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ORIGINAL ARTICLE

Neuronal Nitric Oxide Synthase Mediates the Effect of Ethanol on IgA

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Abstract — **Aims:** We showed previously that the acute effect of ethanol on intestinal immunoglobulin A (IgA) expression might be mediated by endogenous nitric oxide (NO). To extend these findings, this study was designed to investigate a possible role of neuronal NO synthase (nNOS) in the observed phenomenon, using 7-nitroindazole (7-NI), a selective inhibitor of its activity. **Methods:** Adult male Wistar rats were treated with: (a) ethanol (4 g/kg, intraperitoneally, i.p.), (b) 7-NI (25 mg/kg, i.p.) followed by ethanol (4 g/kg, i.p.) 30 min later and (c) 7-NI (25 mg/kg, i.p.) followed by saline 30 min later. Untreated rats were used as controls. The concentrations of serum and intestinal IgA were measured by enzyme-linked immunosorbent assay, while the expression of nNOS was determined using western blot and immunohistochemistry. **Results:** Acute ethanol treatment significantly increased the concentration of IgA in the ileal extracts, whereas it decreased its serum level. Inhibition of nNOS activity by 7-NI abolished this action of alcohol on IgA. Additionally, western blot analysis revealed that the acute alcohol administration induced an increase in the expression of intestinal nNOS. Furthermore, nNOS-immunoreactive cells, observed within the lamina propria of small intestine, were numerous in ethanol-treated rats. **Conclusion:** Taken together, these results extended our previous findings suggesting that nNOS mediates the acute effect of ethanol on IgA and supported an immunomodulatory role of this enzyme isoform.

INTRODUCTION

Clinical and experimental studies have shown that ethanol affects both innate and adaptive immune response and alcoholics have increased risk of infections and certain tumors (Jerrells and Pruett, 1994; Szabo and Mandrekar, 2009). However, the mechanisms by which alcohol exerts immunomodulatory effects are only partially understood. In addition, the investigations dealing with influence of ethanol on mucosal immune system, which is highly specialized and functions largely independently of the systemic immune system, are still limited.

Secretory immunoglobulin A (IgA), predominant immunoglobulin of the mucosal immune system, is produced by plasma cells against pathogenic and commensal microbes in T-cell-dependent and -independent pathways (Fagarasan *et al.,* 2010). In the gastrointestinal tract, IgA-committed B cells, leave Peyer's patches to the draining lymph nodes, via thoracic duct and the blood and migrate to the lamina propria, where they finally differentiate into IgA-secreting cells (Brandtzaeg and Johansen, 2005; Cerutti *et al.,* 2011). In addition, IgA can be synthesized in the isolated lymphoid follicles and lamina propria (Fagarasan *et al.,* 2010). It is generally accepted that secretory IgA forms the first line of adaptive immune defense and maintains mutualism with the indigenous microbiota (Corthésy, 2010; Feng *et al.,* 2011).

It has been reported that ethanol reacts with nitric oxide (NO; Deng and Deitrich, 2007). NO is a multifunctional signaling molecule that modulates a wide range of biological processes, including vasodilatation, neurotransmission and the immune response (Thippeswamy *et al*., 2006). This unstable gas is formed from L-arginine by a family of enzymes NO synthase (NOS): neuronal—nNOS (NOS I), endothelial—eNOS (NOS III) and inducible—iNOS (NOS II). NO generated by iNOS regulates differentiation, proliferation and apoptosis of cells, production of cytokines, expression of costimulatory and adhesion molecules (Bogdan, 2001, 2011). Despite information of NO in mucosal defense and gastrointestinal disorders (Wallace and Miller, 2000), its function in IgA system has been a focus of few recent studies. Thus, the subsets of dendritic cells (Tezuka *et al.,* 2007) and IgA plasma cells (Fritz *et al.,* 2011) that express tumor necrosis factor-α (TNF-α) and iNOS regulate IgA production in the gut.

We showed previously that the acute effect of ethanol on intestinal IgA expression might be mediated by endogenous NO (Budeč *et al*., 2007). To extend these findings, we investigated a possible role of nNOS as an abundant isoform of NOS in rat small intestine (Qu *et al.,* 1999). Acute alcohol treatment elevated intestinal concentration of IgA, while inhibition of nNOS activity by 7-nitroindazole (7-NI) abolished this action. Also, the acute ethanol administration increased the expression of intestinal nNOS protein levels. Moreover, observed nNOS-immunoreactive cells were numerous in the lamina propria of the small intestine of alcohol-treated rats.

