Eur J Nutr (2009) 48:213–220
DOI 10.1007/s00394-009-0004-3

ORIGINAL CONTRIBUTION

Oral treatment with genistein reduces the expression of molecular and biochemical markers of inflammation in a rat model of chronic TNBS-induced colitis

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Received: 23 July 2008 / Accepted: 27 January 2009 / Published online: 21 February 2009

Abstract
Background Inflammatory bowel disease (IBD) in humans has a high incidence in Europe and the USA, whereas in East Asia, incidence has been historically low. The risk of IBD appears to increase in Asian immigrants adopting western lifestyles, suggesting a strong link of environmental/dietary factors in the development of IBD. Exposure to high levels of isoflavones such as genistein (Gen) in traditional East Asian diets has been associated with a decreased risk of developing breast cancer and may also be beneficial for the prevention of IBD.
Aim In this study, the effect of orally administered genistein on the inflammatory response in the TNBS-induced chronic colitis rat model was investigated.
Methods Eighteen male Wistar rats, aged 12 weeks, were randomized to one of three groups ($n = 6$). Two groups received a 2,4,6-trinitrobenzenesulfonic acid (TNBS) enema, then were treated daily by oral gavage with either Gen (100 mg/kg b.w.) or vehicle, for 14 days. The last group served as a control group, not receiving the TNBS enema. At the end of the 14 days, animals were killed and tissues collected. Molecular and biochemical inflammatory markers in the colon, specifically cyclooxygenase-2 (COX-2) and myeloperoxidase (MPO), were analyzed. In addition, to assess the efficacy of Gen treatment, relative wet weights of the accessory sexual organs, specifically prostate and the seminal vesicle, were compared between the groups treated or not with Gen.
Results Wet weights of both prostates and seminal vesicles were significantly ($P < 0.01$) reduced upon Gen administration. In the colon, expression of COX-2 mRNA and protein was reduced ($P < 0.05$) in the Gen treatment group, as compared to the control group, whereas there was no significant inhibitory effect of Gen on the expression of proliferating cell nuclear antigen. In Gen treated animals colon wet weight was not altered, however a decrease in MPO activity ($P < 0.01$) was seen.
Conclusion These results may provide evidence that oral administration of Gen exerts beneficial anti-inflammatory effects in a rodent model of TNBS-induced chronic colitis. While the sample size of this study was small, it nevertheless might encourage the realization of larger blinded randomized controlled studies for the proof of concept.

Keywords Genistein · Colitis · IBD · TNBS · COX-2 · Inflammation

Introduction

Chronic inflammatory bowel disease (IBD), comprising Crohn's disease and ulcerative colitis, is a widespread condition of significant economic importance in Western European countries and the US [35]. IBD has been associated in several studies with lower quality of life and psychiatric distress [5, 15]. In addition, it is commonly followed by the development of colorectal carcinomas as a sequela in patients [4].

IBD incidence rates are significantly lower in East Asia and in several underdeveloped countries although rates have been increasing over the last few years, accompanying the process of "westernization" [18].

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The etiology of IBD is still widely unknown. About 50% of Crohn's disease cases can be attributed to genetic predisposition [10]. Besides genetic factors, several environmental factors are believed to play an important role in the pathogenesis of IBD [25, 31].

Asian immigrants who adopt the western lifestyle and diet show a strong increase in the risk for IBD [20], suggesting an important role for dietary factors in the protection against the development of these diseases. Asian and Western diets differ in several aspects from each other. For instance, the traditional Asian diet contains high amounts of soy and soy products, which are rich in isoflavones, plant-derived polyphenols. The average daily dietary isoflavone intake in East Asian countries such as Japan is 20–50 mg [3, 23] whereas in Europe less than 1 mg is common [8, 19].

