

DOI 10.2478/pjvs-2013-0064

Original article

The expression of mitochondrial, cytoplasmic and extracellular superoxide dismutase in the colonic wall of pigs suffering from swine dysentery

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Abstract

The expression of 3 types of peroxide dismutase (SOD1, SOD2 and SOD3) was studied with Real-Time PCR in the colonic wall of domestic pig suffering from swine dysentery. The expression of enzymes was studied separately in the mucosa and the muscular membrane. It was found that in the mucosa the expression of SOD1 (cytoplasmic) did not change, while the levels of expression of mitochondrial SOD2 and extracellular SOD3 were raised in inflamed colon. More dramatic changes were seen in the muscular membrane where expression of SOD1 rose twice, this of SOD2 rose ca. 5-fold and the expression of SOD3 rose dramatically, even 30-fold. The obtained data are contradictory to findings in other types of colonic inflammation, which were studied either in the whole colonic wall, or in mucosa alone. The results show a very strong reaction of antioxidant systems in the muscular membrane in the enteritis.

Key words: swine dysentery, colon, SOD1, SOD2, SOD3, Real-Time PCR

Introduction

It was found that in the inflammatory bowel disease (IBD), peripheral blood monocytes and intestinal macrophages produce free radicals, called reactive oxygen species (ROS) (Rugtveit et al. 1995). The growing body of evidence suggests that leukocyte-produced free radicals, such as peroxide anion, hydrogen peroxide and hypochlorous acid are actively involved in the pathogenesis of inflammation. The mucosal

damage is suspected to be in large part caused by ROS produced by leukocytes attracted to the inflamed tissue (Weiss 1989). ROS are highly toxic and can damage all cell components, including regulatory and structural proteins, carbohydrates and DNA (Cheeseman and Slater 1993). To control the ROS levels the complex system of antioxidant enzymes exists. The initial part of the system are superoxide dismutases (SOD) which convert superoxide anion into hydrogen peroxide. Three types of SOD exist: the

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cytoplasmic enzyme (SOD1), mitochondrial enzyme (SOD2) and the extracellular form (SOD3). Reports exist that the intestinal inflammation affects negatively the functions of superoxide dismutase rendering tissues vulnerable to damage by ROS (Verspaget et al. 1994). Differences in the concentrations of SODs (mainly SOD2) were found also in the inflamed human stomach tissues in the course of *Helicobacter pylori* infection (Goetz et al. 1996). This data suggest that there is a mutual relation between the gastrointestinal inflammation and the status of SODs concentration/activity.

IBD becomes more and more serious problem in the developed countries. To study this problem many experimental models were designed, mainly in laboratory animals. We developed the experimental model using the domestic pig whose alimentary tract is the most similar to human one from the (patho)physiological point of view. The inflammation is induced by an experimental infection with *Brachyspira hyodysenteriae*, which induces necro-haemorrhagic inflammation of the colon called swine dysentery (Lakomy et al. 2009). The model was successfully used to study various aspects of intestinal inflammation, however, we have found some discrepancies between the published results and the data obtained in the porcine model. These discrepancies regard the changes in the expression, for example some neuropeptides and neuropeptide receptors involved in the inflammation (unpublished data). Hence, we decided to use this model also to study the expression of other substances involved in the inflammation to validate the porcine model against the data obtained in the laboratory animals and humans. This paper presents the results of the study on the changes in the expression of three superoxide dismutases in the colonic tissues of animals with experimental swine dysentery.

Materials and Methods

The study was performed on 8 female pigs of the Large White Polish breed: clinically healthy animals ($n = 4$) and the experimental group ($n = 4$). The study was accepted by the Local Ethical Commission of the University of Warmia and Mazury in Olsztyn, Poland. The procedure of the experimental infection with *B. hyodysenteriae* as well as the sacrifice procedure were described elsewhere (Lakomy et al. 2009). The infected animals were sacrificed when they developed strong, dysenteric diarrhoea with flecks of mucus, fragments of mucosa and blood. The mucosa was thickened, ulcerated and covered with fibrin. Fragments of the colonic wall were taken for histopathological examination which revealed micro-

scopic changes typical for swine dysentery. Fragments of the descending colon wall from control and experimental animals were collected and placed into RNALater (Ambion, USA). Then the mucosa was scraped and the total RNA was isolated with a Total RNA Mini Plus kit (A&A Biotechnology, Poland) from the scraped mucosa and the remaining tissue consisting of the muscular membrane. Reverse transcription was performed with 5 µg of total RNA, 0,5 µg Oligo(dT)18 Primer (1 µl), 4 µl of 5x Reaction Buffer, 20u RiboLock™ RNase Inhibitor (0,5 µl), 200u RevertAid™ Reverse Transcriptase (1 µl), 2 µl 10 mM dNTP mix (all reagents from Fermentas, Lithuania) and water to 20 µl for 60 min. at 42°C. From each sample of cDNA four Real Time PCRs were performed for SOD1, SOD2, SOD3 and GAPDH, each in triplicate. Composition of PCR mix was: 12,5 µl FastStart Universal SYBR Green Master-Mix (Roche, USA), 1 µl cDNA preparation and 1 µl of 5 mM starter mix (reverse and forward, Sigma, USA).

