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Harpagoside suppresses lipopolysaccharide-induced iNOS and COX-2 expression through inhibition of NF-κB activation

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Abstract

Preparations of *Harpagophytum procumbens*, known as devil's claw, are used as an adjunctive therapy for the treatment of pain and osteoarthritis. Pharmacological evaluations have proven the effectiveness of this herbal drug as an anti-inflammatory and analgesic agent. The present study has investigated the mechanism of action of harpagoside, one of the major components of *Harpagophytum procumbens*, using human HepG2 hepatocarcinoma and RAW 264.7 macrophage cell lines. Harpagoside inhibited lipopolysaccharide-induced mRNA levels and protein expression of cyclooxygenase-2 and inducible nitric oxide in HepG2 cells. These inhibitions appeared to correlate with the suppression of NF-κB activation by harpagoside, as pre-treating cells with harpagoside blocked the translocation of NF-κB into the nuclear compartments and degradation of the inhibitory subunit IκB-α. Furthermore, harpagoside dose-dependently inhibited LPS-stimulated NF-κB promoter activity in a gene reporter assay in RAW 264.7 cells, indicating that harpagoside interfered with the activation of gene transcription. These results suggest that the inhibition of the expression of cyclooxygenase-2 and inducible nitric oxide by harpagoside involves suppression of NF-κB activation, thereby inhibiting downstream inflammation and subsequent pain events.

Keywords: COX-2; iNOS; NF-κB; Harpagoside; Devil's claw

1. Introduction

Harpagophytum procumbens (Burch.) DC. Ex Meisn. (Pedaliaceae) is a perennial herbaceous plant growing specifically in Southern Africa. Preparations of its secondary roots are in current use for the treatment of pain and osteoarthritis (Chantre et al., 2000; Chrubasik et al., 2003a,b). Clinical efficacy of *Harpagophytum procumbens* in terms of alleviation of pain and decreased requirements for non-steroidal anti-inflammatory drugs has been demonstrated for root powder and aqueous extract containing at least 50 mg of harpagoside in the daily dosage (Chrubasik et al., 2003b) and evaluated in numerous double blind clinical trials and comparative studies against leading anti-arthritic drugs, such as rofecoxib and diacerrhein (Chantre et al., 2000; Chrubasik et al., 2003a,b).

Extracts of *Harpagophytum procumbens* (devil's claw) showed significant reduction in pain and inflammation induced by chemical, thermal and mechanical stimulus in various experimental test procedures including Freund's adjuvant induced arthritis and the carragenan-induced rat paw oedema (Lanhers et al., 1992; Andersen et al., 2004). The secondary root preparations of *Harpagophytum procumbens* have been shown to suppress interleukin (IL) induced production of metalloproteinases in human chondrocytes in vitro (Schulze-Tanzil et al., 2004); inhibit lipopolysaccharide (LPS)-stimulated expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide (iNOS) in fibroblast cell line L929 (Jang et al., 2003); and also inhibit LPS-induced release of cytokines (tumor necrosis factor (TNF)-α, IL-6, IL-1β) and prostaglandin (PG) E_2 from human monocytes (Fiebich et al., 2001).

Abbreviations: AG, aminoguanidine; COX-2, cyclooxygenase-2; DMSO, dimethyl sulfoxide; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO, nitric oxide; PDTC, pyrrolidine dithiocarbamate

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Devil's claw extracts containing harpagoside exhibited a concentration-dependent suppression of nitrite formation in renal mesangial cells and this inhibition was attributable to inhibition of iNOS expression at the level of its transcriptional activation (Kaszkin et al., 2004). Interestingly, a harpagoside-free extract also markedly inhibited inducible NO synthase expression and in contrast to pure harpagoside exerted strong antioxidative activity (Kaszkin et al., 2004). Recently, it was shown that a methanolic extract of devil's claw inhibited TPA (12-*O*-tetradecanoylphorbol-13-acetate)-induced COX-2 expression in human breast epithelial cells and in mouse skin in vivo by inhibiting DNA-binding of NF-κB activated by TPA (Na et al., 2004). Topical application of the extract also inhibited TPA-induced activation of activator protein-1 and attenuated the expression of its key component c-Fos (Kundu et al., 2005).

