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The effect of oxidative stress upon the intestinal epithelial uptake of butyrate

Pedro Gonçalves, Inês Gregório, Telmo A. Catarino, Fátima Martel *

Department of Biochemistry (U38-FCT), Faculty of Medicine, University of Porto, Porto 4200-319, Portugal

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ABSTRACT

Our aim was to investigate the effect of oxidative stress upon butyrate uptake at the intestinal epithelial level. For this, IEC-6 cells were treated with *tert*-butylhydroperoxide 3000 μM (tBOOH), which increased levels of oxidative stress biomarkers, while maintaining cellular viability. The effect of tBOOH upon uptake of [^{14}C]butyrate ([^{14}C]BT) (10 μM) can be summarized as follows: (a) it caused a reduction in the intracellular accumulation of [^{14}C]BT over time, (b) it strongly reduced total [^{14}C]BT uptake but did not affect Na^+-independent uptake of [^{14}C]BT, and (c) it did not affect the kinetics of [^{14}C]BT uptake at 37 °C, but increased uptake at 4 °C. Moreover, tBOOH increased the efflux of [^{14}C]BT not mediated by breast cancer resistance protein. We thus conclude that tBOOH strongly inhibits Na^+-coupled monocarboxylate cotransporter 1 (SMCT1)-mediated, but not H^+-coupled monocarboxylate transporter (MCT1)-mediated butyrate uptake; moreover, it increases uptake and efflux of butyrate by passive diffusion. tBOOH did not affect the mRNA expression levels of MCT1 and SMCT1 nor their cell membrane insertion. Rather, its effect was dependent on extracellular signal regulated kinase 1/2 and protein tyrosine kinase activation and on the generation of reactive oxygen species by NADPH and xanthine oxidases and was partially prevented by the polyphenols quercetin and resveratrol. In conclusion, tBOOH is an effective inhibitor of SMCT1-mediated butyrate transport in non-tumoral intestinal epithelial cells. Given the important role played by butyrate in the intestine, this mechanism may contribute to the procarcinogenic and proinflammatory effect of oxidative stress at this level.

1. Introduction

Reactive oxygen species are naturally produced as a result of oxygen metabolism. Under physiological conditions, the burden of reactive oxygen species production is largely neutralized by an intricate antioxidant defense system (Wojcik et al., 2010). Increased reactive oxygen species level, also known as oxidative stress, is a result of either increased reactive oxygen species generation and/or a loss of antioxidant defense mechanisms (Khandrika et al., 2009). A major consequence of oxidative stress is damage of tissue via direct oxidation of nucleic acid bases, lipids and proteins, but also via profound alterations in signal transduction pathways, which can severely compromise cellular functions (Okayama, 2005). Not surprisingly, it is associated with numerous pathologies, from atherosclerosis to inflammation and cancer (Halliwell, 2001; Klaunig and Kamendulis, 2004; Stocker and Keaney, 2004).

The gastrointestinal tract is a major target for oxidative stress damage due to constant exposure of reactive oxygen species generated by a large variety of xenobiotics, endogenous toxic substances (e.g., bile acids), as well as microbes and their products (Ames, 1983). Interestingly, the etiology of many gastrointestinal tract diseases, such as colon cancer or inflammatory bowel disease, is associated with an imbalance in the cellular redox system leading to increased levels of reactive oxygen species (Acharya et al., 2010; Almenier et al., 2012; Seril et al., 2003). The effect of oxidative stress on membrane transport mechanisms at the intestinal level remains, however, poorly understood.

Butyrate, a product of intestinal flora fermentation of dietary fibre, plays a key role in colonic epithelium homeostasis, by having multiple regulatory roles at that level, including: being the main energy source for colonocytes; promotion of growth and proliferation of normal colonic epithelial cells; inhibition of colon carcinogenesis; inhibition of colon inflammation; and inhibition of oxidative stress (Hamer et al., 2008; Wong et al., 2006). Butyrate is transported into colonic epithelial cells by two specific carrier-mediated transport systems, the electroneutral H^+-coupled monocarboxylate transporter 1 (MCT1) and the Na^+-coupled monocarboxylate cotransporter (SMCT1) (Gupta et al., 2006). MCT1 (Cuff et al., 2005) and SMCT1 (Gupta et al., 2006) were recently proposed to function as tumor suppressors, most probably due to their ability to mediate the entry of butyrate into colonic epithelial cells. Therefore, factors that

* Corresponding author. Tel.: +351 22 0426654; fax: +351 22 5513624.
E-mail address: fmartel@med.up.pt (F. Martel).

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interfere with butyrate uptake into colonic epithelial cells are potentially detrimental to intestinal health and integrity by promoting oxidative stress, inflammation and colorectal cancer (Hamer et al., 2008; Wong et al., 2006).

Several studies have demonstrated that reactive oxygen species can interfere with protein, including membrane transporters, activity (Akram et al., 2006; Kumar et al., 2007). However, nothing is known concerning the effect of oxidative stress upon the intestinal absorption of butyrate. So, the aim of this work was to investigate the effect of oxidative stress on [^{14}C]butyrate ([^{14}C]BT) uptake by IEC-6 cells. IEC-6 cells are a nontumoral rat intestinal epithelial cell line (Wood et al., 2003). Oxidative stress was generated with *tert*-butylhydroperoxide (tBOOH), a useful model compound to study mechanisms of oxidative stress injury (Deiana et al., 2010; Garcia-Cohen et al., 2000; Griendling et al., 2000; Tupe and Agte, 2010).