METHODS

Animals

Male Wistar rats, 10–12 weeks of age (obtained from the Breeding Colony of the Medical Military Academy, Belgrade), were housed in a temperature-controlled room with light on at 6 h and off at 18 h, provided with standard laboratory diet (Veterinarski zavod, Subotica) and water *ad libitum*.

The protocol was in accordance with local institutional guidelines for the care and use of laboratory animals (O 282-2/10—authorization number of the institution ethical approval). The investigation also conformed to the principles and guidelines of Conseil de l`Europe (published in the Official Daily N. L358/1-358/6, 18 December 1986).

Experimental procedure

On the day of experiment, the animals were weighed and treated with: (a) ethanol (4 g/kg, intraperitoneally, i.p.), (b) 7-NI (25 mg/kg, i.p.) followed by ethanol (4 g/kg, i.p.)

30 min later and (c) 7-NI (25 mg/kg, i.p.) followed by saline 30 min later. Untreated rats were used as controls. The animals were sacrificed by decapitation 30 min after the last injection. Ethanol was diluted in sterile saline to yield a dose of 4 g/kg, whereas 7-NI was dissolved in sterile filtered DMSO (ampules A7248,0005, AppliChem) just before use. The dose of 7-NI, regimen of administration and route of injection were based on previous pharmacokinetic studies (Vassiljev *et al.*, 1998; Bush and Pollack, 2000). To avoid changes associated with the circadian rhythm, the experiments were carried out between 8.00 and 12.00 h.

Tissue preparation and homogenization

For each rat, five ileal segments containing Peyer's patches were excised immediately, opened longitudinally and rinsed in cold saline. All the specimens were weighed and extracted in 0.5-ml ice-cold RIPA buffer, pH 7.6 [containing 50 mM Tris–HCl, 150 mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 5 μg/ml aprotinin, 5 μg/ml leupeptin, 3 μg/ml pepstatin, 1 mM phenylmethylsulphonyl fluoride, 1 mM EDTA] per 0.1 g of tissue. After homogenization, tissue lysates were centrifuged at 15,000*g* for 20 min at 4°C. Supernatants were collected and protein concentration was determined by the BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific Inc., USA). The samples were stored at –70°C until analysis.

IgA determination

The concentration of rat IgA in the serum and intestinal extracts was measured using Rat IgA ELISA Quantitation Set (Bethyl Laboratories, Inc., USA) according to the manufacturer's instructions. Briefly, 96-well microtiter plates were coated with affinity-purified goat anti-rat IgA antibody (in carbonate-bicarbonate buffer pH 9.6) by overnight incubation at 4°C. After washing with Tris-buffered saline (TBS)/0.05% Tween-20 and blocking with 1% bovine serum albumin (BSA) in TBS, 2-fold serial dilutions of the standards (starting from 1000 to 15.6 ng/ml of reference rat serum) and serum or intestinal extracts diluted 1:500 in 1% BSA/TBS/0.05% Tween-20 were added. The plates were then washed and incubated with horse-radish peroxidase (HRP)-conjugated rat IgA detection antibody, diluted 1:80000 in 1% BSA/TBS/0.05% Tween-20. Finally, 3,3′,5,5′-tetramethylbenzidine substrate solution was used as the chromogenic substrate for the HRP detection. After 15 min incubation in the dark, ELISA stop solution was added. Optical densities were measured at 450 nm on an ELISA multiscan plus plate reader (Labsystems, Finland), and IgA concentrations were determined from the reference curve. All the samples were individually tested in duplicate.

Western blot

SDS–PAGE was carried out as described by Laemmli (1970). Total protein extracts were diluted in reducing sample buffer, boiled for 5 min and 60 μg of proteins per lane electrophoresed on 7.5% polyacrylamide gels. The size-separated proteins were transferred onto nitrocellulose membranes (AppliChem GmbH, Darmstadt) using wet blotting system. First, the membranes were blocked in 3% BSA/TBS/0.05% Tween-20 for 60 min and then washed. The membranes were incubated with mouse monoclonal anti-nNOS (NOS1) antibody (Santa Cruz Biotechnology, Inc., USA) diluted 1:100 in 1% BSA/TBS/0.05% Tween-20 and mouse monoclonal anti-actin antibody (Santa Cruz Biotechnology, Inc.) diluted 1:400, for 2 h, followed by three washes with TBS/0.05% Tween-20. Peroxidase-conjugated goat anti-mouse immunoglobulin (Santa Cruz Biotechnology, Inc.) diluted 1:5000 was used as a secondary antibody. The western blots were developed using the enhanced chemiluminescence reagent system (Amersham, GE Healthcare) according to the manufacturer's instructions. The content of nNOS in the tissue extracts was estimated by the densitometry of scanned immunoblot bands using the Image Master Total Lab (GE Healthcare) software. Actin was used as a loading control. Results were expressed as a ratio of nNOS to actin density (arbitrary units).