Despite extensive clinical evaluation of the effectiveness of devil's claw preparations for use as an analgesic and in the treatment of osteoarthritis, and extensive in vitro data demonstrating anti-inflammatory and analgesic properties of *Harpagophytum procumbens*, active principles responsible for the clinical effect and the modes of action are not fully understood. Therefore, we further examined the molecular mechanism of action of harpagoside in modulation of the expression of COX-2 and iNOS proteins in inflammatory conditions induced by LPS stimulation in human hepatocarcinoma HepG2 cells and RAW 264.7 macrophage cell lines.

2. Materials and methods

2.1. Materials

Aminoguanidine, dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS) from *Salmonella minnesota* and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma–Aldrich, Australia. All solvents were of analytical grade and were distilled before use. Silica gel 60H and TLC plate (silica gel 60 F254) were purchased from Merck Pty. Ltd. Dried, sliced, secondary root tubers of *Harpagophytum procumbens* was donated by Finzelberg, GmbH and Co., Germany. Harpagoside was isolated in our laboratory and characterized by high-field nuclear magnetic resonance (NMR) (Varian Gemini 300 MHz, Palo Alto, USA).

2.2. Isolation and identification of harpagoside from devil's claw

Dried, sliced, secondary root tubers (100 g) of *Harpagophytum procumbens* were ground to powder and extracted twice with methanol by stirring at room temperature to obtain crude extract (26 g). The extract was fractionated through the normal phase silica bed using a short column vacuum chromatographic method (Zarate et al., 1992). A stepwise gradient of increasing polarity of mobile phase (200 ml) consisting of a mixture of chloroform/methanol ($CHCl_3$:MeOH ratio; 100:0, 99:1, 98:2, 96:4, 94:6, 92:8, 90:10, 88:12, 85:15 and 80:20) was employed to elute the components. Eluents were analysed by TLC with a mobile phase system consisting of $CHCl_3$:MeOH:H_2O (10:2:0.1). Harpagoside was purified and identified as 8-*O*-*E*-cinnamoylharpagide (Fig. 1). Its structure was characterized by ^{1}H NMR and ^{13}C NMR, and was found to be to that reported in literature (Chaudhuri et al., 1980).

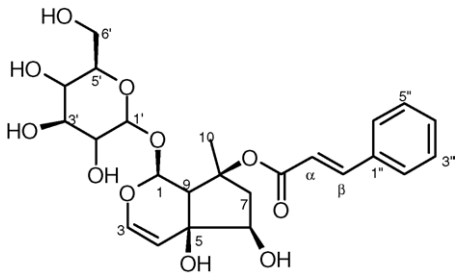

Fig. 1. Structure of harpagoside (8-*O*-*E*-cinnamoylharpagide).

2.3. Cell cultures

RAW 264.7, a mouse macrophage cell line, was obtained from the American Type Culture Collection and the human hepatocellular carcinoma cell line (HepG2) was as a kind gift from Dr. Javed Akhter (University of New South Wales, Sydney, Australia). Cells were cultured as described previously (Aktan et al., 2003; Hong et al., 2004).

2.4. Cell proliferation assay

RAW 264.7 macrophage and HepG2 cells were seeded at a density of 5×10^4 per well onto sterile 96-well flat-bottomed microtiter tissue culture plates (Sarstedt) overnight. The cells were treated with various concentrations of harpagoside (0.1–200 μM) and further incubated for 3 days at 37 °C in a humidified atmosphere with 5% CO_2. The plates were then added the MTS (tetrazolium salt) reagents from the CellTiter96® Aqueous One Solution Cell Proliferation Assay (Promega) and incubated at 37 °C with 5% CO_2 for 4 h and analysed using a microtiter plate reader (model 3550, Bio-Rad) at an absorbance wavelength of 490 nm.

