The nutritional supplement Active Hexose Correlated Compound (AHCC) has direct immunomodulatory actions on intestinal epithelial cells and macrophages involving TLR/MyD88 and NF-κB/MAPK activation

Abdelali Daddaoua a, Enrique Martínez-Plata b, Mercedes Ortega-González c, Borja Ocón d, Carlos J. Aranda c, Antonio Zarzuelo e, María D. Suárez c, Fermín Sánchez de Medina d, Olga Martínez-Augustin c*,

a Department of Environmental Protection, Consejo Superior de Investigaciones Científicas, C/ Profesor Albareda 1, E-18008 Granada, Spain
b Pharmacy Department, Hospital de Poniente, El Ejido, Almería, Spain
c Department of Biochemistry and Molecular Biology II, CIBERehd, School of Pharmacy, University of Granada, Granada, Spain
d Department of Pharmacology, CIBERehd, School of Pharmacy, University of Granada, Granada, Spain

1. Introduction

Active Hexose Correlated Compound (AHCC) is a nutritional supplement used in Japan and other Asian countries, as well as in the United States, as a dietary supplement to boost immune function (Matsushita et al., 1998; Yogita, Maruyama, Wakasugi, & Sukegawa, 2002). It is a mixture of polysaccharides, amino acids and lipids enriched in α-1,4-linked glucans (Kidd, 2000; Matsui et al., 2002; Matsushita et al., 1998) and it is derived from the mycelia of species of Basidiomycetes mushrooms: Shintake (Lentinus edodes) and Shimeji (Lyophyllum shimeji). AHCC has been the subject of multiple in vitro, in vivo and clinical studies in the last few decades (Aviles, Belay, Vance, Sun, & Sonnenfeld, 2004; Daddaoua et al., 2007; Gao et al., 2006; Turner & Chaudhary, 2009; Ye, Wakame, Ichimura, & Matsuzaki, 2004; Yin, Fujii, & Walshe, 2010). Studies in mouse models have shown that AHCC stimulates the immune system modulating the response against pathogens and increasing the survival following infections in mice (Ritz, 2008). These studies include a variety of infectious agents like influenza, Pseudomonas aeruginosa or meticillin-resistant Staphylococcus aureus (Ritz, 2008). An intestinal anti-inflammatory effect of AHCC dependent probably on its prebiotic effect has also been described in rats (Daddaoua et al., 2007). AHCC has been used widely in immunocompromised patients, especially those with cancer, in order to hasten patient recovery from antineoplastic therapy (Kidd, 2000; Matsui et al., 2002; Matsushita et al., 1998; Shah et al., 2011; Sumiyoshi et al., 2010; Turner & Chaudhary, 2009). Recent research indicates that AHCC enhances the immune response by multiple mechanisms, including augmented macrophage and natural killer cell proliferation (Matsushita et al., 1998; Nishioka, Akao, & Wakame, 2009; Ritz, Nogusa, Ackerman, & Gardner, 2006) and a higher production of various cytokines by macrophages and T
lymphocytes (interferon-γ (IFN-γ), interleukin (IL)-8, IL-1β, tumour necrosis factor (TNF-α, IL-2 and IL-12) (Yin et al., 2010).

Intestinal mucosal immunity is thought to be modulated by cytokine release from intestinal epithelial cells, but it is unclear how this crosstalk actually works, especially in relation to the influence of luminal factors, including nutrients and various dietary compounds. Moreover, luminal bacteria are considered to play a pivotal role in this regard, and the microbiota clearly influences intestinal epithelial cell (IEC) gene expression. In IECs the nuclear factor κB (NFκB) is a key player in maintaining intestinal barrier integrity since its stimulation is important for defensin production (Voss et al., 2006). Upon activation by various stimuli, NFκB transcriptionally regulates many cellular genes involved in early inflammatory responses, including cytokines, suggesting a possible role in inflammatory bowel disease (IBD) pathogenesis. However, this activation may be also important to contain effectively the luminal microorganisms and prevent bacterial translocation. Thus the mucosal immune system acts as a double edged sword in the interphase between the lumen and the internal milieu.