Immunohistochemistry

Ileal segments containing Peyer's patches were fixed in formalin and embedded in paraffin. After deparaffinization and dehydratation, the sections were heated in a microwave oven (at 680 W, in 10 mmol/l citrate buffer pH 6.0, for 20 min) for epitop retrieval. Endogenous peroxidase was blocked by 3% H_2O_2 solution in PBS for 10 min. Mouse monoclonal anti-nNOS (NOS1) antibody (Santa Cruz Biotechnology, sc-5302, 1:25) was applied and the sections were incubated overnight in a humidity chamber at 4°C. After washing, immunostaining was performed using Novocastra Peroxidase Detection System (Ready-to-Use) kit (Code No. RE7 110-CE), which employed the streptavidin-biotin technique and DAB Substrate/Chromogen System for visualization. Control sections were processed in parallel with omission of the primary antiserum. The nuclei were counterstained with Mayer's hematoxylin. Immunoreactive cells were analyzed using a computer-supported imaging system connected to the light microscope (Olympus AX70) with an objective magnification of ×40.

Statistical analysis

All values were expressed as mean ± SEM of each group. Statistical significance was assessed applying one-way analysis of variance (ANOVA). *Post hoc* comparisons using the Bonferroni-corrected *t*-test or the Games–Howell test were performed as appropriate. Significance was defined as $P < 0.05$

RESULTS

We tested the hypothesis that nNOS is involved in the acute effect of ethanol on rat IgA, using an inhibitor of its activity, 7-NI. The influence of alcohol on serum IgA, obtained by ELISA, is presented in Fig. 1. The acute ethanol treatment significantly decreased serum concentration of IgA ($P < 0.01$). Intraperitoneal pretreatment with 7-NI prevented this action of alcohol ($P < 0.01$). No significant difference was observed in the serum level of IgA between the group injected with 7-NI + saline and the control group.

In addition to serum, we measured the concentration of IgA in the ileal extracts. ANOVA revealed a significant difference in the intestinal level of IgA among the groups

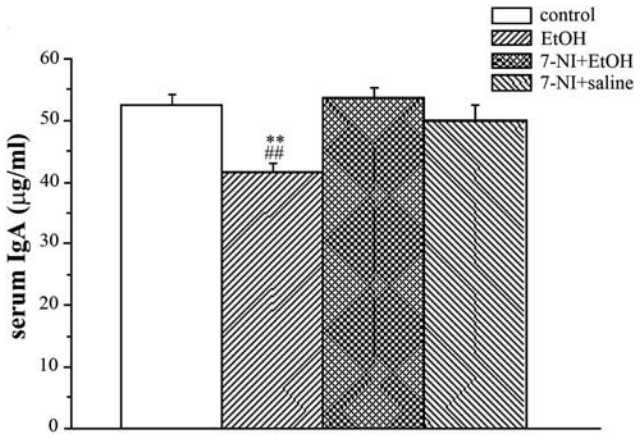

Fig. 1. The levels of serum IgA in rats under various experimental conditions. Data are reported as means ± SEM. Statistical significance was determined using ANOVA followed by Bonferroni-corrected *t*-tests. $^{**}P < 0.01$ compared with the controls; $^{\#\#}P < 0.01$ compared with 7-NI +EtOH. EtOH, ethanol; 7-NI+EtOH, 7-nitroindazole+ethanol.