2.5. Transfection and NF-κB luciferase assay

pNF-κB-Luc (Clontech, USA) was transfected in RAW 264.7 cell line as described in the literature (Aktan et al., 2003) and was readily available in our laboratory. The pNF-κB-Luc plasmid contains multiple copies of the NF-κB consensus sequence fused to a TATA-like promoter region from the Herpes simplex virus thymidine kinase (HSV-TK) promoter. Transfected cells were pretreated for 2 h with either vehicle (DMSO), pyrrolidine dithiocarbamate, PDTC (100 mM) a known inhibitor of NF-κB or harpagoside (0.1–200 μM) prior to 4 h treatment with LPS (100 ng/ml). Unstimulated RAW 264.7 cells acted as a negative control. The cells were subsequently lysed and assayed for luciferase and β-galactosidase activities using the Bright-Glo Luciferase Assay System and Beta-Glo Assay System (Promega, Australia), respectively. The results were expressed

as relative luciferase activity (fold difference compared to negative control).

2.6. Nitrite (nitric oxide) assay

The nitrite assay was carried out as described previously (Aktan et al., 2003). Briefly, RAW 264.7 cells (3×10^6 cells) were cultured in 24-well plates for 2 days at 37 °C in a 5% CO_2 incubator. The cells were pretreated with the vehicle (DMSO) or harpagoside (0.1–200 μM) for 1 h, prior to LPS (100 ng/ml) stimulation for a further 24 h. Aminoguanidine (100 μM), a known inhibitor of NOS enzymes, was used as a positive control (Wolff et al., 1997). LPS untreated cells were used to determine the basal activity. After 24-h incubation supernatants were collected and the amount of NO product quantified using the Griess Reagent System (Promega, Australia).

2.7. Semi-quantitative mRNA analysis for COX-2 and iNOS

Total RNA was extracted from cultured HepG2 cells using TRIzol reagent (Invitrogen, Australia). The relative levels of specific mRNAs were assessed by reverse transcriptase polymerase chain reaction (RT-PCR) following the method reported in the literature (Abe et al., 2002). The sequences of the sense and antisense primers used for amplification were: COX-2 (M90100: 411 bp), 5′-AACAGGAGCATCCTGAATGG-3′ and 5′-GGTCAATGGAAGCCTG-TGAT-3′; iNOS (AF049656: 488 bp), 5′-CAGAGGACCCAGAGACAAGC-3′ and 5′-ACTGGGTGA-ACTCCAAGGTG-3′; β-actin (NM001101: 629 bp), 5′-GGAGTAACCAGGTCGTCCAA-3′ and 5′-GAAGGTGCCCAGAATACCAA-3′.

2.8. Protein extraction and semi-quantitative immunoblotting

Immunoblots were carried out as described previously (Hong et al., 2004). Briefly, HepG2 cells were treated with vehicle (DMSO), PDTC (100 mM) or harpagoside (200 μM) for 2 h prior to being exposed to LPS (10 μg/ml) for 1 or 6 h. Cells were lysed with lysis buffer (Cell Signaling) for determination of COX-2 and iNOS proteins. The nuclear (NF-κB(p65)) and cytosolic (IκB-α) proteins were, respectively, extracted with NE-PER nuclear extraction and cytoplasmic extraction reagents (Pierce). The membrane was incubated with anti-COX-2 (Santa Cruz Biotechnology), anti-iNOS (Caymanchem), anti-IκB-α or anti-NF-κB(p65) primary antibodies (Santa Cruz Biotechnology). The proteins were detected by chemiluminescence and normalized to actin for quantification.

2.9. Statistical analysis

All results are expressed as means ± S.E.M. Data were analyzed by one-factor analysis of variance (ANOVA). If a statistically significant effect was found, the Newman–Keuls test was performed to isolate the difference between the groups. $P < 0.05$ were considered to be significant.