Colonic bacteria are probably handled in the intestinal mucosa to a greater extent by innate immunity mechanisms, which involve ligation by bacterial products of receptors devoted to the recognition of microbe-associated molecular patterns. These include the Toll-like receptors (TLRs). One of the TLRs that has received most attention is TLR4. In vitro, monocyte stimulation with the TLR4 agonist lipopolysaccharide (LPS) generates a significant change of gene expression (Frost, Nystrom, & Lang, 2002; Nau et al., 2002; Shoham, Huang, Chen, Golenbock, & Levitz, 2001), including the production of chemokines and cytokines. After recognition of microbial ligands, TLR signalling is initiated by binding of the adapter molecule MyD88 to the cytoplasmic Toll/interleukin-1 receptor (IL-1R) domain present in all TLRs. Recruitment of IL-1R-associated kinases (IRAK4, IRAK1 and tumour necrosis factor (TNF) receptor associated factor 6 (TRAF6)) results in activation of the mitogen activated protein kinases (MAPK) and NFκB pathways (Akira & Takeda, 2004).

The effects of AHCC on intestinal epithelial cells and monocytes/macrophages have been poorly studied. Some reports indicate immune enhancing effects on macrophages, but the molecular mechanism of action involved has not been assessed. Both intestinal epithelial cells and monocytes/macrophages are involved in the intestinal innate immune response. The study of the effects and mechanism of action of AHCC on these cell types would contribute to support its use as a nutritional supplement. This study aimed to determine the impact of AHCC on IEC (IEC18 and HT29 cells) and monocyte (THP-1) activation. The results show that AHCC has in vitro immunoenhancing effects stimulating the secretion of cytokines (growth regulated oncogene α (GROα), monocyte chemotactic protein (MCP-1), IL-1β, IL-8 and TNF-α) in cells, acting not only on the NFκB pathway but also by combining several inflammatory response pathways.

2. Materials and methods

2.1. Materials

Except were indicated culture media and reagents were purchased from Sigma (Barcelona, Spain).

2.2. Cell culture and treatment

The following cell lines were used in this work: IEC18 rat intestinal epithelial cells (ECACC 88011801), HT-29 human intestinal epithelial cells (ECACC 91072201) and THP-1 human monocytic cells (ECACC 88081201). The cells were cultured in DMEM (25 mM glucose) and RPMI-1640 medium, respectively, supplemented with heat-inactivated foetal bovine serum (10% v/v), 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 2.5 μg/ml amphotericin and 2 mM glutamine.

2.3. AHCC preparation

AHCC was provided by Amino Up Chemical (Sapporo, Japan). The AHCC manufacturing process has been described elsewhere (Miura, Kitadate, Nishioaka, & Wakame, 2010). Commercial AHCC was freshly prepared by dissolving the original powder in complete DMEM at a final concentration of 5 mg/ml. After 10 min of sonication to ensure total dissolution, it was passed through 0.22 μm filters and used immediately.

2.4. Effect of AHCC on cytokine secretion

Cytokine secretion (GROα and MCP-1 for IEC18 cells, IL-8 for HT29 cells and IL-1β, TNF-α and IL-8 for THP-1 cells) was measured in the 24 h supernatant of cells treated with AHCC (0.05, 0.5 and 5 mg/ml). In some cases, specific pathway inhibitors were added prior to AHCC treatment (see below). Cytokines were quantitated by ELISA (Biosource Europe, Nivelles, Belgium and BD Biosciences, Erembodegem, Belgium), following the protocols recommended by the manufacturers.

2.5. Characterisation of the signal transduction pathways using the NFκB and MAPK inhibitors

The cells were treated as above using the concentration of 5 mg/ml of AHCC, after the addition of specific inhibitors, including Bay 11-7082 (IkBα phosphorylation), PD98059 (ERK1/2 MAPK), SB203580 (p38 MAPK), SP600152 (JNK MAPK) and wortmannin (phosphatidylinositol-3 kinase, PI3K). These inhibitors of intracellular signalling were added to the culture 60 min before the addition of 5 mg/ml of AHCC to cells. All inhibitors were used at a concentration of 10 μM except wortmannin, which was used at 1 μM.

