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Withaferin A inhibits NF-kappaB activation by targeting cysteine 179 in IKKβ

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ABSTRACT

The transcription factor NF-κB is one of the main players involved in inflammatory responses during which NF-κB becomes rapidly activated. However to maintain homeostasis, this NF-κB activation profile is only transient. Nevertheless deregulation of NF-κB activity is often observed and can lead to chronic inflammatory diseases as well as cancer. Therefore various research projects focus on the development of therapeutics that target the NF-κB activation pathway. One such compound is Withaferin A from the Ayurvedic plant Withania somnifera. Several reports already described the NF-κB inhibiting, anti-inflammatory capacity of WA, either *in vitro* as well as *in vivo*. However the underlying molecular mechanism remains largely unknown. In this paper we demonstrate a direct interaction of WA with the IKK-complex, more specifically with IKKβ, a kinase which is indispensable for the nuclear translocation of NF-κB. Hereby WA directly inhibits IKK catalytic activity. By mutation of Cys179 in IKKβ we could demonstrate loss of interaction between IKKβ and WA indicating that WA exerts its anti-inflammatory effects by targeting the crucial Cys179 residue located in the catalytic site of IKKβ. Upon docking of WA to a IKKβ homology structure model, WA was found to fit nicely into the groove of IKKβ where it can form hydrogen bond to stabilize its interaction with Cys179.

1. Introduction

The transcription factor nuclear factor-kappaB (NF-κB) is involved in the upregulation of proteins that promote cell survival, stimulate growth, induce angiogenesis and reduce susceptibility to apoptosis [1]. Due to the required control of expression of these NF-κB driven proteins, activation of NF-κB is tightly regulated. In several haematologic cancers, including acute myeloid leukaemia, chronic lymphocytic leukaemia, and multiple myeloma, constitutively active NF-κB has been observed [2,3]. Furthermore prolonged NF-κB activation has also been observed in chronic inflammatory diseases such as asthma, Crohn's disease, rheumatoid arthritis, but also in metabolic disorders including obesity, type 2 diabetes, and atherosclerosis [4]. Consistent with its central role in numerous diseases *via* coordination of inflammatory as well as anti-apoptotic responses, a lot of effort has been invested in the search and development of new therapeutic products exerting NF-κB inhibiting potential. One of these compounds, demonstrated to inhibit inflammatory NF-κB activation and to sensitize cells to cell death, is Withaferin A (WA) (reviewed in [5]).

WA is a steroidal lactone purified from the ancient Ayurvedic nutritional herb, also called Ashwagandha (Latin name Withania somnifera). The roots of this plant from the Solanaceae or nightshade family are used to prepare medicinal Ashwagandha, claimed to possess aphrodisiac, sedative, rejuvenating and life prolonging properties. Its traditional use for treatment of various divergent disorders, including chronic fatigue, rheumatism, cancer, *etc.*, urged Whitania somnifera to be extensively studied to gain better insight in its biological activities. For characterization of the bio-active entities in Ashwaganda, several research groups analysed the chemical constituents of Withania Somnifera. The major constituents of this plant are Whitanolides, C28-steroidal

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lactones built on an intact or rearranged ergostane framework, in which C-22 and C-26 are appropriately oxidized to form a six-membered lactone ring. WA, a highly oxygenated withanolide, was demonstrated as being one of the main active withanolides, exerting a wide variety of activities. Previous research indicated that WA potently inhibits NF-κB activation induced by different inducers in a broad range of cell types [6–10].

Under quiescent conditions, inactive NF-κB is present in the cytoplasm bound to its inhibitor IκB, which masks its nuclear localization signal. Several inflammatory stimuli, including proinflammatory cytokines such as tumour necrosis factor (TNF) and interleukin-1 (IL-1), but also infection with microbial pathogens, rapidly activate NF-κB *via* triggering of cognate receptors inducing initiation of receptor specific signal transduction pathways. The convergence point of these pathways is the activation of the IKK complex [11]. This complex is composed of the adaptor protein IKKγ (also termed NEMO) and two catalytic kinase subunits, IKKα and IKKβ, exposing a high degree of sequence similarity [11–15]. Upon activation IKKβ mediates phosphorylation of IκB proteins leading to their proteasomal degradation and nuclear translocation of NF-κB resulting in enhanced transcription of a wide variety of genes (reviewed in [1]).

Although several lines of evidence clearly demonstrate the NF-κB inhibiting activity of WA, to the best of our knowledge the definite direct target of WA activity has not been identified. Molecular analysis revealed that pretreatment of cells with WA hampers TNF and LPS mediated phosphorylation of the inhibitory subunit IκBα, leading to reduced degradation of IκBα and reduced nuclear translocation of NF-κB. Furthermore, Kaileh and colleagues demonstrated reduced TNF-induced IKKβ kinase activity after pretreatment of the cells with WA, indicating that WA interferes with this signal transduction cascade upstream or at the level of the IKK-complex [6]. *In silico* predictions, involving semi-flexible docking analysis, suggested that WA can form strong intermolecular interactions with the adaptor subunit of the IKK-complex, IKKγ, thereby sterically as well as thermodynamically hindering the formation of the naive IKK complex [16]. In this study we further investigated how WA interferes with NF-κB activation at the level of the IKK complex. No interference of WA with the interaction of IKKβ and its adaptor protein IKKγ could be observed. In contrast, by using a biotinylated version of WA, we confirmed that WA could directly bind with IKKβ at Cys179 located within the catalytic domain of the kinase. *In vitro* studies confirmed that WA directly interferes with the kinase activity of the IKKβ protein.

