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Inducible nitric oxide synthase involvement in the mechanism of action of *Saccharomyces boulardii* in castor oil-induced diarrhoea in rats

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Abstract

The biotherapeutic agent *Saccharomyces boulardii* has been shown to inhibit castor oil-induced diarrhoea in rats in a dose–response fashion, and one of the suggested mechanisms of action included involvement of the nitric oxide pathway. The present study was designed to address this mechanism of action by firstly measuring the effects of *S. boulardii* on the inducible nitric oxide synthase (iNOS) isoform activity in vitro. Second, the effects of *S. boulardii* on the increase in colonic citrulline level associated with castor oil treatment were examined. In vitro, *S. boulardii* showed a dose-dependent inhibition of iNOS activity with an IC_{50} of 0.89 mg/ml. In the rat diarrhoea model, the antidiarrhoeal effect of *S. boulardii* was confirmed using a single oral dose of 12×10^{10} CFU/kg (viable cells). In this model, castor oil significantly elevated citrulline level from 2526 ± 164 to 3501 ± 193 nmol/g in the colon. When the rats were treated with the same antidiarrhoeal single dose of *S. boulardii*, no increase in citrulline level was observed. Moreover, the iNOS inhibitor 1400W at 10 mg/kg and the inhibitor of iNOS expression dexamethasone at 1 mg/kg, administered subcutaneously, blocked the citrulline production induced by the laxative. Taken together, these findings confirm the involvement of inhibition of the inducible isoform of nitric oxide synthase in the mechanism of action of *S. boulardii* in diarrhoea.

Keywords: *Saccharomyces boulardii*; Yeast; Diarrhoea; Castor oil; Nitric oxide; Inducible nitric oxide synthase

The biotherapeutic agent *Saccharomyces boulardii* [1,2] has been shown to decrease the incidence of antibiotic-associated diarrhoea in adults [3,4] and in children [5,6] and to diminish the risk of recurrence of *Clostridium difficile*-associated disease [7,8]. In inflammatory bowel diseases, the yeast improved the symptoms of Crohn's disease [9]. In various animal models, *S. boulardii* has exhibited effects consistent with these clinical findings. In hamsters, *S. boulardii* decreased the disruption of the flora homeostasis induced by clindamycin [10] and reduced the rate of mortality induced by clindamycin in a model of pseudomembranous colitis [11,12]. In gnotobiotic mice, this yeast protected against death due to *C. difficile* infection [13].

We have recently demonstrated that oral administration of *S. boulardii* inhibited castor oil-induced diarrhoea in the rat [14] in a dose-dependant fashion and made the observation that this effect was significantly reduced by L-arginine, a natural substrate of nitric oxide synthase (NOS). These results suggested the possibility of involvement of the nitric oxide (NO) pathway in the mechanism of action of *S. boulardii*.

NO is released through conversion of L-arginine to NO and L-citrulline by three isoforms of NOS [15]. Two isoforms are calcium dependent and constitutive, with one predominantly present in the brain (neuronal NOS,

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nNOS) and the other in the endothelium (endothelial NOS, eNOS). The third isoform, inducible (inducible NOS, iNOS), is calcium independent and found largely in cells of the macrophage line [16,17]. The inducible enzyme (iNOS) is present in very low concentrations in normal states, but is rapidly transcribed at times of immune activation, and active transcription can last for several days with significant increases in the total amount of NO synthesized [15]. The induction of iNOS in tissues can lead to the sustained production of high concentrations of NO that may exert pro-inflammatory effects including vasodilatation, edema, cytotoxicity, and the mediation of cytokine-dependent processes [18,19].

NO is involved in inflammatory bowel diseases [20,21] and in the laxative effect of castor oil [22,23]. Other systems induced by castor oil include the production of prostaglandins [24], changes in permeability [25], and stimulation of platelet-activating factor biosynthesis [26].