3. Results

3.1. Effect of harpagoside on LPS induced-NO synthase in RAW 264.7 macrophage cell lines

We first established the effect of harpagoside on the release of NO in a macrophage cell line. Treatment of RAW 264.7 cells with LPS (100 ng/ml) for 24 h significantly increased the release of NO (37 ± 3 μM) by approximately 12-fold compared to that of the untreated cells (3 ± 2 μM) (Fig. 2). This increased release of NO was inhibited by iNOS inhibitor aminoguanidine (10 μg/ml). Harpagoside reduced NO release in LPS-stimulated cells in a dose-dependent manner, with an IC_{50} value of 39.8 μM but had no effect on NO release in the LPS-untreated cells (Fig. 2). To confirm that the inhibition of NO release by harpagoside was due to its action on the iNOS system rather than by affecting cell viability, a cytotoxicity assay for harpagoside (0.1–200 μM) was examined. Harpagoside treatment retained >90% cell viability in LPS-treated cells (Fig. 3).

3.2. Effect of harpagoside on COX-2 and iNOS mRNA and protein expression in HepG2 cell lines

To examine if harpagoside affected expression of inflammatory enzymes, the effect of harpagoside on mRNA and protein levels was examined. HepG2 cells treated with the pro-inflammatory agent LPS (10 μg/ml) significantly upregulated the mRNA expression of inflammatory genes COX-2 and iNOS by approximately 3.8- and 2.2-fold, respectively (Fig. 4). Immunoblotting showed that the increase in mRNA levels of COX-2 and iNOS corresponded to the increased protein expressions of these enzymes of approximately 1.4- and 1.5-fold, respectively, as shown in Fig. 5. Harpagoside (200 μM) significantly inhibited the induction of COX-2 (Fig. 4a) and iNOS mRNA (Fig. 4b) and their corresponding protein expression

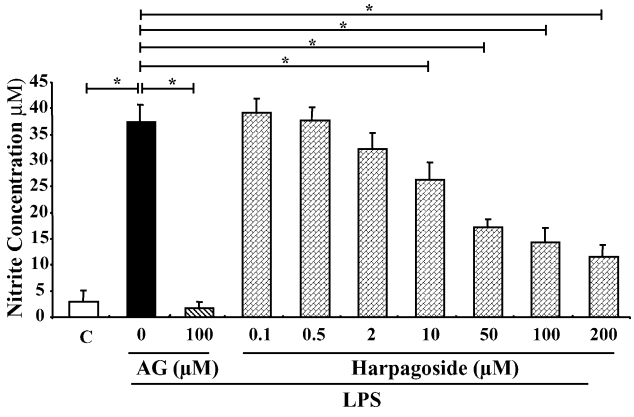

Fig. 2. Dose-dependent effect of harpagoside on LPS induced-NO synthase in RAW 264.7 macrophage cell lines. RAW 264.7 cells were treated for 1 h with either vehicle (DMSO) or harpagoside (0.1–200 μM), after 24-h stimulation with 100 ng/ml LPS. Aminoguanidine (AG: 100 μM), a known inhibitor of nitric oxide synthase enzymes, was used as a positive control. Unstimulated RAW 264.7 cells acted as a negative control. Nitrite, a stable end-product of NO, was measured by the Griess reaction. All values are means ± S.E.M. ($n = 3$) vs. control (C), $^*P < 0.05$.

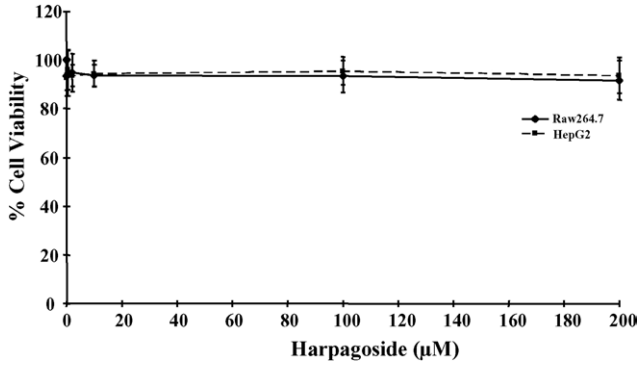

Fig. 3. Cytotoxic effect of harpagoside on HepG2 and RAW 246.7 macrophage cells. RAW 264.7 macrophage and HepG2 cells were seeded at a density of 5×10^4 per well and treated with Harpagoside (0.1–200 μM) over a 3-day period. Cells viability was determined using the CellTiter96® Aqueous One Solution Cell Proliferation Assay. The results are expressed in terms of percentage relative cell viability. All values are means ± S.E.M. ($n = 3$) vs. control.