2. Materials and methods

2.1. Expression vectors

The eukaryotic expression vectors pUT651 and pNFconluc have been described earlier [17]. Eukaryotic expression plasmids encoding NIK, Myr-IKKγ and FLAG-tagged IKKβ were gifts from Dr. R. Beyaert (DMBR-VIB, Ugent, Belgium). Vectors for eukaryotic expression of FLAG-tagged site specific mutants of IKKβ (IKKβ-C179A, IKKβ-C662/716A, IKKβ-S177/181E and IKKβ-S177/181E-C179A) were generously provided by Dr. T. Gilmore.

2.2. Cell lines

Human embryonic kidney HEK293T cells were a kind gift of Dr. M. Hall (Department of Biochemistry, University of Birmingham, UK). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 0.4 mM sodium pyruvate and penicillin/streptomycin. HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 2 mM L-glutamine and antibiotics.

2.3. Reagents

Recombinant human TNF was expressed in *Escherichia coli*, purified to at least 99% homogeneity and had a specific biological activity of 2.3×10^7 IU/mg purified protein, as determined with the international standard (code 87/650; National Institute for Biological Standards and Control, Potters Bar, UK). Withaferin A was obtained from Chromadex (Irvine, USA). Biotinylation of WA was performed by Dr. P. Van der Veken (WA-BIOT; Universiteit Antwerpen, Belgium). Both formulations of WA were stored as 20 mM solutions in DMSO at −20 °C. Polyclonal antibodies anti-IKKβ (H-740) and anti-NEMO (FL-419) were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-FLAG M2 tag antibody, anti-FLAG M2 affinity beads and Biotin were obtained from Sigma (St. Louis, MO). Manumycin A was purchased from Cayman (Ann Arbor, MI). Neutravidin agarose beads were from Thermo Scientific (Rockford, IL).

2.4. NF-κB dependent luciferase reporter gene assay

HEK293T cells were seeded in 24-well plates and transiently transfected by a standard PEI co-precipitation method using 400 ng DNA per well. Each transfection contained 80 ng of pNFconluc plasmid and 80 ng pUT651 plasmid. Twenty-four hours after transfection, cells were pretreated for 1 h with WA or WA-BIOT as indicated and were either stimulated with 1000 IU/ml hTNF or left untreated. After 6 h incubation, cells were lysed in 150 μl lysis buffer and NF-κB-dependent luciferase activity was measured as previously described [17]. Also activity of constitutively expressed β-galactosidase was measured to normalize for differences in transfection efficiency. For analysis of NF-κB activation by overexpression of NIK, Myr-IKKγ or IKKβ, cells were cotransfected with 150 ng plasmids encoding Myr-IKKγ or IKKβ or 15 ng plasmid encoding NIK. Directly after transfection cells were treated with different doses WA for 18 h. Subsequently cells were lysed and processed as described above.

2.5. Affinity purification and co-immunoprecipitation

For identification of endogenous WA target proteins, HEK293T or HeLa cells were plated at 10^7 cells on 15-cm tissue culture dish and incubated for 24 h at 37 °C. Cells were treated with biotinylated WA (5 μM) or left untreated for 2 h. Cells were lysed in 1 ml lysis buffer (5 mM Tris pH 7.6, 1% Triton-X 100 and 5 mM EDTA, supplemented with Complete™ protease inhibitor cocktail from Roche and phosphatase inhibitors) and incubated with Neutravidin beads overnight. Beads were washed 5 times with lysis buffer. Total protein lysates as well as co-precipitating proteins were separated by SDS–PAGE and electrotransferred onto a nitrocellulose membrane. Blots were probed using the appropriate antibodies and the immunoreactive proteins were detected using the Odyssey imaging system (LI-COR Biosciences, Lincoln, USA).

Structural analysis of WA targets was performed by overexpression of the wild type protein and its site specific mutants. Therefore 1.5×10^6 HEK293T cells were plated on 10-cm Petri dish and transfected by PEI precipitation method with 1 μg expression plasmids encoding the protein of interest. Twenty-four hours after transfection cells were lysed in 500 μl of the above described lysis buffer. Samples were handled further as previously described.

For co-immunoprecipitation of the endogenous IKK complex, HEK293T cells were seeded at 10^7 cells on 15-cm tissue culture dish and incubated for 24 h at 37 °C. Cells were lysed in 2.5 ml lysis buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4,