High soy-isoflavone intake is thought to contribute to the low rates of breast cancer in Asia [1], a disease that is related to estrogen-receptor regulated gene expression. Epidemiological studies support an association in Asian-American and Japanese populations between high soy consumption and a reduced risk for breast cancer development [32, 34]. The underlying biological mechanisms in breast cancer protection could present an analogy to the situation in IBD:

One of the most abundant and the best-characterized dietary isoflavones is genistein (Gen). Like other isoflavones, Gen is capable of binding to the estrogen receptor (ER), with a preference for ERβ, the predominantly expressed ER subtype in the gastrointestinal tract [17], and to trigger mechanisms of estrogen action [6]. However, there is evidence that Gen exhibits differential effects in classic estrogen-sensitive target organs such as the mammary gland [13]. The estrogenic potency of Gen is low when compared to endogenous 17β-estradiol (E2) but the consumption of food and/or dietary supplements can result in the ingestion of substantial quantities of Gen.

Studies have shown that both E2 and specific ERβ ligands are capable of attenuating symptoms in a transgenic in vivo model of IBD (HLA-B27 rat) [11, 12]. For Gen, an anti-inflammatory effect in TNBS-induced ileitis has been observed [26] and in a recent study, Martín et al. [21] were able to show that the phytoestrogen resveratrol was effective in a rodent model of TNBS-induced colitis. These data allow the assumption that the consumption of phytoestrogens such as Gen might have an important nutritional influence on IBD rates in East Asia and that Gen could well be a potent candidate in the prevention and/or treatment of IBD. On the other hand, in a recent study, our laboratory was able to demonstrate that lifelong exposure to a phytoestrogen-enriched diet did not have a preventive effect but even enhanced the extent of acute chemically induced colitis [27]. Altogether, these findings provide reason for further investigation, to specifically elucidate the role of Gen in the pathogenesis of IBD.

In the present study, we report on the investigation of the effects of orally administered Gen on markers of inflammation in a rat model of chronic TNBS-induced colitis. Following the induction of colitis with TNBS, animals were assigned to one of two groups, either receiving daily Gen or vehicle by oral gavages for 14 days. A third group did not receive TNBS or Gen and served as a control group. mRNA and protein expression of COX-2 and PCNA, myeloperoxidase (MPO) activity, as well as relative colon wet weights served as markers of chronic inflammation. Relative wet weights of prostates and seminal vesicles were compared to non-treated animals as an indicator for efficacy of the Gen treatment.

Materials and methods

Experimental animals

Male Wistar rats (total sample size = 18) were obtained from Janvier (Le-Genest St-Isle, France) and kept under controlled conditions (temperature 20 ± 1°C, humidity 50–80%, illumination 12L/12D). All animals had free access to water and a special phytoestrogen-depleted rodent diet (Ssniff GmbH, Soest, Germany). The animals were randomly allocated to three different treatment groups (Control, TNBS, TNBS/Gen; for each $n = 6$).

The animal experiment was approved by the Committee on Animal Care and by the local institution, and complied with accepted veterinary medical practice.

Induction of colitis

At the age of 12 weeks, colitis was induced by TNBS according to procedures previously described [14, 22]. In brief, following a 12 h fast, rats were deeply anaesthetized with ketamine and xylazine hydrochloride and an enema of 100 µl ethanol–water (50:50 v/v) containing TNBS (2,4,6-trinitrobenzenesulfonic acid; Sigma–Aldrich, Taufkirchen, Germany) was instilled rectally 7–8 cm ascending from the anus at the point of the splenic flexure, using a rubber catheter (Freka®-Ernährungssonde, OD 2 mm, Fresenius, Bad Homburg v.d.H., Germany). Control animals were administered a 100 µl solvent enema (ethanol–water 50:50 v/v). Following TNBS administration (at 40 mg/kg b.w.) or solvent-only, animals were kept in a head-down position for 2 min to avoid leakage of the solutions.