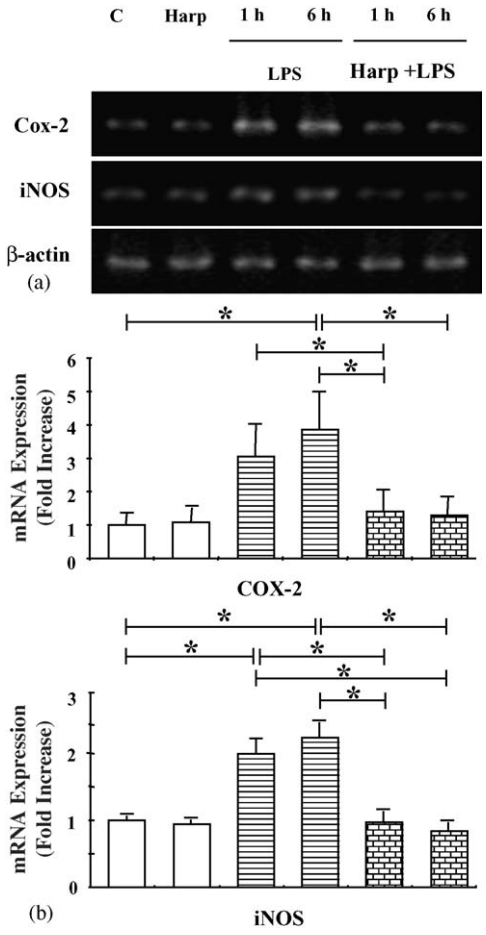

Fig. 4. Effect of harpagoside on COX-2 (a) and iNOS (b) mRNA expression in HepG2 cell lines. HepG2 cells were pretreated in the absence and presence of harpagoside (Harp: 200 μM) for 2 h before LPS treatment (10 μg/ml), then exposed to LPS for the indicated times (1 and 6 h). Total mRNAs were prepared from the cell pellets using TRIzol. The relative levels of mRNAs were assessed by RT-PCR. Results were normalized to β-actin. Levels in control were arbitrarily assigned a value of 1.0. All values are means ± S.E.M. ($n = 3$) vs. control (C), $^*P < 0.05$.

