

SHORT COMMUNICATION

Evidence that Marine-derived, Multi-mineral, Aquamin Inhibits the NF- κ B Signaling Pathway *In Vitro*

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It is well established that nuclear factor kappa B (NF- κ B) is a central regulator of the immune response and that dysregulation of NF- κ B contributes to the pathogenesis of many autoimmune and inflammatory diseases. The food supplement Aquamin is a natural multi-mineral derived from the red algae *Lithothamnion corallioides*, rich in calcium, magnesium and 72 other trace minerals. This study describes an anti-inflammatory role for Aquamin in inhibiting NF- κ B activation through reducing the phosphorylation and degradation of its upstream inhibitor I κ B α . Aquamin inhibition of NF- κ B activation results in significantly reduced cyclo-oxygenase-2 gene expression following treatment of macrophage cells with lipopolysaccharide. These data suggest that nutritional supplements such as Aquamin may play an important role in regulating the inflammatory response by modulating the nuclear factor kappa B signalling pathway. Copyright © 2011 John Wiley & Sons, Ltd.

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INTRODUCTION

Nuclear factor kappa B (NF- κ B) is the master regulator of the immune response, its dysregulation has been shown to contribute to the pathogenesis of a wide range of autoimmune and inflammatory diseases (D’Acquisto *et al.*, 2002). Recent reports suggest that NF- κ B is a promising target for the anti-inflammatory activity of many natural compounds including flavonoids, curcumin, green tea polyphenols, resveratrol and grape seed procyanidins (Luqman and Pezzuto, 2010). NF- κ B also controls the expression of a number of key pro-inflammatory genes including cyclo-oxygenase-2 (COX2). COX2 is a well established target for non-steroidal anti-inflammatory drugs (NSAIDs) frequently employed to provide relief from inflammation and pain.

Growing evidence suggests that mineral-rich seaweed extracts may play an important role in the regulation of inflammation (Smit, 2004). The food supplement Aquamin is a natural, seaweed-derived, multi-mineral rich in calcium and magnesium and trace amounts of 72 other minerals. Aquamin has been shown previously to provide relief from the symptoms of osteoarthritis (Frestedt *et al.*, 2008, 2009) and to be of benefit in bone (Aslam *et al.*, 2010a) and digestive health (Aslam *et al.*, 2010b). It has also been shown recently that Aquamin attenuates lipopolysaccharide (LPS)-induced tumour necrosis factor alpha (TNF α) and interleukin-1 beta (IL-1 β) secretion *in vitro* (Ryan *et al.*, 2011).

This study aims to elucidate whether Aquamin can inhibit the NF κ B signalling pathway and the expression of the downstream pro-inflammatory gene, COX2, following LPS-stimulation in macrophages.

MATERIALS AND METHODS

Aquamin (GRAS 000028) is a natural product obtained from the skeletal remains of the red marine algae *Lithothamnion corallioides* harvested under approved licence off the coasts of Ireland and Iceland and prepared under ISO and HACCP certification. Aquamin contains 12% calcium, 1% magnesium and measurable levels of 72 other trace minerals as previously reported (Ryan *et al.*, 2011). Approximately 0.5 mg/mL of Aquamin is equivalent to a physiological level of extracellular Ca²⁺.

Lipopolysaccharide from *E. coli* (O55:B5) was purchased from Sigma (St Louis, MO). The murine macrophage cell line RAW 264.7 was cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). The NF- κ B luciferase reporter plasmid and the pRLTK plasmid were purchased from Promega (Madison, WI). The RAW cells were transfected with reporter constructs using Turbofect (Fermentas). The luciferase activities of the whole cell lysates were analysed using a dual-luciferase reporter assay system as described previously (Dyer *et al.*, 2000). Co-transfection of the *Renilla* luciferase expression vector pRL-TK (Promega) was used as an internal control for all reporter assays. For all samples, the data were normalized for transfection efficiency by dividing firefly luciferase activity by that of the *Renilla* luciferase.

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To prepare whole cell lysates, the cells were lysed in RIPA buffer containing 50 mM Tris-HCl pH 7.4, 1% NP40, 0.25% deoxycholate, 1.50 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate and 1x Complete Protease Inhibitors (Roche). Equal quantities of whole cell lysates were resolved by electrophoresis on a denaturing SDS polyacrylamide gel and transferred to a nitrocellulose membrane. Following immunoblotting with DyLight conjugated secondary antibodies (Pierce), the membrane was analysed using an Odyssey infra red scanner (LiCor). The antibodies used for immunoblotting were anti-I κ B α , anti-phospho-I κ B α (Cell Signalling Technologies) and anti- β -actin antibody (Sigma).