Animal treatment

Starting 24 h after TNBS administration, the animals were treated once daily with Gen (100 mg/kg b.w.) or vehicle per os for 14 days using a gavage canula (Hauptner, Solingen, Germany). Gen was dissolved in a vehicle solution (0.9% NaCl, 2% Tween 80, and 0.5% methyl cellulose (both Sigma–Aldrich) in water). Application volume was 5 ml/kg b.w.

Study end

Body weights were obtained just prior to sacrifice. Fourteen days after initial enema application and subsequent daily treatment, animals were lightly anesthetized with CO_2 and then killed by decapitation. The distal 10 cm portion of colons, the prostates, and the seminal vesicles were collected during necropsy.

Tissue preparation and analysis of organ wet weights

After cleaning with cold saline (0.9%), wet weights of the collected organs were measured and the organ weight relative to body weight was calculated. Then, pieces of colon were snap frozen in liquid nitrogen for protein and mRNA preparations.

Western blot analysis of colonic protein expression

Pooled frozen colon tissue was powdered and homogenized in a buffer (623.5 mM Tris pH 8 EDTA) containing enzyme inhibitors (5 mg/ml aprotonin, 5 mg/ml leupeptin, 1 mg/ml pepstatin-A, 5 mg/ml antipain, 100 mM pefac in 0.5 M EDTA, pH 8). Protein concentrations were determined (DC Protein Assay, Bio-Rad, Munich, Germany). Equal amounts of sample (40 µg protein) were loaded on 4–12% Bis-Tris NUPAGE® Novex Gels (Invitrogen Life Technologies, Karlsruhe, Germany). After electrophoresis and separation, proteins were transferred onto nitrocellulose membranes and blocked with 5% milk powder in phosphate buffered saline (PBS) solution (100 mM; pH 7.4) at room temperature for 1 h. The immobilized proteins were quantitatively detected using specific antibodies for Actin (A5060, Sigma–Aldrich), COX-2 (M-19, sc-1747, Santa Cruz Biotechnology, Inc., CA, USA), and PCNA (M0879, Dako, Glostrup, Denmark). Polyclonal Rabbit Anti-Goat, Rabbit Anti-Mouse, and Swine Anti-Rabbit Immunoglobulins/HRP (P0160, P0260, and P0217, Dako) were used as species-specific HRP-conjugated secondary antibodies. Blot signals were visualized by the chemoluminescent POD-Substrate (Lumi-Light Plus, Roche Diagnostics, Mannheim, Germany) and a Fluorchem Luminescent Imager (Alpha Innotech, CA, USA). The protein bands were quantified by densitometry using the software ImageJ 1.38 (National Institutes of Health, USA, http://rsb.info.nih.gov/ij/). To account for inhomogeneous protein loading of the slots the ratios of the reference protein (Actin) to the target proteins (COX-2 and PCNA) were calculated.

RNA preparation, reverse transcription, and real-time RT-PCR

Total RNA was isolated from frozen tissues using a standard TRIzol® (Invitrogen) protocol followed by first-strand cDNA synthesis with the SuperScript® Kit (Invitrogen). Real-Time PCR was performed in the iCycler thermal cycler (Bio-Rad). The protocol comprised 50 cycles of 94°C, 58°C/60°C (PCNA/COX-2), and 72°C for 1 min each. Specific primers were designed with primer3 software (Whitehead Institute for Biomedical Research, Cambridge, USA) based on the cDNA sequences available at the EMBL database: 1A: up: 5′-CGTCACAGCCCATG CATTCG-3′, dw: 5′-CTGTTCATCCTGTTCCAGCTC-3′. COX-2: up: 5′-GCTCAGCCATACAGCAAATCC-3′, dw: 5′-GGGAGTCGGGCAATCATCAG-3′. PCNA: up: 5′-GA GCAACTTGGAATCCCAGAACAGG-3′, dw: 5′-CCAAG CTCCCCACTCGCAGAAAACT-3′. All PCRs were run in triplicates for each sample and threshold cycles (CT) were measured. A ΔCT value was calculated for each sample by subtracting the CT value of the reference gene (1A) from the CT value of the gene of interest (COX-2 or PCNA). 1A has been used as internal standard in several studies investigating the effects of estrogenic compounds in tissues [13, 27]. All samples were normalized to the ΔCT value of a control sample (ΔΔCT). The relative expression of COX-2 and PCNA was calculated using the expression $2^{-\Delta\Delta CT}$ and is reported as arbitrary units.