Sequences of starters were: SOD1: forward ACA-CAAGGCTGTACCAGTGC, reverse TTTGCCAG-CAGTCACATTGC, SOD2: forward CCCAAAG-GGGAATTGCTGGA, reverse GACGGATACAG-CGGTCAACT, SOD3: forward ACTCCTGCCAT-GCTGACG, reverse TGCCAGATC-TCCGTCCTTT. The housekeeping gene was porcine GAPDH. The starters were: forward TTCCAC-CCACGGCAAGTT and reverse GGCCTTTCCAT-TGATGACAAG. Starters for SODs and GAPDH were designed using sequences of porcine SOD1 (NM_001190422.1), SOD2 (NM_214127.2), SOD3 (DQ915492.1) and GAPDH (NM_001206359.1) sequences available in Gen Bank. The starters were designed with a Primer-BLAST software (<http://ncbi.nlm.nih.gov>). The reaction was performed with the following thermal profile in 7500 Fast Real-Time PCR System (Applied Biosystems, USA): initial denaturation 10 min. 95°C, denaturation 15 sec. 95°C and annealing 1 min. 60°C for 40 cycles. Data for SODs expression were normalized against GAPDH using software 7500 v. 2.0.2 (Applied Biosystems, USA). Data were statistically analyzed with Graph-Pad Prism 5 software using one-way ANOVA and Tukey's post-test.

Results

The swine dysentery did not change expression of SOD1 in the colonic mucosa (Fig 1A). The dysentery-associated colitis induced marked increase in the expression of mitochondrial SOD2 (Fig. 1B) and two-fold increase of extracellular SOD3 expression

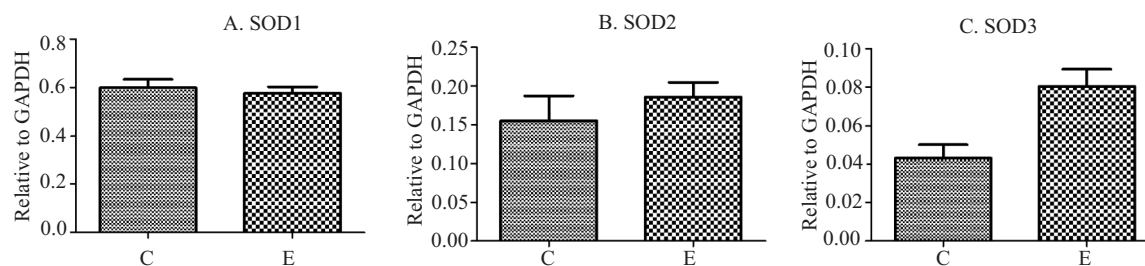


Fig. 1. Expression of SOD1 (A), SOD2 (B) and SOD3 (C) in the mucosa of colon in control (C) and dysenteric (E) animals. Results displayed as mean \pm SEM.

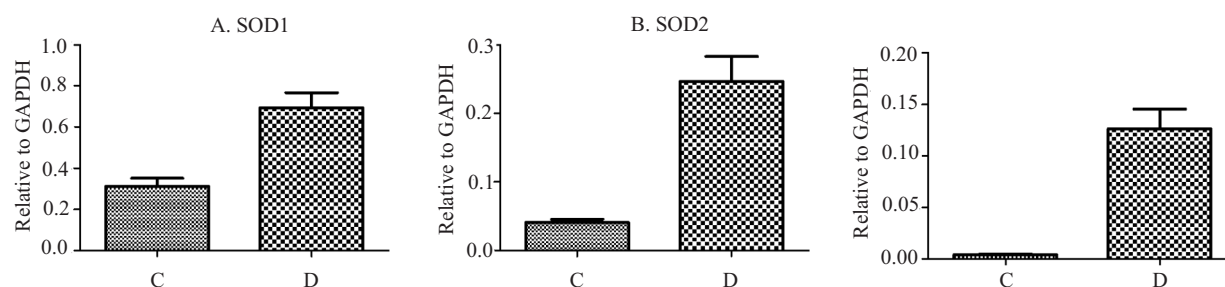


Fig. 2. Expression of SOD1 (A), SOD2 (B) and SOD3 (C) in the muscular membrane of colon in control (C) and dysenteric (E) animals. Results displayed as mean \pm SEM.

(Fig. 1C). However, the increases were found to be statistically insignificant. Dramatic changes in the expression of SODs were found in the muscular membrane. In dysenteric animals SOD1 expression rose more than two-fold (Fig. 2A), while expression of SOD2 rose about six-fold (Fig. 2B). Tremendous rise was encountered in case of SOD3, where it was about 30-fold (Fig. 2C) in dysenteric animals. In the muscular membrane the differences between control and dysenteric animals were statistically significant.