For real time PCR analysis cells were lysed in Side-step lysis buffer (Stratagene) and cDNA synthesized using the Transcriptor cDNA synthesis kit (Roche). Real time PCR was performed using primers for COX2 and the Universal Probe Library system (Roche) and analysed on a Light Cycler 480 (Roche).

RESULTS AND DISCUSSION

The effect of Aquamin on NF- κ B transcriptional activity was analysed using a NF- κ B luciferase reporter plasmid in the RAW 264.7 macrophage cell line. NF- κ B transcriptional activity was induced by stimulating cells with LPS, a well characterized trigger of inflammation and activator of NF- κ B. As demonstrated in Fig. 1A, the RAW264.7 cells exhibited a robust NF- κ B transcriptional response when compared with unstimulated control cells. Cells that were pretreated with Aquamin (0.5 mg/mL) for 1 h prior to LPS stimulation exhibited a clear reduction in the level of NF- κ B transcriptional activity when compared with untreated cells ($p < 0.05$). This demonstrates that Aquamin is an inhibitor of NF- κ B activity in LPS treated macrophages.

NF- κ B transcriptional activity is inducible, and under normal conditions is tightly regulated. In unstimulated cells NF- κ B is maintained in the cytoplasm through interaction with the inhibitory protein, inhibitor of kappa B alpha (I κ B α). In stimulated cells, I κ B α is phosphorylated by the upstream I κ B kinase (IKK) kinase complex. Phosphorylation of I κ B α triggers its

ubiquitination and proteasomal degradation. In the absence of I κ B α , NF- κ B is free to translocate to the nucleus where it may bind its cognate binding sites in the promoter regions of target genes and activate transcription. Thus, the phosphorylation and degradation of I κ B α is a key event in the activation of NF- κ B transcriptional activity. This paper analysed the effect of Aquamin treatment on the ability of LPS to induce the phosphorylation and degradation of I κ B α to gain further insight into the mechanism of NF- κ B inhibition. As Fig. 1B shows, pretreatment of cells with Aquamin (0.5 mg/mL) for 1 h prior to LPS stimulation resulted in a significant inhibition in the phosphorylation and degradation of I κ B α when compared with the untreated cells. This indicates that Aquamin is acting at or upstream of the IKK kinase complex in the NF- κ B pathway.

The study next sought to determine the effect of Aquamin on the expression of COX2 in LPS stimulated RAW 264.7 macrophages to further characterize its anti-inflammatory properties. Real time PCR analysis of the LPS-stimulated cells demonstrated a significant inhibition of COX2 expression in cells pretreated with Aquamin (0.5 mg/mL) for 1 h prior to stimulation relative to the untreated cells (Fig. 2). These data suggest that the previously observed anti-inflammatory properties of Aquamin may result from reduced NF- κ B activation and transcription of target genes such as COX2.

The naturally occurring food supplement, Aquamin, is rich in calcium and magnesium as well as trace amounts of other minerals. Many of these minerals including magnesium, copper, manganese, selenium and zinc are now recognized as important regulators of inflammation. Magnesium may influence inflammation through reducing the serum level of the pro-inflammatory protein C-reactive protein. Copper, zinc and manganese are essential cofactors of the antioxidant enzyme superoxide dismutase. Selenium is a vital constituent of glutathione peroxidase. Consequently, many of the antioxidant-related minerals that compose Aquamin may be anti-inflammatory in nature.

This study has elucidated a role for Aquamin in regulating NF- κ B and COX2 activity in LPS-stimulated murine macrophages. These findings suggest that in