MPO activity

Analysis of MPO activity served as a quantitative marker of neutrophil granulocyte infiltration into the inflamed colon tissue. The activity of the enzyme was assessed according to the method of Grisham et al. [9] in tissue lysate produced from the pellet of the protein isolation step (see above). This pellet was homogenized in 10 volumes of 50 mM PBS (pH 6.0) and 0.5% HETAB (Sigma–Aldrich) and snap frozen in liquid nitrogen. The homogenate was kept on ice until formation of another pellet upon sedimentation. The supernatant was then used for the analysis of MPO.

Supernatant (1 µl) was diluted in 100 µl of TMB reagent Biosource, Nivelles, Belgium) and changes of absorbance over 5 min were measured with a spectrophotometer (Kinetic Microplate Reader, MWG Biotech

GmbH, Ebersberg, Germany) at 650 nm at 25°C. One unit of MPO activity was defined as the amount of enzyme present that produced a change in absorbance of 1 U/min at 25°C. Results were quantified as U/μg protein.

Statistical analysis

All data are expressed as arithmetic means with their standard deviations. Statistical significance of differences was calculated either using a two-tailed *t* test, Mann-Whitney U-test, or using a one-way ANOVA followed by a Tukey HSD test where appropriate. Significance was established at $P < 0.05$. It was differentiated between $P < 0.05$, $P < 0.01$, and $P < 0.001$ levels of significance.

Results

Relative wet weights of the accessory sexual organs were strongly affected by Gen. Animals treated with Gen had significantly ($P < 0.01$) decreased weights of both prostate (Fig. 1a) and seminal vesicle (Fig. 1b).

Fourteen days after the induction of colitis, there was visible local thickening of the colon wall in the animals that received an enema of TNBS but not in those of the control group that received solvent only (not shown). Associated with this finding was a significantly ($P < 0.05$) increased relative wet weight of the inflamed colon portions in the TNBS-stimulated animals (+69.5%). There was no detectable difference in colon-wet weights between animals of the TNBS and the TNBS/Gen group. The results of the colon weight analysis are depicted in Fig. 2a.

Measurement of myeloperoxidase activity (Fig. 2b) showed significant ($P < 0.01$) induction upon stimulation with TNBS in animals that did not receive Gen treatment. Administration of Gen resulted in suppressed MPO activity that was significantly ($P < 0.01$) lower when compared with the untreated TNBS-animals.

To further investigate the effects of Gen on inflammatory markers at a molecular level, both COX-2 and PCNA mRNA and protein expressions in the colon were investigated using Real-Time RT-PCR and western blot analysis. COX-2 mRNA expression was highest in the TNBS group. Upon treatment with Gen, COX-2 mRNA expression

