PhD Thesis

Sigma-1 receptor-mediated protection in TNBS-induced rat colitis

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List of abbreviations:

3-NT	3-nitrotyrosine
ANOVA	analysis of variance
CD	Crohn's Disease
CU	colitis ulcerosa
DMSO	dimethyl sulfoxide
DTNB	5,5-dithio-bis-(2-nitrobenzoic acid)
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
FLV	fluvoxamine
GI	gastrointestinal
GSH	glutathione
HMGB1	high mobility group box 1
HO	heme oxygenase
IBD	Inflammatory Bowel Diseases
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
IP_3R	inositol-1,4,5-trisphosphate receptor
MAM	mitochondria-associated ER membrane
MPO	myeloperoxidase
NF-κB	nuclear factor kappa B
NOS	nitric oxide synthase
Prdx1, -2, -4, -6	peroxiredoxin-1, -2, -4, -6
ROS	reactive oxygen species
SASP	sulfasalazine
SEM	standard error of mean
SOD	superoxide-dismutase
SSRI	selective serotonin reuptake inhibitor
TNBS	2,4,6-trinitrobenzenesulfonic acid
UCHL-1	ubiquitin C-terminal hidrolase L1
σ1R	sigma-1 receptor
σ2R	sigma-2 receptor
σR	sigma receptor

Introduction

Inflammatory Bowel Diseases (IBD) are chronic ailments of the gastrointestinal (GI) tract. The two main forms of the disease are Crohn's Disease (CD) and colitis ulcerosa (CU). Based on epidemiological studies IBD is more common in developed countries and seems to be steady in the last decade, however developing countries undergo a higher incidence currently. It is increasingly clear that several factors, such as genetic predisposition, environmental factors, immunological processes and the body's own microbiome contribute to the development of IBD. Several immunological processes and mediators are involved in the pathogenesis of IBD, such as the elevated levels of interleukin-6 (IL-6) and the increased expression of nuclear factor kappa B (NF-κB). Furthermore, the pro-inflammatory high mobility group box 1 nuclear mediator, the role of ubiquitin C-terminal hidrolase L1 deubiquitinating enzyme, the carbon monoxide generating heme oxygenase (HO) and the nitric oxide synthesizing nitric oxide synthase (NOS) enzymes. Additionally, oxidative stress seems to play a role in the pathogenesis of IBD since this disease is characterized with an increased reactive oxygen species (ROS) production. For the elimination of ROS the antioxidant system is responsible. Of these, thiol-containing glutathione (GSH), superoxide-dismutase (SOD) and members of the peroxiredoxin (Prdx) enzyme family are the most important.

Sigma receptors (σRs) are intracellular chaperones localized in the mitochondria associated endoplasmic reticulum (ER) membrane. The main functions of σRs are the regulation of Ca^{2+} homeostasis through the interaction with inositol 1,4,5-trisphosphate receptor (IP_3R) and the protection against ER stress. Several stress conditions or the presence of its agonist induces translocation of the receptor itself from the MAM to nuclear and plasma membrane, thus the receptor confers its protective effect in the whole cell. σRs are basically consists of two subtypes, the σ1R and σ2R. σ1R has greater consideration in research compared to σ2R. Recently, σ1R emerged as a potential therapeutic option in inflammatory conditions and in the therapy of sepsis.

Interestingly, a vast number of pharmacological compounds bind to σ1R with high affinity, such as benzomorphans, antipsychotics, antihistamines, antidepressants, antifungal agents and selective serotonin reuptake inhibitors (SSRI). Fluvoxamine (FLV) is an SSRI antidepressant, with a high binding affinity to σ1R. FLV is described in several animal models that it confers anti-inflammatory effects.

Aims

In our study we aimed to investigate the role of σ1R in 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced rat colitis.
We were seeking answers for the following questions:

- Does the σ1R exist in a detectable level in rat colon, and does TNBS induction affect its density? Furthermore, whether our σ1R-associated treatments have an impact on the density and expression of the receptor itself?

- Which doses of the σ1R ligands (agonist: FLV, antagonist: BD1063) are considered as effective based on their impacts on the lesion caused by TNBS?

- Moreover, what molecular mechanisms led to the protective anti-inflammatory effect caused by the effective dose of FLV?

In our experimental design, FLV agonist modelled the activation, BD1063 antagonist caused σ1R inactivation. Furthermore, in our combined treatment we treated the rats with the effective doses of the two ligands, and we presumed that the changes in the biochemical parameters are σ1R-regulated if the presence of the antagonist abolished the protective effect of the agonist.

Materials and methods

σ1R radioligand binding assay in rat colon

Competition binding studies were done in Wistar Hannover rat colon membrane homogenates with [^{3}H](+)-pentazocine or unlabeled ligands. The equilibrium dissociation constant (K_d) and receptor density (B_{max}) were determined by saturation binding experiments.

Experimental design and the induction of TNBS colitis model

Male Wistar Hannover rats (225-250 g) were used in our experiments. Animals were randomly divided into 3 groups: absolute control (no treatment, n=12), 50% ethanol (intracolonic (i.c.), n=12), and TNBS (10 mg dissolved in 50% ethanol i.c., n=85). Colitis was induced based on Morris et al's method. Briefly, animals were fasted overnight then TNBS enema was done with an 8 cm polyethylene cannula through the rectum. We further divided the TNBS-induced colitis group into 10 groups and treated them with the following drugs: fluvoxamine (FLV, σ1R

agonist) i.c. with different doses (10 mg/kg, 1 mg/kg, 0.1 mg/kg and 0.01 mg/kg dissolved in 3% dimethyl sulfoxide (DMSO); BD1063 (σ1R antagonist) 1 mg/kg and 0.1 mg/kg (dissolved in 0.9% physiological saline); FLV + BD1063 (combined treatment with the two effective doses of the ligands: FLV 1 mg/kg + BD1063 0.1 mg/kg); physiological saline (vehicle of BD1063); DMSO (3%, vehicle of FLV); sulfasalazine (SASP, positive control, per os 2 x 25 mg/kg). Ligand treatments were done once a day at the same time for 3 consecutive days. Animals were fasted 5 hours before i.c. treatments. 72 hours after TNBS instillation animals were sacrificed and the last 8 cm of their colons were removed and a photo were taken from each colon segments for further macroscopic analysis. Tissues than were frozen in liquid nitrogen and used for further biochemical measurements.