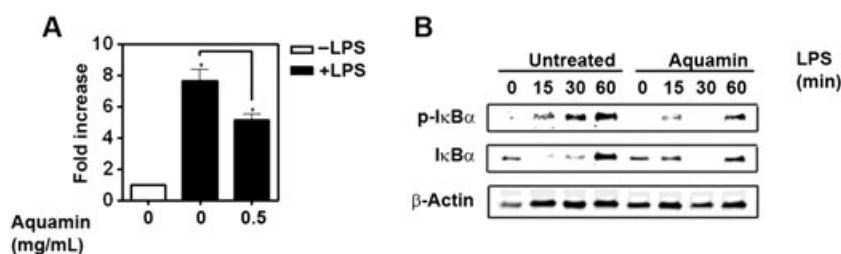


Figure 1. (A) Aquamin inhibited NF- κ B activity in LPS treated RAW 264.7 macrophage. RAW 264.7 macrophage cells were transiently transfected with a NF- κ B luciferase reporter plasmid for 24 h followed by treatment with Aquamin (0.5 mg/mL) for 1 h prior to stimulation with 100 ng/mL LPS for 8 h before luciferase activity was measured. Cotransfection of the *Renilla* luciferase expression vector pRLTK was used as an internal control for the normalization of transfection across all samples. Induction of reporter activity is presented as the fold increase over unstimulated cells. Data are presented as the mean of triplicate samples \pm SEM and are representative of three independent experiments: * $p < 0.05$. (B) Aquamin inhibited LPS-induced I κ B α phosphorylation and degradation. RAW 264.7 macrophage cells were untreated or pretreated with 0.5 mg/mL Aquamin for 60 min prior to stimulation with LPS for the indicated time. Whole cell lysates were prepared and resolved by SDS PAGE. Immunoblot analysis of total I κ B α and phospho-I κ B α (Ser32/33) protein levels was performed using specific antibodies as indicated. Protein loading was assessed by immunoblotting with anti- β -actin antibody. Data are representative of three independent experiments.

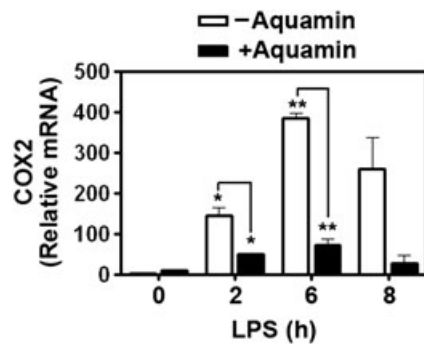


Figure 2. Aquamin inhibits LPS-induced COX2 expression. RAW 264.7 macrophage cells were treated with 0.5 mg/mL Aquamin or left untreated for 1 h prior to stimulation with 100 ng/mL LPS. Total RNA was extracted at 2, 6 and 8 h post LPS-treatment and COX2 mRNA levels analysed by real time PCR using primers specific for COX2 mRNA. COX2 mRNA levels were normalized to 18 s RNA and expressed relative to untreated cells. Data are presented as the mean of triplicate samples \pm SEM. * $p < 0.05$; ** $p < 0.003$.

addition to Aquamin-mediated inhibition of IL-1 β and TNF α (Ryan *et al.*, 2011), inhibition of NF- κ B and COX2 may be an additional mechanism by which Aquamin exerts its anti-inflammatory effects. Further studies are needed in animals and humans to confirm these *in vitro* findings.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

REFERENCES

- Aslam MN, Kreider JM, Paruchuri T *et al.* 2010a. A mineral-rich extract from the red marine algae *Lithothamnion calcareum* preserves bone structure and function in female mice on a Western-style diet. *Calcif Tissue Int* **86**: 313–324.
- Aslam MN, Paruchuri T, Bhagavathula N, Varani J. 2010b. A mineral-rich red algae extract inhibits polyp formation and inflammation in the gastrointestinal tract of mice on a high-fat diet. *Integr Cancer Ther* **9**: 93–99.
- D'Acquisto F, May MJ, Ghosh S. 2002. Inhibition of nuclear factor kappa B (NF- κ B): an emerging theme in anti-inflammatory therapies. *Mol Interv* **2**: 22–35.
- Dyer BW, Ferrer FA, Klinedinst DK, Rodriguez R. 2000. A noncommercial dual luciferase enzyme assay system for reporter gene analysis. *Anal Biochem* **282**: 158–161.
- Frestedt JL, Kuskowski MA, Zenk JL. 2009. A natural seaweed derived mineral supplement (Aquamin F) for knee osteoarthritis: a randomised, placebo controlled pilot study. *Nutr J* **8**: 7–14.
- Frestedt JL, Walsh M, Kuskowski MA, Zenk JL. 2008. A natural mineral supplement provides relief from knee osteoarthritis symptoms: a randomized controlled pilot trial. *Nutr J* **7**: 9.
- Luqman S, Pezzuto JM. 2010. NFkappaB: a promising target for natural products in cancer chemoprevention. *Phytother Res* **24**: 949–963.
- Ryan S, O'Gorman DM, Nolan YM. 2011. Evidence that the marine-derived multi-mineral Aquamin has anti-inflammatory effects on cortical glial-enriched cultures. *Phytother Res* **25**: 765–767.
- Smit AJ. 2004. Medicinal and pharmaceutical uses of seaweed natural products: A review. *J Appl Phycol* **16**: 245–262.