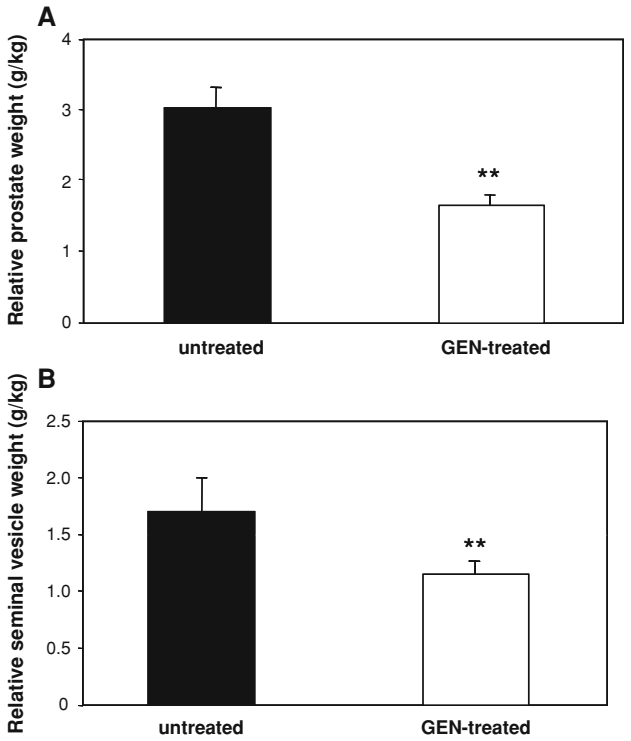

Fig. 1 Effect of 14-day oral treatment with genistein (Gen) on the wet weights of the prostate (**a**) and the seminal vesicles (**b**) of male Wistar rats (g/kg b.w.). Data shown are mean values ± SD ($n = 6$). **Mean values were statistically different for comparison *v*. The respective control group ($P < 0.01$); Mann–Whitney *U* test

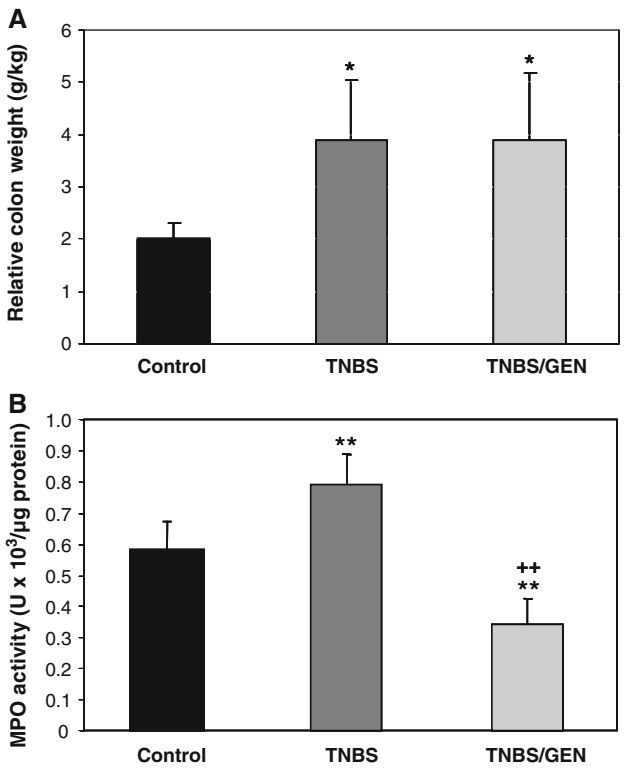

Fig. 2 Effect of 14-day oral treatment with Gen after TNBS stimulation on colonic wet weight (**a**) and colonic myeloperoxidase (MPO) activity (**b**). Data shown are mean values ± SD ($n = 6$). $^{*(*),+(+)}$Mean values were statistically different for the following comparisons: $^{*(*)}v$. Control values, $^{+(+)}v$. TNBS values, $^{*,+}P < 0.05$, $^{**,++}P < 0.01$; ANOVA. *Control* group not stimulated with TNBS (solvent-only), *TNBS* group stimulated with TNBS but not treated with Gen (vehicle-only), *TNBS/GEN* group stimulated with TNBS and treated with Gen

(Fig. 3a) was only marginally increased after TNBS stimulation when compared with the control group. This increase was significantly ($P < 0.05$) lower than in the untreated TNBS group. These results were supported by western blot analysis (Fig. 3b, c).