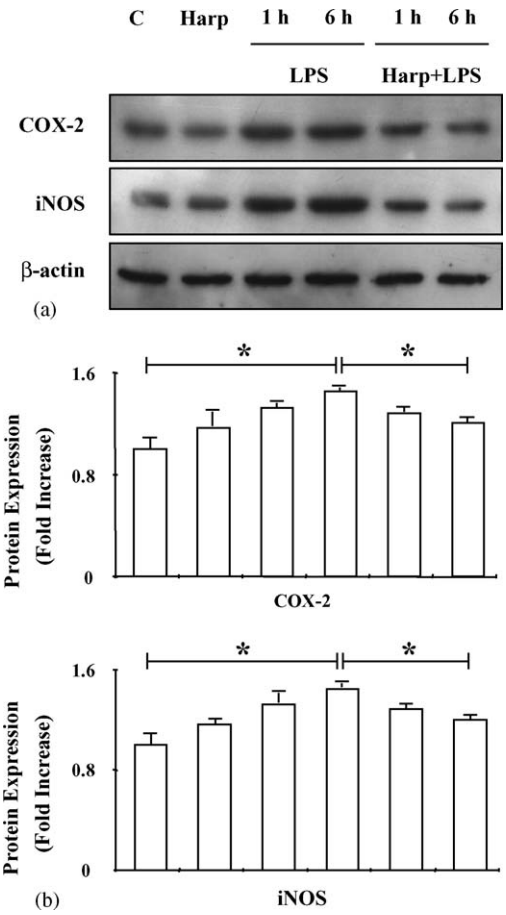

Fig. 5. Effect of harpagoside on COX-2 (a) and iNOS (b) protein expression in HepG2 cell lines. HepG2 cells were pretreated in the absence and presence of harpagoside (Harp: 200 μM) for 2 h before LPS treatment (10 μg/ml), then exposed to LPS for the indicated times (1 and 6 h). Protein extracts from cell pellets were subjected to SDS-PAGE followed by the Western blot analysis using anti-COX-2 and anti-iNOS antibodies. Protein contents were determined using the Bradford assay method with BSA as a standard. The results were normalized to β-actin. Levels in control were arbitrarily assigned a value of 1.0. All values are means ± S.E.M. ($n = 3$) vs. control (C), $^*P < 0.05$.

(Fig. 5a and b) in LPS-treated cells, indicating that harpagoside attenuated the synthesis of these proteins at both the transcriptional and translational levels.

3.3. Effect of harpagoside on inhibiting LPS-induced NF-κB luciferase reporter activity

To further explore the molecular mechanism by which harpagoside inhibited the synthesis of inflammatory proteins COX-2 and iNOS, we investigated the effect of harpagoside on the activity of the nuclear transcription NF-κB system. It is known that pro-inflammatory agents such as LPS activate the NF-κB pathway leading to the induction of COX-2 (D'Acquisto et al., 1997) and iNOS production (Kleinert et al., 2003). RAW 264.7 cells transfected with NF-κB luciferase reporter plasmid exhibited approximately three-fold increased luciferase signal after treatment with LPS (100 ng/ml), representing increased transcriptional activity compared to untreated cells (Fig. 6). Cells pretreated with harpagoside exhibited dose-dependent

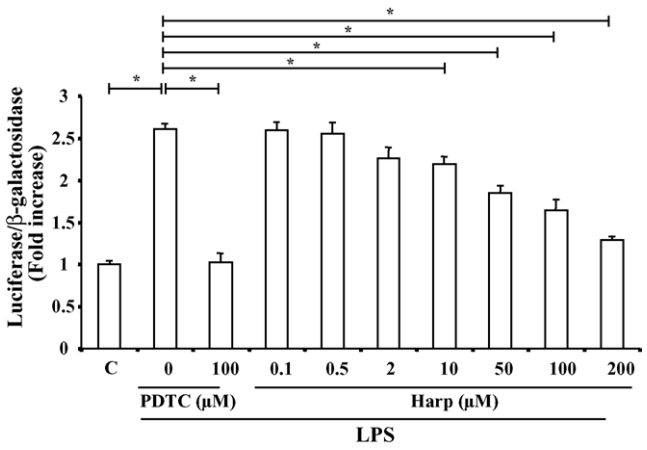

Fig. 6. Effect of harpagoside on LPS-induced NF-κB luciferase reporter activity in Raw 264.7 macrophage cell lines. RAW 264.7 cells transiently transfected with pNF-κB-Luc and pSV-β-galactosidase were pretreated for 1 h with either vehicle (DMSO), pyrrolidine dithiocarbamate, PDTC (100 mM) a known inhibitor of NF-κB or harpagoside (Harp: 0.1–200 μM), prior to 4 h of treatment with LPS (100 ng/ml). Unstimulated RAW 264.7 cells acted as a negative control. Cells were then harvested and luciferase and β-galactosidase activities were assessed. Results are expressed as relative luciferase activity (fold difference compared to negative control. All values are means ± S.E.M. ($n = 6$). vs. control (C), $^{*}P < 0.05$.

inhibition of the LPS-induced NF-κB transcriptional activity in transfected RAW 264.7 cells, with an IC_{50} of 96.4 μM, as shown in Fig. 6.