2.6. Western blot

For Western blot experiments IEC18, HT-29 and THP-1 cells were cultured with AHCC for different periods of time (0, 15, 30, 60, 90 min, 3, 6 and 24 h), then the cells were collected and processed as described. Briefly, the samples were homogenised in lysis buffer (0.1% w/v SDS, 0.1% w/v sodium deoxycholate, 1% v/v Triton X-100 in PBS) with protease inhibitor cocktail 1:100 (v/v). Then homogenates were sonicated and centrifuged at 7000g for 5 min at 4 °C. Protein concentration was determined by the bicinchoninic acid assay (Smith et al., 1985). Samples were boiled for 5 min in Laemmli buffer, separated by SDS–PAGE, electroblotted to activated PVDF membranes (Millipore, Madrid, Spain), and probed with the corresponding antibodies. The bands were detected by enhanced chemiluminescence (PerkinElmer, Waltham, MA) and quantitated with NIH software (Scion Image). The composition of the Laemmli buffer (5×) was: 312 nM SDS, 50% v/v glycerol, 1% v/v 2-mercaptoethanol, 22.5 mM EDTA trisodium salt, 220 mM Tris and traces of bromphenol blue (pH = 6.8). The primary antibodies used were from Cell Signalling (Danvers, MA) except the JLA20 antibody against α-actin, which was obtained from the Development Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences.
2.7. Gene silencing

IEC18 cells were pretreated in some experiments with shRNA specific for MyD88 and TLR4 (Santa Cruz Biotechnologies, Heidelberg) for gene knockdown, following the manufacturer’s instructions. Briefly, IEC18 cells were plated on six well plates and grown for 24 h until 50% of confluence. Before infection, IEC18 medium was supplemented with polybrene 5 μg/ml and cells were incubated for 10 h. Control, MyD88 and TLR4 shRNA lentiviral particles were also separately pretreated with polybrene 5 μg/ml for 30 min, added to the culture medium and incubated overnight. On the third day, fresh medium was substituted for lentiviral particles containing medium and the cells were cultured until confluence for 24 h. Finally, IEC18 cells were split (1:5), cultured again for 24 h, and selected with a range of 5–10 μg/ml of puromycin dihydrochloride.

2.8. Statistical analysis

All results are expressed as mean ± SEM. Differences among means were tested for statistical significance by one-way ANOVA and a posteriori least significance tests on preselected pairs. All analyses were carried out with the SigmaStat 3.5 program (Systat, San Jose, CA). Differences were considered significant at p < 0.05.

3. Results

3.1. AHCC induces cytokine secretion in IECs in a concentration dependent fashion

The effect of AHCC on IECs was tested by examining the production of GROα and MCP-1 by the rat colonic epithelial cell line IEC18 grown as monolayers in standard culture conditions. AHCC was added at three different concentrations (0.05, 0.5 and 5 mg/ml) and cytokine levels were determined after 24 h. In both cases AHCC exerted a significant stimulatory effect (Fig. 1A).

Next it was aimed to confirm the stimulatory effect of AHCC on human IECs, namely HT-29 cells. The data shown in Fig. 1B indicate that IL-8 (considered the human orthologue of rodent GROα) was induced in a concentration dependent fashion in these cells. The observed effect at 5 mg/ml was maximal and the potency of the effect was similar to that observed in IEC18 cells.