Expression of PCNA, a molecular marker of cell proliferation, was also increased on both mRNA and protein levels following the TNBS stimulus. Similar to COX-2, this increase was statistically significant at the mRNA level ($P < 0.05$). Treatment with Gen on the other hand had no significant inhibitory effect on either mRNA or protein expression of PCNA (Fig. 4a–c).

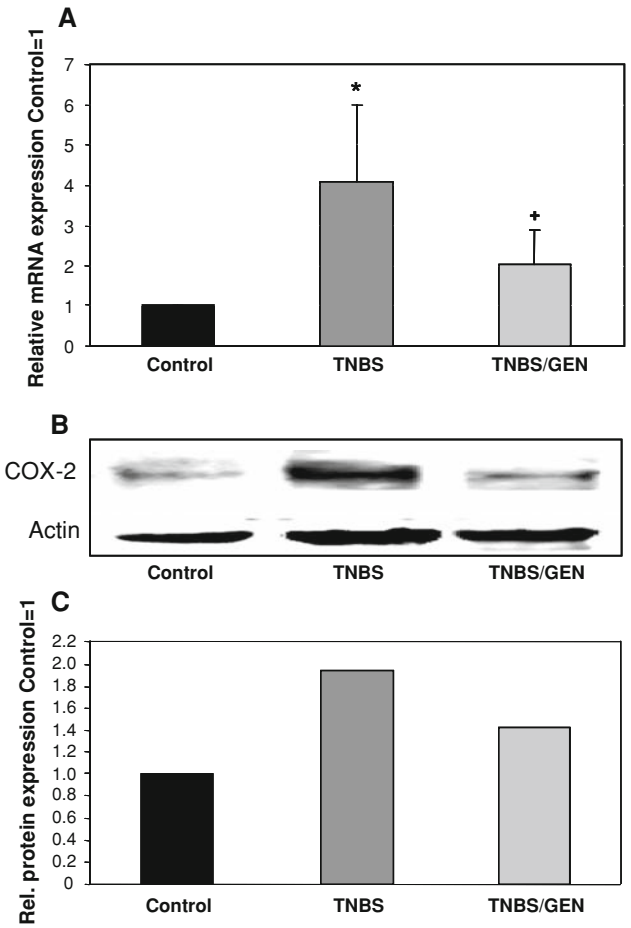

Fig. 3 Analysis of the effect of 14-day oral treatment with Gen after TNBS stimulation on cyclooxygenase-2 (COX-2) mRNA and protein expression in the colon. **a** mRNA expression of COX-2 determined by Real-Time RT-PCR. **b** Protein expression of COX-2 detected by western blot analysis. **c** Densitometric analysis of the shown western blot. Data shown are mean values ± SD. *,+Mean values were statistically different for the following comparisons: *v*. Control values ($P < 0.05$), +*v*. TNBS values ($P < 0.05$); dependent *t* test. *Control* group not stimulated with TNBS (solvent-only), *TNBS* group stimulated with TNBS but not treated with Gen (vehicle-only), *TNBS/GEN* group stimulated with TNBS and treated with Gen, *Rel.* relative