3.4. Effect of harpagoside on NF-κB(p65) and IκB-α protein expression in HepG2 cell lines

It is known that in the inflammatory pathway upon stimulation of the NF-κB system, IκB-α is phosphorylated by IκB kinases and subsequently degraded to release NF-κB, which is then translocated to the nucleus where it activates various genes (Hayden and Ghosh, 2004). Thus induction of NF-κB activation by LPS increases p65 protein level in the nucleus, whereas for the cytosolic IκB-α, protein level is reduced in inverse proportion to that of p65, as confirmed in Fig. 7. To evaluate the stage at which harpagoside blocked NF-κB activation, cytosolic and nuclear extracts of the cells were analyzed by Western blot for IκB-α and p65 protein levels, respectively. Pre-treatment of HepG2 cells with harpagoside (200 μM) prior to exposure to LPS resulted in a near normalization of IκB-α in the cytosol compared to that of LPS-untreated (control) cells (Fig. 7). This indicated that translocation of IκB-α from the cytosol to the nucleus following LPS treatment was blocked by harpagoside.

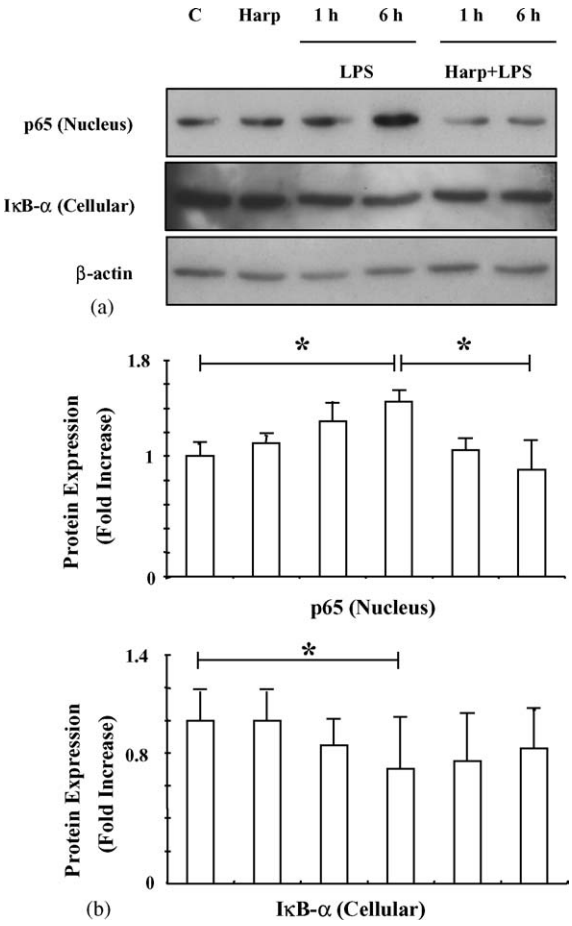

Fig. 7. Effect of harpagoside on NF-κB(p65) and IκB-α protein expression in HepG2 cell lines. Cells were pretreated with harpagoside (Harp: 200 μM) for 2 h before LPS treatment (10 μg/ml), then exposed to LPS for the indicated times. Nuclear and cytosolic fractions were subjected to SDS-PAGE followed by Western blotting analysis using anti-NF-κB(p65) (a) and anti-IκB-α (b) antibodies. Protein contents were determined using the Bradford assay method with BSA as a standard. The results were normalized to β-actin. Levels in control were arbitrarily assigned a value of 1.0. All values are means ± S.E.M. ($n = 3$) vs. control (C). $^{*}P < 0.05$.

4. Discussion

The present study shows for the first time that harpagoside, a major component of *Harpagophytum procumbens*, effectively suppressed the synthesis of two inducible enzymes responsible for inflammation, COX-2 and iNOS at both the transcriptional and translational levels, as seen by the suppression of mRNA and protein levels in LPS-treated HepG2 cells. These inhibitions appeared to correlate with the suppression of NF-κB activation by harpagoside, as pre-treating cells with harpagoside blocked the translocation of NF-κB(p65) into the nuclear compartments and the degradation of the inhibitory subunit IκB-α. Harpagoside exhibited dose-dependent inhibition of LPS-stimulated NF-κB promoter activity in the gene reporter assay, indicating that harpagoside interferes with the activation of gene transcription. Whether harpagoside prevents the degradation of IκB-α from the NF-κB binding complex requires further study through electro mobility shift assay to provide a more precise mode of action of harpagoside with regard to the inhibition of the NF-κB system.