2. Materials and methods

2.1. IEC-6 and Caco-2 cell culture

The IEC-6 and Caco-2 cell lines were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and were used between passages numbers 18–37 (IEC-6 cells) and 40–43 (Caco-2 cells). The cells were maintained in a humidified atmosphere of 5% CO_2 – 95% air. IEC-6 cells were cultured in Dulbecco's Modified Eagle's Medium:RPMI 1640 medium (1:1), supplemented with 10% fetal bovine serum, 0.1 U/ml insulin, 5.96 g HEPES, 2.2 g $NaHCO_3$, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (all from Sigma, St. Louis, MI, USA). Caco-2 cells were cultured in Minimum Essential Medium containing 5.55 mM glucose and supplemented with 15% fetal calf serum, 25 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (all from Sigma). Culture medium was changed every 2–3 days and the culture was split every 7 days. For subculturing, the cells were removed enzymatically (0.25% trypsin-EDTA, 5 min, 37 °C), split 1:3, and subcultured in plastic culture dishes (21-cm^2; ϕ 60 mm; Corning Costar, Corning, NY, USA). For determination of cell viability, measurement of glutathione levels and [^{14}C]BT uptake studies, cells were seeded on 24-well plastic cell culture clusters (1.9 cm^2; Ø 15.4 mm; TPP®, Trasadingen, Switzerland), and the experiments were performed 8–9 days after the initial seeding. For measurement of lipid peroxidation (thiobarbituric acid reactive substances assay) and protein carbonyl groups, cells were seeded on 12-well plastic cells culture clusters (3.9 cm^2; Ø 21.4 mm; TPP®) and the experiments were performed 8–9 days after the initial seeding.

2.2. Treatment of cells with tert-butylhydroperoxide (tBOOH)

Before each experiment, the cell culture medium was removed and the wells were washed with Glucose-Krebs (GK) buffer at 37 °C, containing in mM: 125 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 25 $NaHCO_3$, 1.6 KH_2PO_4, 0.4 K_2HPO_4, 5.5 glucose and 20 HEPES, pH 7.4 (GK-HEPES buffer). IEC-6 or Caco-2 cells were then incubated for 1 h at 37 °C with tBOOH (100, 1000 or 3000 μM) in GK-HEPES buffer. The effect of antioxidants and inhibitors of intracellular signalling pathways was tested by incubating IEC-6 cells in GK-HEPES buffer containing these compounds (or the respective solvents) for 20 min followed by incubation with tBOOH 3000 μM for 1 h in the presence of these compounds (or the respective solvents).

2.3. Evaluation of tBOOH-induced oxidative stress

The magnitude of oxidative stress induced by tBOOH was indirectly evaluated by measuring total, oxidized and reduced glutathione levels and generation of lipid peroxidation products and protein carbonyl groups.

2.3.1. Measurement of total, oxidized and reduced glutathione levels

IEC-6 cells were seeded on 24-well plates and submitted to treatment with tBOOH. Measurement of intracellular total glutathione levels was carried out according to a previously published method (Capela et al., 2007). Briefly, cultured cells were scraped and proteins precipitated with perchloric acid 5%, then centrifuged for 10 min at 4 °C and the supernatant was neutralized with an equimolar solution of $KHCO_3$. Total glutathione content was measured by the rate of colorimetric change of 0.7 mM 5,5-dithiobis(nitrobenzoic acid) at 415 nm in the presence of 0.4 U of glutathione redutase and 0.24 mM NADPH, using a microplate reader. Oxidized glutathione was also quantified, using 2-vinylpiridine to block free SH groups. Reduced glutathione levels were calculated according to the following reaction: total glutathione=reduced glutathione+2 oxidized glutathione.

2.3.2. Measurement of lipid peroxidation products (thiobarbituric acid reactive substances assay)

IEC-6 cells were seeded on 12-well plates and submitted to treatment with tBOOH. The extent of lipid peroxidation, which can be determined as the formation of malondialdehyde after the breakdown of polyunsaturated fatty acids, was measured by the thiobarbituric acid reactive substances assay (Fernandes et al., 1995). Briefly, 300 μl of cell suspension was precipitated with 200 μl of 50% trichloroacetic acid and centrifuged for 1 min at 6000 rpm. 300 μl of the supernatant were added to an equal volume of 1% thiobarbituric acid and the mixture was heated for 40 min at 95 °C, allowed to cool and the absorbance measured at 535 nm.

2.3.3. Measurement of protein carbonyl groups

IEC-6 cells were seeded on 12-well plates and submitted to treatment with tBOOH. Carbonyl (CO) groups (aldehydes and ketones) are produced on protein side chains when they are oxidized. Protein carbonyl content is the most used marker of protein oxidation (Dalle-Donne et al., 2003). The detection of protein carbonyl groups involves their reaction with 2,4-dinitrophenylhydrazine, which leads to the formation of a stable 2,4-dinitrophenyl hydrazone product, followed by the spectrophotometric quantification of the acid hydrazones (Levine et al., 1990). Briefly, carbonyl content was measured in the resultant pellet, that was treated with 0.5 ml of 2,4-dinitrophenylhydrazine (10 mM in HCl 2 M) or 0.5 ml of HCl 2 M for the blank. Samples were incubated for 1 h at room temperature, vortexing every 10 min. 0.5 ml of TCA 20% was added to each tube which was allowed to stand for 15 min at 4 °C. The resultant pellet was washed 3 times with ethanol–ethyl acetate (1:1), centrifuged at 13,000 rpm for 2 min at 4 °C and dissolved in 1 ml guanidine 6 M overnight. The solution was then centrifuged at 3000 rpm for 15 min. Absorbance was read at 340 nm and carbonyl content was calculated using the extinction coefficient of 22 000 M/cm.