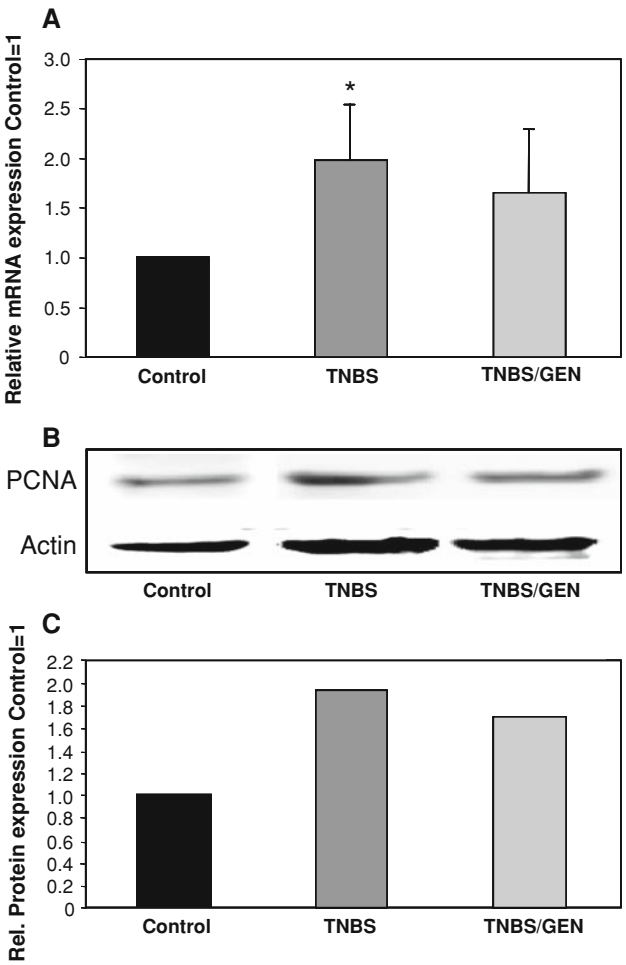

Fig. 4 Analysis of the effect of 14-day oral treatment with Gen after TNBS stimulation on proliferating cell nuclear antigen (PCNA) mRNA and protein expression in the colon. **a** mRNA expression of PCNA determined by Real-Time RT-PCR. **b** Protein expression of PCNA detected by western blot analysis. **c** Densitometric analysis of the shown western blot. Data shown are mean values ± SD. *Mean values were statistically different for comparison *v*. Control values ($P < 0.05$); *Control* group not stimulated with TNBS (solvent-only), *TNBS* group stimulated with TNBS but not treated with Gen (vehicle-only), *TNBS/GEN* group stimulated with TNBS and treated with Gen, *Rel.* relative

Discussion

This study was designed to investigate whether oral administration of Gen has beneficial anti-inflammatory effects in TNBS-induced chronic colitis, an in vivo rodent model. A dose of 100 mg/kg b.w. Gen was chosen for treatment based on previous studies investigating the hormonal activity of Gen in different rat strains [13]. Administration occurred by means of oral gavages, as the route of administration seems to be crucial for efficacy in animal models of IBD: unpublished studies of our laboratory in the TNBS-induced colitis model as well as in HLA-

B27 transgenic rats showed no anti-inflammatory effect of subcutaneously administered Gen.

Prior to the assessment of inflammatory parameters, we investigated whether there was a systemic response to Gen treatment, by measuring the relative wet weights of the prostates and seminal vesicles. These are accessory sexual organs that are very sensitive to treatment with estrogenic compounds. In our study, the wet weights of both organs significantly decreased following the administration of Gen, thereby indicating that the treatment was indeed effective (Fig. 1a, b). Similarly, the TNBS insult effectively induced an inflammatory reaction in the colon.

Colon wet weight significantly increased in both groups that were stimulated with TNBS when compared to the control group, probably due to localized thickening of the colon wall, water retention, and cellular infiltration [16].

However, treatment with Gen did not result in an effect on the colon weight increase (Fig. 2a). Consequently, we investigated molecular and biochemical markers of colitis.

MPO activity is another commonly studied and sensitive marker for inflammatory reactions in several tissues. It is an indicator for leukocyte infiltration, which is commonly found in inflamed tissues [33]. As expected, MPO activity was significantly induced by stimulation with TNBS (Fig. 2b). Interestingly, this stimulatory effect was completely blocked in those animals that received Gen treatment, thereby suggesting an anti-inflammatory effect of Gen.