In the present study, we assessed the possibility of involvement of the NO pathway in the effects of *S. boulardii* using in vitro and in vivo approaches. First, the effect of the yeast on the in vitro activity of the inducible isoform on NOS isolated from mouse macrophages was examined. Second, the effect of *S. boulardii* on colonic mucosal citrulline level increased by castor oil during diarrhoea in the rat was investigated. Citrulline is a stable by-product of NO synthesis and is produced in equimolar amounts with NO. Citrulline level was measured as a marker of NO production because direct assay of NO is not practical due to its very short lifetime.

Materials and methods

Animals

Male Wistar rats were obtained from Elevage Janvier (Genêt-St Isle, France). The rats weighed between 180 and 200 g, after a minimum of 7 days of acclimatization to the housing conditions (temperature of 22 ± 2 °C; humidity $50 \pm 20\%$; A04 food from Safe (Epinay, France); and a 12L:12D photoperiod). Rats were fasted the night before ingestion of castor oil but were not deprived of water. Rats were randomly assigned to cages with an automatic watering system at the beginning of the experiment. All the experiments were carried out in accordance with the recommendations of the IASP (International Association for the Study of Pain) Committee for Research and Ethical Issues Guidelines.

Inducible NO synthase assay

Inducible NO synthase (iNOS) assay was done according to Tayeh and Marletta [27] by Cerep (France). iNOS (NOS II, EC 1.14.13.39) was isolated from murine macrophage (RAW 264-7) cells immunostimulated by lipopolysaccharides and interferon-γ (Cayman Chemical, Ann Arbor, MI, USA). Crude extracts of the macrophage were centrifuged at 100,000*g* to purify the enzyme in the supernatant in a two-step procedure involving an affinity column and an anion exchange column [28]. The activity of iNOS was quantified spectrophotometrically using the oxyhemoglobin assay [28,29]. This approach has been validated by Western blotting of the iNOS protein. The test compound, reference compound or water (control) is mixed in a buffer containing 40 mM Tris–HCl (pH 7.8), 0.5 mM NADPH (nicotinamide adenine dinucleotide phosphate), 4 μM FAD (flavin adenine dinucleotide), 12 μM BH_4 (tetrahydro-L-biopterin), 3 mM DTT (dithiothreitol), and 28 nM [^{3}H]L-arginine. The reaction is then initiated by the addition of the enzyme (0.5–3 μg) and the mixture is incubated for 30 min at 37 °C. For basal control measurements, the enzyme is omitted from the incubation medium. Following incubation, the reaction is stopped by adding an ice-cold buffer containing 20 mM Tris–HCl (pH 5.0), 2 mM EDTA (ethylenediaminetetraacetic acid), and 2 mM EGTA (ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid). [^{3}H]Citrulline is then separated from [^{3}H]L-arginine using an ion exchanger Dowex column (AG50X80). Collected [^{3}H]citrulline is quantified in a scintillation counter (LS series, Beckman) using a scintillation cocktail (Formula 989, Packard). The results are expressed as percent inhibition of the control enzyme activity. The standard inhibitory reference compound is 1400W that is tested in each experiment at several concentrations to obtain an inhibition curve from which its IC_{50} value (concentration causing a half-maximal inhibition of control values) is determined by non-linear regression analysis of the inhibition curve. *S. boulardii* was suspended in distilled water and tested using a broad concentration range, 0.03–0.1–0.3–1.0–3.0 mg/ml, with repeated measures. Yeast viability in the culture medium was checked in the same conditions and compared before and after incubation and no difference was observed. A second experiment was done with yeast autoclaved at 121 °C for 15 min to inactivate the yeast.

Castor oil-induced diarrhoea

Single doses of the yeast were given orally 1 h before the administration of 0.25 ml of castor oil, also by oral route, according to the protocol of our previous study [14]. The floor of the cage consisted of a grid, and all feces expelled from the rat fell through this grid onto a plate. The occurrence and severity of diarrhoea were recorded every hour for 5 h. Diarrhoea was scored cumulatively, according to an adaptation of the method of Hedge et al. [30] as follows: 0 for normal feces or no feces, 1 for well-shaped wet feces, 2 for shapeless feces, and 3 for unshaped feces with large amounts of liquid.