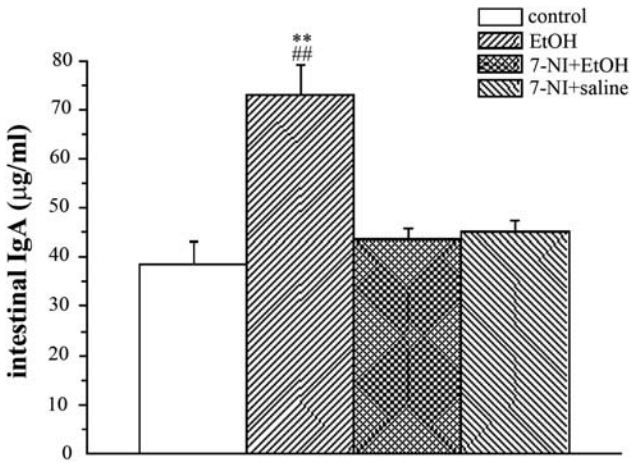

Fig. 2. The concentrations of IgA in the ileal extracts under various experimental conditions. Data are reported as means ± SEM. Statistical significance was determined using ANOVA followed by Bonferroni-corrected *t*-tests. $^{**}P < 0.01$ compared with controls; $^{\#\#}P < 0.01$ compared with 7-NI+EtOH. EtOH, ethanol; 7-NI+EtOH, 7-nitroindazole+ethanol.

($F = 13.911$, $P < 0.001$). As shown in Fig. 2, the acute alcohol administration significantly increased the concentration of IgA in the intestinal samples compared with untreated animals ($P < 0.01$). Inhibition of nNOS activity by 7-NI abolished this effect of ethanol ($P < 0.01$). Treatment with 7-NI followed by saline did not significantly alter intestinal IgA level.

To further investigate a role of nNOS in the action of ethanol on IgA, we determined expression of this enzyme isoform in the ileal extracts. Using western blot analysis, we detected two immunoreactive bands of ~155 and 145 kDa (Fig. 3a). Densitometry of both scanned bands demonstrated that the acute ethanol treatment significantly enhanced ($P < 0.05$) the intestinal nNOS expression compared with the control group (Fig. 3b). There was no difference in nNOS expression between the group treated with 7-NI + ethanol and the controls (Fig. 3b).

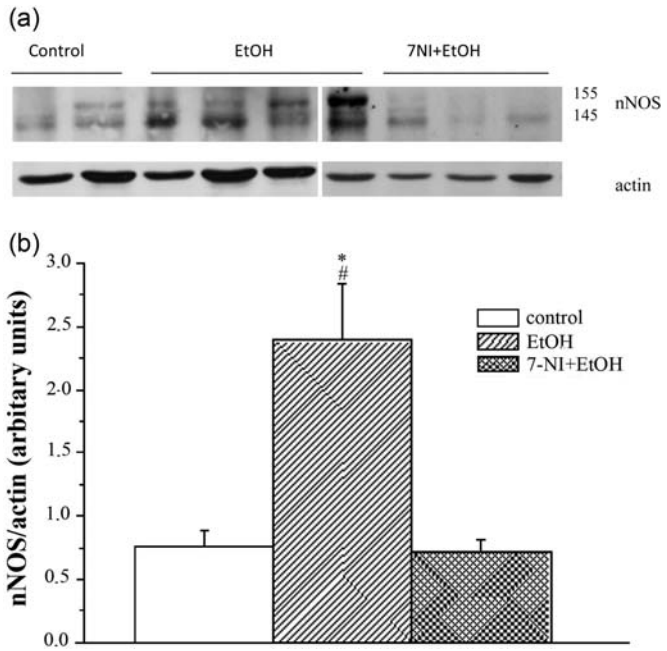

Fig. 3. (**a**) Immunoblotting of nNOS in the intestinal extracts. Two immunoreactive bands of ~155 and 145 kDa were detected. Actin was used as loading control. (**b**) Densitometric analysis of nNOS-immunoreactive bands. Data are expressed as means ± SEM. Statistical significance was determined using ANOVA followed by the Games-Howell *post hoc* test. $^{*}P < 0.05$ compared with controls; $^{\#}P < 0.05$ compared with 7-NI+EtOH. EtOH, ethanol; 7-NI+EtOH, 7-nitroindazole+ethanol.

In order to localize the cells that express nNOS, we performed immunohistochemistry. As expected, nNOS-immunoreactive cells were detected within the muscle layer under various experimental conditions (data not shown). In addition, cytoplasmatic staining of nNOS was also identified within a few cells of the ileal lamina propria in the control animals (Fig. 4a). These cells of the ileal lamina propria were abundant in the ethanol-treated rats (Fig. 4b).

DISCUSSION

We confirmed and extended our previous findings (Budeč *et al*., 2007) suggesting a role of NO in the effect of ethanol on intestinal IgA.