3.2. AHCC evoked cytokine secretion in IEC18 cells depends on NfkB, PI3K and p38 MAPK

It was aimed to identify the signal transduction pathways involved in cytokine induction by AHCC using a pharmacological approach. As shown in Fig. 2A, the NfkB inhibitor, Bay11-7082, completely obliterated GROα and MCP-1 secretion in AHCC treated IEC18 cells, suggesting a determinant role of this transcription factor. In addition, there was a substantial inhibitory effect of the PI3K blocker wortmannin on GROα (76%, p < 0.05), but not MCP-1. We also looked at MAPK pathways (Fig. 2B). A significant involvement of the p38 MAPK is suggested by the marked suppression of the AHCC response obtained with the specific inhibitor SB203580. The specific JNK inhibitor SP600125 had a weak but significant effect on GROα secretion but appeared to fully prevent MCP-1 release. There was no significant change with the use of PD98059, an ERK1/2 blocker.

3.3. AHCC induces cytokine secretion in IEC18 cells via MyD88 and TLR4

In order to test the possible involvement of MyD88 and TLR4 in AHCC effects in IEC18 cells, the respective gene expression was knocked down with specific shRNAs and cytokine secretion was compared to that obtained with control shRNA treated cells. The results are shown in Fig. 3. LPS, used as positive control for MyD88/TLR4 dependent cytokine secretion, elicited a strong response under control conditions which was reduced significantly with either shRNA. MyD88 knockdown resulted in ~50% inhibition of both GROα and MCP-1 secretion (Fig. 3A). TLR4 silencing had however a much more powerful effect on LPS evoked MCP-1 release than on GROα (Fig. 3B). The effect of AHCC (5 mg/ml) on cytokine secretion was roughly half of that obtained with 10 μg/ml of LPS, in line with previous experiments. However, this effect was almost completely obliterated when MyD88 or TLR4 expression was downregulated.

3.4. AHCC induces cytokine secretion in THP-1 cells in a concentration dependent fashion

The effect of AHCC in THP-1 cells, a human monocyte cell line, grown in suspension in standard conditions was tested. IL-8 was also increased in these cells but with a higher potency than in IECs, since the effect was quite marked at the low concentrations of 0.05 and 0.5 mg/ml (Fig. 4A). IL-1β and TNF-α were also...
upregulated by AHCC, with maximal secretion occurring at 5 mg/ml (Fig. 4B and C).

3.5. Activation of different signalling pathways by AHCC in HT-29 and THP-1 cells

Both the MAPK and the NFκB signalling pathways have been shown to be implicated in the production of IL-8 in monocytes/macrophages (Beinke & Ley, 2004). We assessed the functioning status of these pathways in HT-29 and THP-1 cells exposed to AHCC using Western blotting and phosphospecific antibodies and the effects of specific inhibitors on IL-8 secretion (Fig. 5).

As shown in Fig. 5A, AHCC evokes the phosphorylation of IκB-α, pertaining to the NFκB pathway, and of the MAPKs JNK, ERK and p38, in HT-29 cells. IκB-α is phosphorylated in Ser32 by IκB kinase in the canonical pathway of activation of NFκB, resulting in the release and nuclear translocation of the NFκB dimers. Bay 11-7082 inhibits this phosphorylation step and therefore blocks the NFκB canonical activation pathway. In HT-29 cells this resulted in complete ablation of the IL-8 response to AHCC. All three MAPKs were also involved according to the inhibitory effects of the respective blockers, in the order p38 > ERK > JNK. The impact of Bay11-7082 or SB203580 was maximal, so that no additional inhibition was possible.

The situation was similar in THP-1 cells (Fig. 6), except that the increase in phosphorylation in IκB-α and ERK was lower and that in JNK and p38 very intense. Despite this observation, the effect of ERK inhibition was more pronounced than that of p38. The JNK blocker SP600125 was the only probe that prevented completely AHCC evoked IL-8 secretion, even compared to Bay11-7082, which had an important but incomplete inhibitory effect.
4. Discussion

AHCC is used widely in humans in a number of countries and it has been extensively studied in animal models. Interest in AHCC is growing, on the basis of the advantages of nutritional supplements in the management of human disease. In particular, AHCC has well-documented immunomodulatory properties and the possible role of AHCC in inflammatory bowel disease treatment was of particular interest. Our group originally studied the colonic anti-inflammatory properties of this agent based on its high content in non-absorbable glucids, which suggested prebiotic properties. Using the rat trinitrobenzenesulfonic acid model of colitis, AHCC showed significant therapeutic effects that were associated to the expected modulation of colonic microbiota, with increased bifidobacteria and lactic acid bacteria and decreased clostridia (Daddaoua et al., 2007). Thus AHCC does appear to function as a prebiotic. However, these data do not exclude the possibility that AHCC may have additional, unrelated mechanisms of action in intestinal inflammation. Hence the present study aims to respond to this question by assessing effects exerted by AHCC in vitro in the complete absence of bacteria (or bacterial components).

