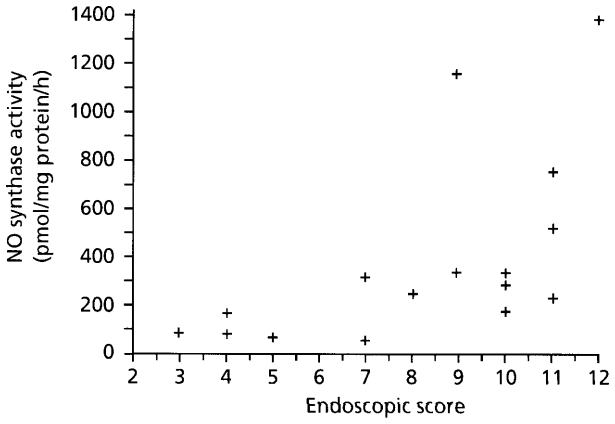

Fig. 3. NOS activity as a function of endoscopic score in ulcerative colitis patients

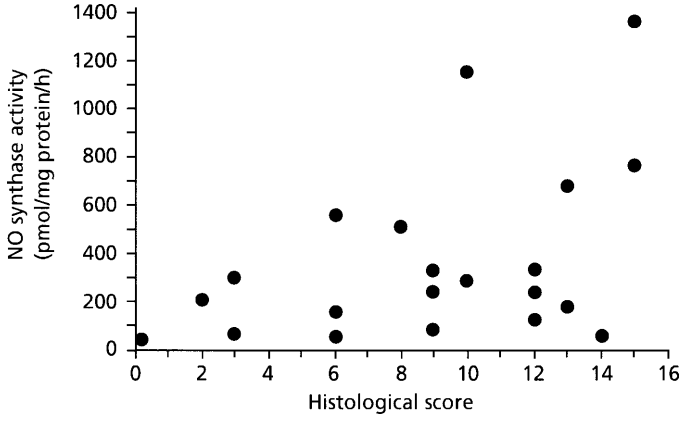

Fig. 4. NOS activity as a function of histological score in ulcerative colitis patients

Discussion

The data presented in the present work clearly showed a several fold increase in NOS activity in colon biopsies originating from patients with both ulcerative colitis and Crohn's disease. The methodology used allowed us to measure NOS activity in biopsies with as little as 0.2 mg proteins. The radiochemical methods used were based on the fact that radioactive L-arginine is labelled on the guanido carbon. Therefore, this carbon is lost in radioactive urea after arginase catalysis leading to unlabelled L-ornithine (Blachier et al., 1991). This point is very important since when using this technique, we were able to exclude the synthesis of radioactive L-citrulline through the stepwise conversion of L-arginine into L-ornithine (catalysed by arginase) and conversion of L-ornithine into L-citrulline (catalysed by ornithine carbamoyltransferase).

Our results confirm previous results for the increase of NOS activity observed in ulcerative colitis (Boughton-Smith et al., 1993; Rachmilewitz et al., 1995).

Furthermore, our data performed on a large number of biopsies demonstrate that biopsies from patients with Crohn's disease are characterised by an increase in NOS activity. Indeed, controversial data have been reported from two laboratories which found either no increase (Boughton-Smith et al., 1993) or an increase (Rachmilewitz et al., 1995) in NOS activity in patients with Crohn's disease. The increase in NOS activity was found to be mainly due to an increase in the inducible form of the enzyme. Interestingly, biopsies from controls were clearly characterised by NOS activity of the inducible type. This result can be compared to what is found in the normal human airway epithelium (Guo et al., 1995). These authors found a continuous expression of inducible NOS. Epithelial airway cells, like epithelial colon cells, are exposed to environmental antigens. Ca^2-independant NOS activity has already been detected in isolated intestinal absorptive cells using the pig as a model (M'Rabet-Touil et al., 1993).

In the present study, in patients with ulcerative colitis, a correlation was established between NOS activity in biopsies and inflammatory parameters such as local parameters (the macroscopic aspect of colon mucosa during endoscopy, polynuclear infiltration upon histological examination of colon mucosa). No correlation was however found with parameters of systemic inflammation. This latter point favors a local role of NO. Concerning patients with Crohn's disease, no correlation was found between NOS activity and clinical status. It should be noted, however, that fewer parameters were evaluated in patients with Crohn's disease than in patients with ulcerative colitis in which endoscopic and histological parameters were determined in addition to clinical status. Furthermore, Crohn's disease is more complex from an immunological point of view.

The present study did not allow the identification of the cell-type(s) which are responsible for the observed increase of NOS activity. Indeed, colon biopsies contain several cell-types including colonocytes, macrophages, neutrophils, etc. NOS activity which can be considered as a measurement of NO synthesis capacity does not take into account parameters such as the reaction of NO with other oxygen species (such as superoxide anion) or trapping of NO by hemoglobin (Beckman and Koppenol, 1996). With these reservations in mind, our results confirmed the increase of NOS activity in ulcerative colitis and Crohn's disease, involving the inducible form of this enzyme. We also report a correlation between the inducible NOS activity and intensity of the disease for ulcerative colitis but not for Crohn's disease.

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