As a serum and mucosal immunoglobulin, IgA has an important role in immunity (Woof and Kerr, 2006). Recent findings have provided evidence that intestinal IgA response to commensals is specific and can protect against pathogens (Round and Mazmanian, 2009) supporting the concept that IgA represents a link between innate and adaptive immunity (Macpherson *et al.*, 2011). Following the acute ethanol treatment, increased concentration of IgA in the ileal extracts was consistent with the increased number of IgA-immunoreactive cells in the lamina propria as well as with enhanced intestinal expression of this immunoglobulin that we reported previously (Budeč *et al.*, 2005, 2007). Because serum IgA is integrally linked with mucosal IgA in the rat (Brown and Kloppel, 1989), we have also examined the influence of ethanol on IgA in circulation. Decreased level of serum IgA in ethanol-injected animals could be explained by specific rat

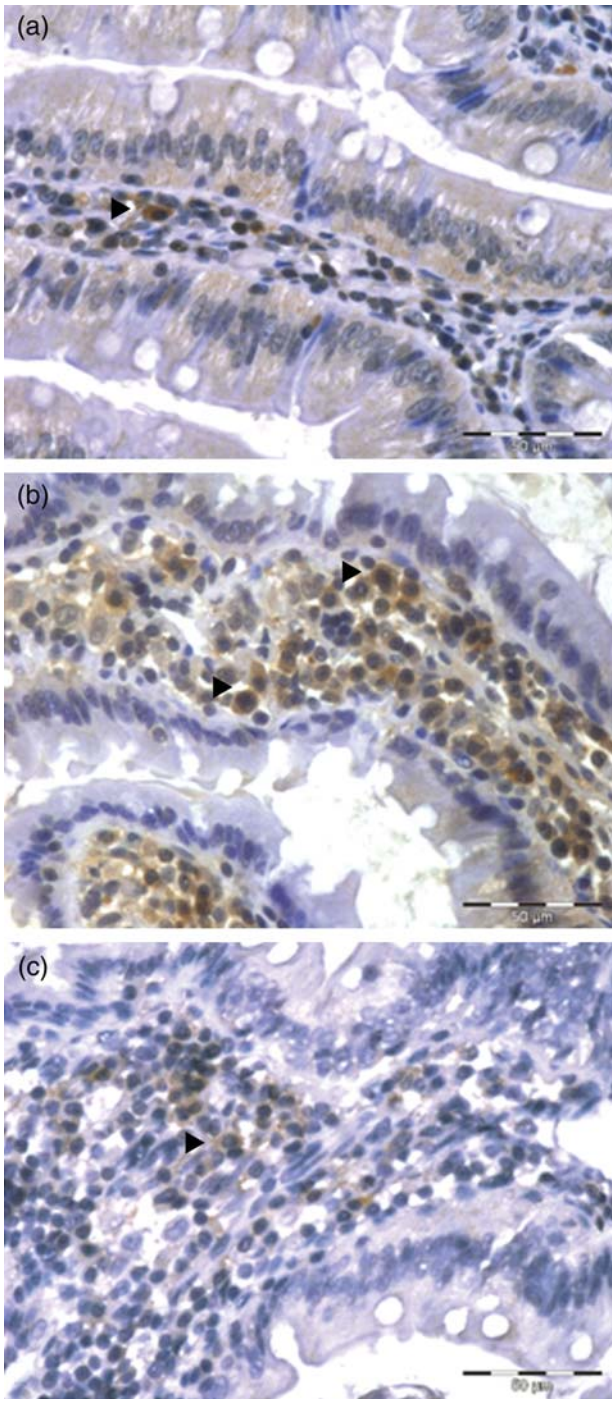

Fig. 4. Expression of nNOS, indicated as cytoplasmatic staining (arrow heads), within the ileal lamina propria in different groups: (**a**) control, (**b**) EtOH- and (**c**) 7-NI+EtOH-treated animals. nNOS-ir cells are numerous in EtOH-injected rats (b). EtOH, ethanol; 7-NI+EtOH, 7-nitroindazole+ethanol. Scale bar = 50 μm.

IgA system, characterized by a rapid clearance of plasma polymeric IgA by the liver and transport into bile (Brown and Kloppel, 1989). It has been reported that chronic alcohol consumption does not alter the number of IgA plasma cells in Peyer's patches (Lopez *et al.,* 1997) but decreases it in the intestinal lamina propria (Lopez *et al.,* 1994).

