ROLE OF OXIDATIVE STRESS IN MEDIATING INTESTINAL iNOS EXPRESSION

J. Markovs[1], N. Berzina[2], D. Svirina[1], S. Isajevs[1], G. Knipse[1]
Faculty of Medicine, University of Latvia[1]
Agency of Latvian University „Institute of Biology of Latvian University" [2]
darjasvirina@inbox.lv

ABSTRACT

This study investigated changes in the expression pattern of iNOS as well as morphological changes in the intestinal wall during chicken exposure to excess of ascorbic acid. In control animals iNOS expression have been identified primarily in villus enterocyte brush border, as well as in enteroendocrine cells, in mucosal macrophages and in myenteric neurons. In chicken intestinal mucosa during high-dose ascorbic acid administration the level of oxidative stress marker - malondialdehyde was increased. Oxidative stress upregulates iNOS expression by apoptotic enterocytes. This indicates that NO acts as a mediator of apoptosis. The subchronic ascorbic acid treatment caused an increase in the number of IEL and mucosal macrophages highly expressing NF-κB and iNOS. These results show that the excess ascorbic acid can upregulate the expression of iNOS in intestinal mucosa and suggest that this regulation is probably mediated by oxidative stress-induced NF-κB activation. There would be a high probability that excess nitric oxide alters neuroendocrine signaling in the small intestine during long-term oxidative stress. The results reported here reveal a tight relationship between oxidative stress and compensatory upregulation of iNOS in the gut mucosa, thus enhancing reactive nitrogen species generation and apoptosis.
KEY WORDS: ascorbic acid, oxidative stress, iNOS, caspase-3, serotonin.

INTRODUCTION

Ascorbic acid as an electron donor is a potent water-soluble antioxidant in animals. However, the excess of ascorbic acid cause an increase in intracellular reactive oxygen species. The small intestine which is the primary site for ascorbic acid absorption is at particular risk for ascorbic acid-induced oxidative stress. Oxidative stress alters neuronal and enteroendocrine signaling, leading to functional adaptation in the inflamed bowel (6). Nitric oxide (NO) a highly reactive, diffusible and unstable radical, is an important signaling molecule regulating the severity of inflammation. On the other hand, under normal circumstances NO contributes to blood pressure regulation, neuronal communication and immune defense. Synthesized in excessive amounts NO have many potentially toxic effects, which are mediated by its oxidation products (e.g. peroxynitrite). Production of NO from L-arginine is catalized by special enzymes NO synthases (NOS). There are 3 types of NOS : endothelial NOS - eNOS, neuronal NOS - nNOS and inducible NOS – iNOS. eNOS and nNOS are constitutive enzymes and are able to generate insignificant amount of NO, by the side of iNOS (8).

As yet there is no consensus to the role of high-output NO generated by iNOS in intestinal injury associated with oxidative stress. The aim of this study was to investigate changes in the expression pattern of iNOS as well as morphological changes in the intestinal wall during chicken exposure to excess of ascorbic acid.

METHODS

Animals were divided into two groups of 10 heads in each. Birds of the control group were fed a wheat/barley full-fed basal diet containing all necessary nutrients (ascorbic acid - 50mg/kg of diet). The chicken of the experimental group provided with the same basal diet plus ascorbic acid (10g/kg of diet) from hatching to 30 days.

Oxidative stress was measured as ileal mucosal malondialdehyde (MDA).

For histological examinations, 1 cm lengths of the intestinal samples (ileum) were taken and were fixed in 10% neutral buffered formalin. Paraffin-embedded tissue was cut in 4-micron-thick sections and was stained with haematoxylin and eosin and periodic acid–Schiff (PAS) reagent. Morphometric analysis of tissue was performed using a light microscope (Leica, Wetzlar, Germany) and Motic (Motic, China) coupled to an image analysis software (Image Pro-Plus or Motic Image respectively). Total area of gut mucosa in transverse section (TAMS) was measured. Mean intra-epithelial (IEL) count per 100 villous enterocytes was computed. Neuronal diameter was calculated using Image Pro-Plus analysis software.

According to an immunohistochemical method described elsewhere, tissue sections were stained for visualization of immunoreactive serotonin, active and nonactive caspase-3 and iNOS positive cells. A negative control without primary antibody was included in each staining run. The total number of immunopositive cells was counted and the final result was expressed as cells/mm^2. The number of enteroendocrine cells, enterocytes and macrophages staining positively with caspase-3 and iNOS, as well as the number of vesiculated serotonin-positive EC cells, shown as total number of cells/mm^2 in separate fields of the section, was determined by counting cells in the gut mucosa.