Surprisingly, in rat mesangial cells harpagoside failed to attenuate NF-κB translocation to the nucleus (Kaszkin et al., 2004) in contrast with the finding in the present study, which has clearly demonstrated that harpagoside inhibited the translocation of NF-κB(p65) into the nuclear fraction in HepG2 cells. This discrepancy suggests that the ability of harpagoside to attenuate NF-κB activity is cell and/or stimulus specific. Cell type and stimulus dependence was in fact demonstrated for tran-

scriptional regulation of COX-2, which is mediated distinctly by the binding of inducible transcriptional factors to *cis*-acting elements in the COX-2 promoter (Yang and Bleich, 2004). Interestingly, the whole extracts of *Harpagophytum* were shown to attenuate NF-κB translocation to the nucleus in rat mesangial cells (Kaszkin et al., 2004) indicating the involvement of other active components in the extract. Nevertheless, the same study demonstrated clearly that the harpagoside present in the *Harpagophytum* extracts was important in order to inhibit NO production (Kaszkin et al., 2004) but is not necessarily responsible for down regulation of iNOS expression, as an extract free of harpagoside showed marked inhibition of iNOS expression. In contrast to pure harpagoside, this extract was shown to exert strong antioxidative activity (Kaszkin et al., 2004).

In the light of this observation the ability of harpagoside to attenuate the NF-κB activity and down regulation of COX-2 and iNOS expression observed in the present study is not likely to be due to an antioxidant effect.

Harpagoside showed no inhibition of COX-1 enzyme activity at either 21 or 200 μM, whilst it exhibited weak inhibition (<20%) of COX-2 enzyme activity at 200 μM using the COX Inhibitor Screening Assay kit (Cayman Chemicals) (results not shown). This finding supports an earlier observation that *Harpagophytum procumbens* root powder (containing 3% total iridoid glycosides) given to healthy volunteers at a daily dose of 2.0 g for 21 days showed no effect on whole blood eicosanoid production, including 6-keto-$PGF_{1\alpha}$, PGE_2, thromboxane (TXB_2) and LTB_4, and blood clotting (Moussard et al., 1992), indicating that devil's claw does not inhibit COX-1 activity.

Thus, suppression of the synthesis of inflammatory proteins COX-2 and iNOS by harpagoside, and possibly other inflammatory proteins, thereby blocking the production of inflammatory mediators such as PGE_2, could explain at least in part the effectiveness of devil's claw extracts used clinically in the treatment of pain and for inflammatory conditions such as rheumatoid arthritis and osteoarthritis. This inhibition of protein synthesis of harpagoside is likely to be due to its modulatory effect on the transcription factor NF-κB activity. This in fact is further supported by the finding that *Harpagophytum procumbens* extracts inhibit cytokine production, particularly TNF-α, suggesting that extracts of *Harpagophytum procumbens* interfere with a common target in the NF-κB pathway.

In conclusion, the present study has demonstrated that harpagoside is involved in the suppression of NF-κB activation, as shown by the reduction of luciferase activity in a gene reporter assay. *Harpagophytum procumbens* comprises at least four major iridoid glycosides, including harpagide, harpagoside, procumbide, and procumboside. Of these, harpagoside and harpagide were suggested as active components of the root of the *Harpagophytum procumbens*. Exploration of the structure–activity relationships of these iridoids glycosides on an inflammation-induced gene reporter assay, such as LPS-induced NF-κB luciferase assay, could elucidate structural features required for active principles acting against NF-κB target and could be used to develop leads for the design of drugs for the management of pain and inflammation.

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