Discussion

SOD1 is an enzyme binding Cu and Zn located in the cellular cytoplasm and in mitochondrial inter-space. Knock-out mice lacking SOD1 gene develop until birth and postnatally, but have shortened life-span and show many age-related degenerative changes, like macular degeneration, cataracts, muscle loss and hepatocellular degeneration (Muller et al. 2007). In contrary to our findings, where expression of SOD1 was found to not change in the dysenteric colonic mucosa, concentration of this enzyme was found to decrease in *H. pylori* gastritis (Goetz et al. 1996). Also in IBD the decrease of SOD1 level was reported consistently (Rugtveit et al. 1995). The significance of SOD1 for the process of inflammation is unclear. However, it seems that the decreased level of SOD1

is involved in the mechanism of inflammation, as in TNBS-induced colitis SOD1 injected subcutaneously ameliorated the inflammation proving its beneficial influence (Segui et al. 2004). The lack of decrease of SOD1 expression in the dysenteric porcine colonic mucosa must be somehow correlated with the intensity of inflammatory process.

SOD2 is the mitochondrial enzyme which is a first defense wall against ROS formed during the normal metabolism of the cell. The common sense would expect it to be the enzyme important just for the basic metabolism of the cells and not to be involved in the reaction to inflammation. Knockout mice lacking Mn-SOD do not survive until birth and this cannot be ameliorated with overexpression of other dismutases. They show extensive mitochondrial damage in many tissues (Copin et al. 2000). Surprisingly, mitochondrial Mn-SOD is involved in the process of inflammation defense (for review see Li and Zhou 2011). The expression of this enzyme may be altered by some factors associated with inflammation, including cytokines, such as TNF α , IFN γ , or IL-1 (Masuda et al. 1988). Similarly to our findings in the porcine dysenteric colon in gastritis evoked by *Helicobacter pylori* the inflamed human gastric mucosa contained more SOD2 than was found in control samples (Goetz et al. 1996). In IBD the massive production of ROS, oxygen and nitrogen metabolites, is very characteristic (Conner et al. 1996). In inflamed

cells in IBD the level of Mn-SOD is decreased what suggests this enzyme as possible therapeutic target (Kruidenier et al. 2003).

SOD3 is a dismutase secreted to the extracellular space. It is believed to be involved in the cardiovascular disorders (Gongora et al. 2006) and in lung diseases (Ganguly et al. 2006). However, the involvement of SOD3 in the inflammation may be illustrated by the protective effect of SOD3 on the collagen-induced arthritis in mice (Yu et al. 2008). The strong increase of SOD3 expression in the inflamed porcine colonic mucosa may suggest the protective process taking place in the dysenteric porcine colon.

To date there were no reports describing changes in the expression of SODs in the muscular membrane of the gastrointestinal tract. We have shown astonishing changes in the expression of all three dismutases in the muscular layer of the colon in the course of dysentery. SOD1 expression rose twice, SOD2 expression rose ca. 5-fold and SOD3 expression rose ca. 30-fold. It is rather thought that the enteritis is largely confined to the mucosa, while sparing submucosa and with muscular layer largely not involved. However, our results show clearly that the muscular layer reacts vividly to the inflammatory process. The question is, which elements of the tissues of the muscular layer are responsible for this rise in SODs expression. This problem will require further studies.

To date there were no reports on the expression of superoxide dismutases in the porcine inflamed colon. The obtained data are largely contradictory to those reported in IBD or *H. pylori* infection. It may suggest that the processes involved in *B. hyodysenteriae*-induced inflammation may be different from those in IBD and gastric ulcer. The reasons for this may be associated with different severity of inflammation present in naturally-occurring gastroenteritis in humans and experimentally-evoked intestinal inflammations in laboratory animals. Also in pigs used in the present study the experimental infection with *B. hyodysenteriae* and resulting enteritis is difficult to control and the disease progress and its severity may be assessed only on the basis of clinical signs and pathomorphologic examination. It is possible, that the expression of dismutases change in the course of the disease reflecting the changing severity of the illness. Studies on the reaction of human colonic xenografts in immune-compromised mice to infection with *Entamoeba histolytica* and *Shigella flexneri* revealed both the time-dependent and factor-dependent changes in the expression of mitochondrial superoxide dismutase. Infection with *E. histolytica* evoked 5-fold increase of SOD2 expression 4 hours from injection which dropped to 3.5-fold level of expression (relative to control) at 24 hours from infection. On the other hand the

reaction of human colonic xenografts to *S. flexneri* infection was completely different. Four hours from infection the increase of SOD2 expression was 7-fold which rose dramatically to over 13-fold 24 h post infection (Zhang and Stanley 2004). It seems that the experimental model based on the infection with enteropathogens may generate results difficult in interpretation. Models based on other enteritis-evoking factors, like TNBS (tri-nitro-benzene sulphonic acid) colonic infusion, seem to be more predictable. However, TNBS is also believed to evoke enteritis by breaking down the hydrophobic barrier of the colonic mucosal epithelium and thus, activating the endogenous microorganism evoking the enteritis (Guarner 2003).

As it often happens, the obtained results raise more questions which will require further studies to address them. The problem of differential answer of the mucosa and muscular membrane, as well as the time course of SODs expression response in the swine dysentery will be objects of our future interest.

Acknowledgements

Supported by grant N N308 233638 from the Polish National Science Center.

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