All statistics were performed using the program SPSS. Means and standard deviations and significance values were calculated. Results are presented as mean ± S.E.M. Statistical comparisons were performed using Student's t-test. Statistical significance was set at $p< 0.05$.

RESULTS AND DISCUSSION

iNOS expression within villus enterocyte microvilli at high constitutive level has been reported. The resulting spatially distributed localization of iNOS supports NO release into the intestinal lumen. This provides an efficient mechanism for nonspecific host defense. We speculate that iNOS in villus enterocyte brush border is continusly induced by antigens in the luminal environment. At the same time, cytoplasmic immunoreactivity for iNOS was most intense in enterocytes that are prone to become apoptotic next in the course of cellular turnover in the top of the villi. It has been found previously that NO provides antimicrobial oxids of nitrogen and at high concentrations can be also proapoptotic (3). The extrusion of apoptotic enterocytes into the intestinal lumen leads to enhanced permeability of the epithelial layer. As cell loss leaves a defect in the epithelium that ultimately has to be protected from bacterial invasion, we hypothesized that NO, secreted by apoptotic cells may provide short-term local defense against infection.

Caspase-3 staining of the ileum mucosa showed that a few apoptotic cells were present in villus tip in control animals and statistically significant increase in the number of caspase-3 positive apoptotic enterocytes in experimental group was observed with a significant localization of apoptotic cells in the upper third of the intestinal villi (4.4 ± 1.3 versus 10.4 ± 1.4 cells/mm^2, $p=0.009$) (Fig. 1).

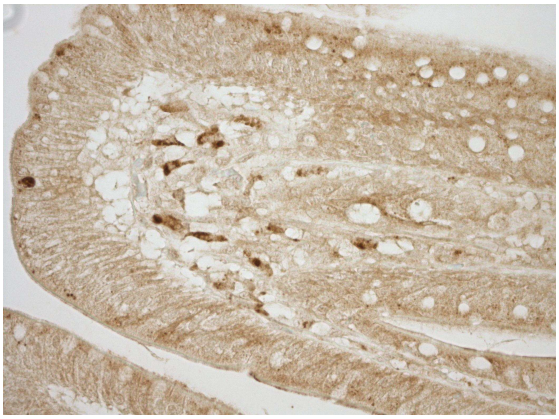

Figure 1.**Caspase-3-positive staining in apoptotic enterocytes and macrophages of experimental animals**.

All of the above-mentioned changes could be attributed to an increased production of reactive oxygen species and reactive nitrogen species. Because these species are potent oxidizing agents, they can destroy cellular antioxidant defense mechanisms, resulting in lipid peroxidation, apoptosis and inhibition of cell proliferation (1). Hence oxidative and nitrosative stress when they work together, may account for observed delayed gut maturation in the ascorbic acid-treated chickens.

Tissue level of malondialdehyde was 40 percent higher in experimental animals when compared with control group.

The mean IEL count per 100 enterocytes was 19 in control animals. The mucosa of experimental animals revealed intra-epithelial lymphocytosis, with 24 lymphocytes per 100 enterocytes ($p<0.05$). From these results it is concluded that high-dose ascorbic acid administration leads to the local immune activation, possibly by increasing the epithelial permeability to luminal bacteria.

Moreover the number of iNOS expressing intestinal macrophages increased in experimental animals, but the results were statistically insignificant. This could be due to macrophage activation in response to bacteria. Subchronic high dose ascorbic acid consumption also leads to an increase in the number of caspase-3 positive mucosal macrophages. It may be that one mechanism by which the loss of mucosal macrophages may occur is by a proportion of activated macrophages continuously undergoing apoptosis in the event of microbial phagocytosis (9). It has shown earlier, that macrophages have an increased susceptibility to NO-mediated apoptosis. Our results demonstrated high expression of iNOS in mucosal macrophages, also implying that nitrosative stress may play a role in the macrophage apoptosis during the phagocytosis of bacteria. Ascorbic acid induced stimulation of iNOS and NF-κB expression in mucosal macrophages has also been detected. These findings are in agreement with recently reported role of NF-κB in mediation of iNOS expression (5).