Our results confirm that AHCC produces immunomodulatory effects in epithelial cell lines of both rat and human origin (IEC18 and HT-29). IEC18 cells were selected because they are nontumoral and they can be used to study changes in signal transduction using transfection techniques (Beinke & Ley, 2004; Lopez-Posadas et al., 2010). HT-29 cells were used additionally with two objectives: first, to confirm that AHCC has effects on colonic cells (IEC18 cells have ileal phenotype), and second, to extend our observations to human cells. Of course, intestinal epithelial cells were initially focused on because these are an obvious putative target of intraluminal agents such as AHCC. Of note, AHCC stimulated rather than decreased the secretion of proinflammatory cytokines. 

Fig. 3. Effect of MyD88 and TLR4 gene knockdown on AHCC evoked cytokine secretion in IEC18 cells. IEC18 cells transfected with shRNA for Myd88 (A) or TLR4 (B) or a control vector were treated with AHCC (5 mg/ml) and cytokine concentration was measured in the culture medium ELISA after a 24 h incubation. LPS treated cells were included as a control. GROα basal secretion was 7186.0 and 732.5 pg/ml (A and B respectively); MCP-1 basal secretion was 36327.7 and 21298.1 pg/ml (A and B respectively). *p < 0.05 vs. the respective control; †p < 0.05 vs. control shRNA.

Fig. 4. Effect of AHCC on cytokine secretion in THP-1 cells. (A) IL-8 secretion; (B) IL-1β; (C) TNF-α secretion. Cells were treated with different concentrations of AHCC (0.5, 1 or 5 mg/ml) and cytokine concentration measured in the culture medium by ELISA after a 24 h incubation. Results are expressed as mean ± SEM of three different experiments (n = 3 in each experiment). †p < 0.05 vs. control (C).
in these cells. This was a concentration dependent effect that was maximal at 5 mg/ml. Furthermore, the magnitude of the response was quite substantial, since it was approximately half of that achieved with 10 µg/ml of LPS, a submaximal concentration.

It was hypothesised that the high sugar content of AHCC could make it a putative TLR4 ligand, based on the same occurrence in the prototype TLR4 agonist, LPS. Hence we used shRNA gene silencing to modulate LPS and AHCC cytokine secretion in IEC18 cells. As expected, LPS effects were significantly inhibited when either MyD88 or TLR4 were downregulated. MyD88 is an intracellular signalling protein involved in the effects of all TLRs except TLR3 (Brikos & O’Neill, 2008). Some of the effects of the other TLRs are also independent of MyD88. Interference with TLR4 expression is therefore expected to produce similar results to those of MyD88 downregulation. This was indeed the case with AHCC, indicating that AHCC ligates TLR4 and further signals through MyD88. It is interesting to note that inhibition of LPS effects was less prominent. This may be due to incomplete gene downregulation coupled to the stronger stimulus of LPS vs. AHCC. Since Myd88 silencing had a stronger inhibitory effect that TLR4 knockdown, it is plausible that AHCC may affect other Myd88 dependent receptors, as has been suggested recently for TLR2 (Nishioka et al., 2009).

MyD88 binds to several intermediate proteins including IRAK1/4, TRAF6 and TAK1, finally converging on activation of the IKK complex, which in turn phosphorylates IkB-α, resulting in the release of the active NFκB dimers. Our data indicate that IkB-α phosphorylation is required for AHCC effects, consistent with TLR4/MyD88 signalling. Although involved, MAPKs and PI3K appear to play a secondary role (except for the latter in GROα secretion), since the effect of Bay11-7082 is maximal. Remarkably, the effects on HT-29 were very similar, although MAPK involvement was of a higher magnitude, such that full inhibition could be achieved simply by targeting these kinases.