Interactions between ethanol and NO have been well recognized (Deng and Deitrich, 2007; Toda and Ayajiki, 2010). Thus, alcohol increases plasma concentration of NO (Baraona *et al*., 2002) and alters the expression and activity of NOS (Deng and Deitrich, 2007). On the other hand, nNOS can oxidize ethanol to α-hidroxyethyl radical (Porasuphatana *et al*., 2001) and peroxynitrite interacts with ethanol to form ethyl nitrite (Deng *et al*., 2004).

Our previous work demonstrated that blockade of NO production by non-specific inhibitor of NOS, L-NAME, prevented the effect of ethanol on IgA (Budeč *et al*., 2007). To further identify isoform of NOS involved in this action of alcohol, we used 7-NI, which selectively inhibits the activity of nNOS. Pretreatment of rats with 7-NI abolished ethanol-induced changes suggesting that NO, produced by nNOS, mediated the observed effect in the current study. These data are consistent with previous reports showing that nNOS is responsible for the actions of ethanol such as conditioned place preference in mice (Itzhak *et al*., 2009) and release of adrenocorticotropic hormone in male rats (Seo and Rivier, 2003). The failure of 7-NI to modulate the levels of serum and intestinal IgA indicates that nNOS has no role in the regulation of IgA under steady-state conditions. Likewise, 7-NI did not affect anti-SRBC antibody titer and the parameters of cellular immunity in non-stressed animals, whereas it altered the immunosuppressive effect of restraint stress (Sharma *et al.,* 2004).

There is evidence that nNOS accounts for >80% of the constitutive NOS activity in rat small intestine (Qu *et al*., 1999). Using western blot analysis with monoclonal nNOS antibody, we detected two bands of ~155 and 145 kDa, in the ileal extracts. Similarly, Huber *et al*. (1998) identified nNOS-ir bands of ~155, 145 and 135 kDa, as different splice variants of nNOS, in a soluble fraction of rat small intestine. Compared with the control group, the acute ethanol administration increased intestinal expression of nNOS supporting the role of this NOS isoform in its action on IgA. Chronic alcohol exposure does not affect ileal nNOS content (Krecsmarik *et al*., 2006), but there are no studies examining acute ethanol effect on this enzyme isoform in the gastrointestinal tract.

Although nNOS was initially described in neurons, it has been detected subsequently in other cells, including: neutrophils (Greenberg *et al*., 1998) and dendritic cells (Adler *et al*., 2010). In our study, many nNOS-stained cells within the intestinal lamina propria-effector site of mucosal immunity were observed after alcohol treatment in accordance with the immunomodulatory function of this enzyme isoform. Thus, nNOS contributes to elimination of *Giardia lamblia* infection (Li *et al*., 2006) and is important for survival, bacterial clearance and regulation of cytokine response during peritonitis and sepsis in mice (Cui *et al*., 2007). Furthermore, nNOS modulates maturation of human dendritic cells *in vitro* in an autocrine pathway (Adler *et al*., 2010).

Over recent years, landmark studies have demonstrated a role of iNOS in IgA system. This isoform of NOS, expressed with TNF-α in a special subset of dendritic cells in response to commensal bacteria, regulates T-cell-dependent and -independent class-switch recombination of IgA in mice (Tezuka *et al.,* 2007; Tezuka and Ohteki, 2010). Moreover, the most recent investigation has described a multifunctional

population of IgA+ plasma cells expressing TNF-α and iNOS in the small intestinal lamina propria, important in IgA production and homeostasis maintaing (Fritz *et al.,* 2011).

Our results substantiated neuro-immune crosstalk in the gastrointestinal tract (Ben-Horin and Chowers, 2008). Morphological analysis showed increased number of IgA-producing cells close to the basement membrane and nerve fibers in response to lipopolyssacharide within mouse intestinal lamina propria (Hisajima *et al.,* 2005). Also, specific staining with NH2-terminal nNOS antibody of submucosal nerve cell bodies and fibers was found within the rat lamina propria around crypts and in the intestinal villi (Saur *et al.,* 2002). In a view of our and previously mentioned observations, we assume that NO produced by nNOS within the lamina propria following acute ethanol administration affects IgA in a paracrine pathway.

Taken together, the results obtained extend our previous findings and suggest for the first time that nNOS is required for the acute effect of ethanol on rat IgA. Further studies are needed to shed light on the immunomodulatory role of nNOS.

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