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Advanced oxidation protein products decrease the expression of calcium transport channels in small intestinal epithelium *via* the p44/42 MAPK signaling pathway

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ABSTRACT

Advanced oxidation protein products (AOPPs), novel protein markers of oxidative damage, accumulate in the plasma of patients with inflammatory bowel disease (IBD). Osteoporosis, which is closely related to the regulation of intestinal calcium transport channels (CTCs), is a prevalent extraintestinal complication of IBD and is associated with oxidative stress. However, the underlying mechanisms are unknown. The present study aimed to verify whether AOPPs inhibit CTCs in the small intestinal epithelium and to identify the underlying mechanisms that may contribute to IBD-associated osteoporosis. Normal Sprague-Dawley rats were treated with AOPP-modified rat serum albumin. The calcium ion level in serum was not significantly altered, while the duodenal expression of CTCs (*e.g.* transient receptor potential vanilloid [TRPV6], calbindin-D9k [CaBP-D9k], plasma membrane Ca^{2+}-ATPase 1 [PMCA1], and Na^{+}/Ca^{2+} exchanger 1 [NCX1]) were decreased. In contrast, the levels of the related hormones that regulate calcium absorption including parathyroid hormone (PTH), 25-$(OH)D_3$, and 1,25-$(OH)_2D_3$ were increased, although the trend toward an increase in PTH levels was not significant. In order to further investigate the effects of AOPP exposure, we also evaluated the expression of CTCs (including the voltage-dependent L-type calcium channel [CaV1.3], TRPV6, CaBP-D9k, PMCA1, and NCX1) in cultured human colorectal adenocarcinoma cells (Caco-2). The expression levels of total CTC protein and mRNA, except for CaV1.3, were significantly down-regulated in a concentration- and time-dependent manner. Moreover, phosphorylation of p44/42 mitogen-activated protein kinase (MAPK) was observed *in vivo* and *in vitro*. The p44/42 inhibitor U0126 reversed the down-regulation of CTCs induced by AOPPs in the Caco-2 monolayer. Our results indicate that AOPPs down-regulate the expression of CTCs through p44/42 MAPK signaling mechanisms in the small intestinal epithelium. These data provide new insights regarding the molecular basis of AOPP-induced reductions in intestinal CTCs, and are relevant to understanding the mechanisms of IBD-associated osteoporosis. Further studies are needed to explore these mechanisms in greater detail.

Introduction

Advanced oxidation protein products (AOPPs), a new family of oxidized protein compounds, were first identified in the plasma of uremic patients undergoing maintenance dialysis (Witko-Sarsat et al., 1996). The AOPPs are a class of dityrosine-containing and cross-linking protein products formed primarily as a consequence of oxidative stress by the reaction of plasma proteins with chlorinated oxidants, such as chloramines or hypochlorous acid (HOCl) (Witko-Sarsat et al., 1996, 1998). Because the accumulative concentration of plasma AOPPs are closely correlated with the level of dityrosine (a hallmark of oxidized protein), the AOPPs have been considered as a novel marker of oxidant-mediated protein damage (Witko-Sarsat et al., 1996, 1998). Increased levels of AOPPs, which are also closely related to the severity of disease, have recently been found in diabetes (Kalousova et al., 2002; Martin-Gallan et al., 2003; Sebekova et al., 2012), coronary artery disease (Skvarilova et al., 2005), cancer (Kosova et al., 2007; Chang et al., 2008), liver

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cirrhosis (Zuwala-Jagiello et al., 2011), and chronic inflammatory bowel disease (IBD) (Baskol et al., 2008; Krzystek-Korpacka et al., 2008). This implies that the accumulation of AOPPs may be relevant in mechanisms underlying numerous pathophysiologic conditions.

Osteoporosis (OP), a systemic skeletal disease characterized by the destruction of bone microstructure and loss of bone mineral density (BMD), is a leading cause of morbidity in patients with IBD. Bone loss and OP in patients with IBD was first reported in the 1970s (Genant et al., 1976). There is emerging evidence to suggest that the risk of fracture is increased in IBD patients (Lin et al., 1996; Card et al., 2004). The involvement of oxidative stress – characterized by an oxidant/antioxidant imbalance – has become recognized as a ubiquitous occurrence in the development of IBD and in case of IBD complicated with OP (Almenier et al., 2012; Bianchi, 2010). Previous studies (Baskol et al., 2008; Krzystek-Korpacka et al., 2008; Alagozlu et al., 2012) have also illustrated enhanced generation of AOPPs are considered as novel markers of oxidative stress in IBD. Nevertheless, the role of AOPPs in the pathophysiological mechanisms underlying IBD in patients with OP complications is not well understood.

Evidence suggests that the reduction of intestinal calcium absorption is an important cause of BMD loss in IBD patients (Bianchi, 2010), ultimately leading to OP (Omi and Ezawa, 2011). The distinct processes by which calcium ions (Ca^{2+}) can be absorbed across the intestinal epithelium include paracellular and transcellular pathways (Hoenderop et al., 2005). The calcium transcellular pathway consists of the following three steps (Hoenderop et al., 2005; Nakkrasae et al., 2010): (1) apical calcium entry *via* transient receptor potential vanilloid (TRPV) 5 and 6 (TRPV5 and TRPV6) Ca^{2+} channels and the voltage-dependent L-type calcium channel, CaV1.3; (2) cytoplasmic diffusion in a calbindin-D9k (CaBP-D9k) – bound form; and (3) basolateral extrusion *via* plasma membrane Ca^{2+}-ATPase 1 (PMCA1) and the Na^{+}/Ca^{2+} exchanger 1 (NCX1). In addition, Ca^{2+} balance is regulated by several well-characterized hormones, such as parathyroid hormone (PTH) and vitamin D_3 metabolite 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) (Hoenderop et al., 2005).