Although only the surface of the intestinal epithelium is expected to be in contact with luminal agents such as bacteria, bacterial products or nutrients, including AHCC, interaction with other cell types present in the mucosa, below the intestinal epithelium surface, cannot be excluded, especially in situations of impaired barrier function and increased permeability, such as inflammatory bowel disease. Thus the effects of AHCC on monocytes/macrophages using the THP-1 cell line as a model were examined. AHCC concentration dependently evoked proinflammatory cytokine secretion, just like it did in epithelial cells. The results agree with recent results showing that AHCC induces IL-1β in human monocytes promoting Th17 and Th1 responses, thus enhancing the intestinal immunity (Lee, Lee, Fujii, & Kang, 2012). On the other hand, an in vivo study indicated that AHCC increases the frequency of CD4(+) and CD8(+) T cells producing IFN-γ alone, TNF-α alone or both (Yin et al., 2010). Although this study used different cell types, it is consistent with the augmented TNF-α production by AHCC described here. A pharmacological analysis of signal transduction operative in THP-1 cells showed results very similar to those obtained with IEC18 and specially HT-29 cells, i.e. almost complete dependence of IkB-α phosphorylation and/or MAPK activation, particularly JNK.

Fig. 5. Involvement of MAPKs and NFκB in AHCC evoked IL-8 secretion in HT-29. Cells were pretreated with different inhibitors (described in the text) just before the addition of AHCC (5 mg/ml). Activation pathways were assessed by Western blot and by the inhibition of cytokine secretion as measured by ELISA in the culture medium after a 24 h incubation. α-Actin was used as a loading control. Data are mean ± SEM from at least three independent experiments. *p < 0.05 vs. control group; †p < 0.05 vs. AHCC group. IKBT: IkB-α total.
In conclusion, the results suggest that AHCC activates TLR4 in intestinal epithelial cells and monocytes, eliciting the expected downstream signalling steps leading to NF-κB/MAPK activation and the secretion of proinflammatory cytokines. The results, on the other hand, prompt the question of the relevance for the treatment of a chronic inflammatory disease. Although counterintuitive, this mechanism is compatible with the hypothesis that inflammatory bowel disease may result not from an overactive immune system but to a relatively weak one. Failure of the mucosal barrier to contain the bacterial flora results in translocation and a robust inflammatory response. Thus inflammation may be secondary to a primary defect in the mucosal defence. For instance, Nenci et al. observed that conditional suppression of intestinal epithelial expression of IKK-γ (also known as NEMO) or IKK-α/β, resulting in reduced activation of the NF-κB pathway, produced a severe inflammatory response (Nenci et al., 2007). In a different study, GM-CSF administration has been shown to be protective in experimental colitis acting on innate immunity mechanisms (Sainathan et al., 2008). Accordingly, GM-CSF knockout mice are more susceptible to inducible colitis (Xu, Hunt, & Bao, 2007). Similarly, lack of expression of CXCL1, considered the main chemokine responsible for neutrophil recruitment in the colon, is associated with augmented colitis (Shea-Donohue et al., 2008). In line with these findings, neutrophil depletion itself aggravates colitis (Kuhl et al., 2007). Thus AHCC may work in part by enhancing mucosal barrier function and defence.

Finally, it is worthwhile to comment that AHCC has been widely used to overcome the side-effects of chemotherapy in cancer patients. Chemotherapy affects the intestinal mucosa producing mucositis and loss of mucosal integrity (van Vliet, Harmsen, de Bont, & Tissing, 2010). Although it is always difficult to translate in vitro results to in vivo effects, the action of AHCC on the mucosal barrier could also provide a molecular and cellular explanation for its beneficial effects on the management of side-effects induced by chemotherapy in patients with cancer.

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**References**