Measurement of the lesions

Inflammatory lesions were analysed via a software, developed by our laboratory, which is based on planimetrics (Stat_2_1_1, Szeged). The extent of the lesions were calculated based on the ration between the area of the whole 8 cm colon and the inflammatory sites.

Measurement of the activity of MPO enzyme

Myeloperoxidase (MPO) is a marker of inflammatory processes, which activity correlates with the severity of inflammation and with the accumulation of neutrophil granulocytes. The determination of the activity of MPO was done via spectrophotometric analysis.

Determination of the expression of σ1R, UCHL-1, iNOS, NF-κB p65 and HMGB1

The expression of σ1R, UCHL-1, iNOS, NF-κB p65 and HMGB1 were measured via Western blot. To develop our bands we used the ECL method which is based on the action of horseradish peroxidase enzyme. The results were analysed with Quantity One software and were normalized with β-actin loading control.

Measuring the levels of IL-6, eNOS, 3-NT and Prdx1, -2, -4, -6 and determination of the activity of SOD enzyme

The levels of the mentioned parameters were measured by specific ELISAs. The activity of the SOD enzyme was determined by a specific kit. In our measurements we followed the attached instructions of the manufacturers.

Measuring the activity of the HO enzyme

The activity of the HO was measured in a reaction where the forming bilirubin correlates with the activity of the enzyme. The levels of the forming bilirubin were done by spectrophotometric method.

Measuring the levels of total GSH

The thiol containing GSH levels were determined by 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) method followed by spectrophotometric analysis.

Protein determination

The determination of the total protein levels was done via Bradford method using spectrophotometry.

Statistical analysis

The results are shown in mean ± standard error of mean (SEM). Statistical analysis was done via analysis of variance (ANOVA) method followed by Bonferroni or Holm-Sidak post hoc tests. Statistical significance was determined when $p < 0,05$.

Results

In our study, we found a medium σ1R density in case of the absolute control group, which did not get any treatment. After TNBS instillation the colonic tissue showed a significant decrease in σ1R density. Furthermore, we showed that the effective dose of FLV significantly elevated the total binding capacity compared to TNBS group and significantly increased the expression of the receptor. Additionally, 1 mg/kg dose of FLV significantly decreased the severity of the colonic lesions, while treatment with BD1063 significantly worsened inflammation. Regarding colonic damage in case of the simultaneous administration of the two ligands (combined treatment) the presence of BD1063 abolished FLV-induced protection. According to our combined treatment we hypothesized that the FLV-induced protective mechanism was modulated by σ1R in TNBS-induced colitis. Then the molecular mechanism which was exerted by FLV and σ1R activation was investigated via measuring inflammatory mediators and oxidative stress/antioxidant elements. Based on our results we presumed the role of the decreased IL-6 levels, the elevated UCHL-1 expression, the increased HO activity, and the decreased expression of two nuclear mediators, NF-κB p65 and HMGB1 in FLV-induced protection. Furthermore, we found an FLV-induced decrease in iNOS expression and an

increase in eNOS levels. In case of the oxidative stress related factors we presumed the role of the decrease in 3-NT levels and the increase in the levels of GSH and Prdx1.

Summary

I. σ1R is detectable in rat colon with medium density.

II. σ1R showed a decreased density in TNBS-induced colitis compared to control group. Treatment with the 1 mg/kg effective dose of FLV significantly elevated the density of the receptor. Furthermore, the effective dose of FLV caused higher σ1R expression in the colon.

III. The effective dose of the σ1R agonist FLV significantly reduced colonic ulcers compared to TNBS-induced colitis group. The combined treatment with the two effective doses of the ligands showed that the presence of BD1063 abolished FLV-induced protection, thus we presumed that the protection in our case was mediated by the activation of σ1R.

IV. Based on our results, the σ1R-induced protective mechanism was presumably emerged by the alleviation of inflammatory markers, such as the reduced MPO activity and IL-6 levels, and the elevated expression of UCHL-1. Moreover, the reduced levels of 3-NT and the increased levels of GSH might contributed to the protection exerted by FLV.

Publications:

MTMT code: 10060398

Publications directly related to the thesis:

1. **Almási N.**; Török S.; Dvorácskó S.; Tömböly C.; Csonka Á.; Baráth Z.; Murlasits Z.; Valkusz, Z.; Pósa A.; Varga C.; Kupai K. Lessons on the Sigma-1 Receptor in TNBS-Induced Rat Colitis: Modulation of the UCHL-1, IL-6 Pathway. Int. J. Mol. Sci. 2020, 21, 4046.
IF: 4,556

2. **Almási N.**; Török S.; Valkusz Z.; Tajti M.; Csonka Á.; Murlasits Z.; Pósa A.; Varga C.; Kupai K. Sigma-1 Receptor Engages an Anti-Inflammatory and Antioxidant Feedback Loop Mediated by Peroxiredoxin in Experimental Colitis. Antioxidants 2020, 9, 1081.
IF: 5,014

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