The influence of Gen on inflammation was further assessed by analyses of COX-2 mRNA and protein expression levels in the colon. COX-2 is the inducible isoform of the two cyclooxygenase enzymes that catalyze key steps in prostaglandin biosynthesis from arachidonic acid [7]. COX-2 expression is normally low in most healthy tissues but is high in many tumors and rapidly induced in inflamed tissues following cytokine discharge via the NFκB pathway [29, 30].

The NFκB pathway can be modulated by estrogenic compounds: steroid estrogens are capable of inhibiting the activation of the NFκB pathway via binding to an ER [24]. Since Gen has the potential to bind to the ER and to elicit estrogenic responses [28] it was hypothesized that treatment with Gen might inhibit the expression of COX-2 via an ER-dependent mechanism, thereby reducing prostaglandin levels and subsequently the extent of colonic inflammation.

As depicted in Fig. 3a, Real-Time RT-PCR revealed clear increases in COX-2 mRNA levels in the inflamed colon tissue of the TNBS group animals ($\approx$4 fold). Resembling the data obtained by MPO analysis, the induction was significantly inhibited in the TNBS/Gen group, though mRNA levels were still elevated when compared to the control group ($\approx$2 fold). Similarly, western blot analysis revealed increased levels of COX-2 protein expression in the TNBS animals that were slightly lower in Gen-treated animals (Fig. 3b, c). The effects on the protein level were smaller in magnitude ($\approx$1.3 and 1.1 fold, respectively) in comparison with those on the mRNA level, suggesting that the inflammatory response seen at the transcriptional level might not have been directly translated into functional protein.

To assess effects of TNBS and Gen on processes of proliferation in the colon we measured the expression of the proliferation marker PCNA on mRNA and protein levels. As expected, PCNA expression was induced ($\approx$2 fold) in the animals of the TNBS group on both mRNA (Fig. 4a) and protein level (Fig. 4b, c), indicating that cell proliferation is a likely mechanism for the replacement and repair of damaged colon tissue. However, administration of Gen had no significant inhibitory effect on TNBS-induced PCNA induction. These results therefore do not support a positive role of Gen in the prevention of IBD-related colorectal carcinomas but cannot rule this out either. Hence, additional specific studies using models of colon cancer should be performed to clarify this.

The promising results of this study should not be taken as a generalized guideline for the dietary use of all phytoestrogens, as our previous study [27] suggests that lifelong phytoestrogen exposure might even increase acute inflammation. In addition, dietary factors other than isoflavones could be responsible for the low risk of developing IBD in East Asia: for example, n-3 polyunsaturated fatty acids that are abundant in fish have been shown to reduce early mucosal inflammation [2]. Furthermore, it has to be noted that this study was a basic research animal study comprising a small sample size. It is difficult to predict if and how results from such studies translate into the human clinical situation. Large cohort studies in both animals and humans should be able to provide an answer for this; the present small study can only be a starting point to encourage realization of such studies.

In conclusion, our experimental study provides evidence that oral treatment with Gen exerts beneficial anti-inflammatory effects in a rodent model of TNBS-induced chronic colitis. It might therefore be a suitable treatment of IBD. Whether it plays a role in the prevention of cancerous IBD-sequelae in the colon remains to be determined. Further studies are needed to investigate the efficacy of Gen treatment in IBD, to reveal the underlying mechanisms of action and to elucidate the seemingly contradictious effects of isoflavones in the prevention and treatment studies using the model of TNBS-induced colitis. In addition, larger, blinded, randomized, placebo-controlled studies will be necessary to predict transferability to the human clinical situation.

Acknowledgments This work was supported by DFG grant DI 716/10-3. The authors wish to thank Dr. Eric Houdeau (Institut National de la Recherche Agronomique, Toulouse, France) for providing valuable background information, Burkhardt Schleipen (German Sport University Cologne) for surgical assistance during this study and Martina Velders (German Sport University Cologne) and Dr. Lisa Rehm (Abbott Laboratories, Abbott Park, IL, USA) for careful and critical reading of the manuscript.

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