It may be more appreciate to consider AOPPs as pro-inflammatory factors that play a crucial role in widespread biological events, and not just as markers of oxidative protein damage. The AOPPs are capable of inducing vascular-endothelial dysfunction (Chen et al., 2008) and accelerating atherosclerosis by enhancing oxidative stress and increasing expression of inflammatory factors (Liu et al., 2006). The AOPPs have been reported to induce podocyte apoptosis (Zhou et al., 2009) and intestinal epithelial cell death (Xie et al., 2014). These effects are dependent on the generation of reactive oxygen species (ROS) by activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which results in activation of mitogen-activated protein kinases (MAPKs). Moreover, it has been proposed that ROS may regulate Ca^{2+} homeostasis in several cell types through calcium ion channels, such as voltage-gated Ca^{2+} channels (CaV), transient receptor potential vanilloid (TRPV) channels, and plasma membrane calcium ATPases (PMCA) channels after oxidative stress stimulation (Bogeski et al., 2011; Waring, 2005). Xiao et al. (2010) further demonstrate that a redox imbalance in the duodenum was associated with down-regulated transcription of intestinal calcium absorption-related genes, which thereby reduced calcium absorption. However, it remains unknown if AOPPs affect the expression of calcium transport channels, or indeed, the serum calcium concentration during oxidative stress.

In the present study, we therefore hypothesized that AOPPs alter the expression of calcium transport channels (CTCs) and contribute to the induction of oxidative stress, thereby suggesting that there is a correlation between IBD and the regulation of CTCs. We evaluated the expression of CTCs (TRPV6, CaBP-D9k, PMCA1, and NCX1) in isolated tissues and the serum levels of calcium and several other mineral ions in AOPP-challenged rats. In addition, serum levels of PTH, 25-$(OH)D_3$, and 1,25-$(OH)_2D_3$ were simultaneously assessed in these rats. We also measured the effects of AOPPs on the expression of calcium transporters (CaV1.3, TRPV6, CaBP-D9k, PMCA1, and NCX1) in Caco-2 cells. Our aim was to better understand the mechanisms through which AOPPs affect the expression of CTCs in intestinal epithelium *via* the p44/42 MAPK signaling pathway.

Materials and methods

AOPP-albumin preparation

Advanced oxidation protein product-rat serum albumin (AOPP-RSA) was prepared *in vitro* by the incubation of RSA (Sigma, St. Louis, MO, USA) with HOCl (Fluke, Buchs, Switzerland), as described previously (Witko-Sarsat et al., 1996; Zhou et al., 2009; Li et al., 2007). In brief, 100 mg/mL of RSA was exposed to 200 mmol/L of HOCl for 30 min at room temperature and dialyzed overnight against phosphate-buffered saline (PBS) to remove the free HOCl (Li et al., 2007). The AOPP preparation was passed through a Detoxi-Gel column (Pierce, Rockford, IL, USA) to remove any contaminated endotoxin. The endotoxin levels in the preparation were tested using a Limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD, USA) (endotoxin levels were below 0.025 EU/mL). The content of the AOPP in the preparation – as determined by the absorbance at 340 nm of the reaction mixture of the samples and acetic acid (Witko-Sarsat et al., 1996; Zhou et al., 2009; Li et al., 2007) – was 51.6 ± 3.4 nmol/mg protein in AOPP-RSA *versus* 0.30 ± 0.03 nmol/mg protein in the unmodified RSA.

Caco-2 cell culture

Caco-2 human colon adenocarcinoma cells were obtained from the Committee on Type Culture Collection (Chinese Academy of Sciences, Shanghai, China). Cells were cultured and maintained in high-glucose (4500 mg/L D-glucose) Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Grand Island, NY, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Life Technologies, Newcastle, Australia), 1000 U/L penicillin, 100 mg/L streptomycin, and L-glutamine (2 mmol/L) in a 5% CO_2 and 95% air incubator at 37 °C. Cells were passaged using Hank's balanced salt solution (HBSS) containing 0.25% trypsin and 1 mM EDTA.

Caco-2 cell treatment and cell extract preparation

Cells were plated at 1.0×10^5 cells per well in six-well plates (Corning Inc., Corning, NY, USA) for Western blotting analysis (including assessment of MAPK phosphorylation) and mRNA expression, and plated at 5.0×10^4 cells per well in 24-well plates (Corning Inc.) for immunofluorescent staining. The growth medium was changed every other day. After growing to near confluence, cells were serum-deprived overnight before the addition of AOPP to the culture medium, as indicated later. To assess dose- and time-dependent changes, cells were incubated either in control medium, RSA (100 μg/mL), or AOPP-RSA (100–600 μg/mL) for 24 h, or with AOPP-RSA 400 μg/mL for different periods of time (0 h, 1 h, 3 h, 6 h, 12 h, 24 h). For the detection of the MAPK activation, cells were treated with 400 μg/mL AOPP-RSA for 0 min, 5 min, 10 min, 15 min, 20 min, 30 min, 60 min, 120 min, and 180 min. Inhibition experiments were performed by pre-treatment for 60 min with SP600125 (20 μM, a JNK MAPK inhibitor) or U0126 (10 μM, a p44/42 MAPK inhibitor). The effective concentrations of AOPP-RSA, SP600125, and U0126 were obtained from the literature. For immunofluorescent staining, the cells were stimulated by the control medium, by RSA (100 μg/mL), or by AOPP-RSA (400 μg/mL) for 24 h.