Results from iNOS immunostaning showed that changes in epithelial iNOS activity after excess ascorbic acid exposure occurred mainly in the enteroendocrine cells rather than in the absorptive cells. Serotonin-secreting EC cells are regarded as the predominant neuroendocrine cells of the bowel (6). It is well known that serotonin is a powerful vasoconstrictor. Since the NO is secreted together with the serotonin we speculate that NO improve the mucosal blood flow and ameliorate the serotonin bioavailability. In experimental group the number of serotonin-positive EC cells with vesiculated cytoplasm increased (78.90 cells/mm^2) as compared with control group (12.40 cells/mm^2, $p<0.05$**)** These findings can be explained by the stimulation of piecemeal degranulation of EC-cells (2).

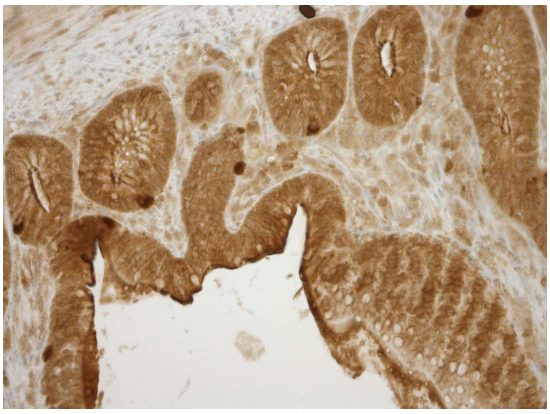

Figure 2. **iNOS immunohistochemical staining is clearly positive in brush border of villus enterocytes and enteroendocrine cells in the control animals**.

This may be due to luminal acidification with ascorbic acid. As has been shown earlier, intestinal luminal acid stimulates serotonin release from EC cells (11). It is known that oxidative stress can give rise to hypertension by superoxide interacting with NO, thereby decreasing NO availability for smooth muscle relaxation function (7). This in turn, led to compensatory up regulation of iNOS in enteroendocrine cells during long term exposure to high-dose ascorbic acid (Fig.2). Consequently, chronic exposure to excess NO may reduce the modulatory effects of serotonin due to the formation of its chemical derivatives (10).

Our results showed a 7 percent reduction in body mass of experimental animals. Furthermore, prolonged exposure of high-dose ascorbic acid resulted in upregulation of mucosal macrophages (Fig. 3), and decrease in the diameter of myenteric neurons (52.65 ± 1.593 versus 38.21 ± 0.8973 μm, $p<0.001$) (Fig.4), and TAMS (7.3 ± 0.21 versus 11.29 ± 0.55 mm^2, $p=0.0002$).

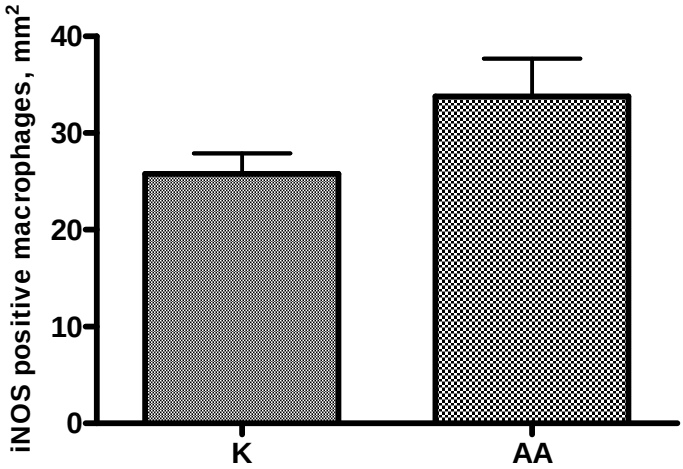

Figure 3. **The number of iNOS positive macrophages in the ileal mucosa of control (K) and experimental (AA) animals**.

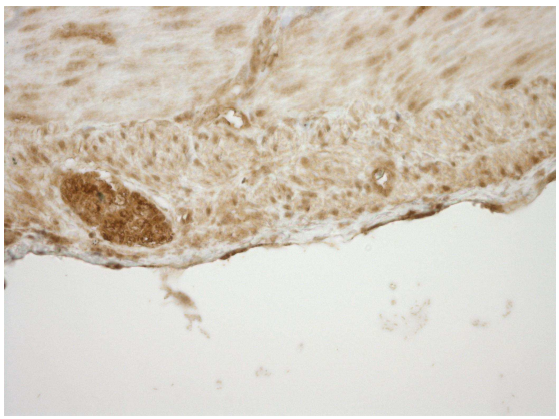

Figure 4. **iNOS positive myenteric neurons of experimental animals**.

Taken together these results indicate that high-dose ascorbic acid-induced oxidative stress upregulates gut iNOS. In addition, iNOS most likely contributes to delayed gut maturation and growth retardation of 30 days-old chickens through nitrosative stress.

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