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Novel effect of helenalin on Akt signaling and Skp2 expression in 3T3-L1 preadipocytes

Corinth A. Auld, Robin G. Hopkins, Karishma M. Fernandes, Ron F. Morrison *

Department of Nutrition, The University of North Carolina at Greensboro, Greensboro, NC 27402, USA

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Abstract

We have previously shown that the F-box protein, Skp2, is highly regulated during preadipocyte proliferation and plays a mechanistic role in p27 degradation during cell cycle progression. Data presented here demonstrate that the anti-inflammatory, anti-carcinogenic phytochemical, helenalin is a potent inhibitor of periodic Skp2 protein accumulation during early phases of 3T3-L1 adipocyte differentiation. Furthermore, helenalin was shown to completely block p27 degradation, cyclin A accumulation, and G_1/S transition resulting in G_1 arrest. Helenalin was also shown to block Skp2 mRNA accumulation in a concentration-dependent manner and to completely suppress hormonally induced Skp2 promoter activity suggesting transcriptional mechanisms were involved. Examination of signaling events previously determined to be important for Skp2 upregulation during adipogenesis revealed impaired Akt phosphorylation immediately preceding the inhibitory effect of helenalin on Skp2 mRNA accumulation. These studies demonstrate a novel effect of helenalin on Skp2 regulation and growth factor receptor signaling during early stages of adipocyte differentiation.

Keywords: Adipocyte; Obesity; Cell cycle; Proliferation; Signal transduction; Helenalin

The sesquiterpene lactone, helenalin, is a component found in the aerial portion of the flowering plant, arnica montana L. Arnica is a native plant of mountainous regions in Europe, specifically Russia, and has long been used as an anti-inflammatory treatment for external wounds (e.g., bruises). Early evidence suggests that helenalin is an effective cardiotonic agent, improving cardiac strength in an in vivo animal model through increases in cyclic AMP [1]. However, helenalin is principally used in cellular studies as an inhibitor of NFκB p65, a function that is important for its known anti-inflammatory effects. While it has been suggested that this occurs through inhibition of IKK activity and subsequent IκB degradation [2], others have demonstrated that helenalin modifies p65 directly via alkylation, thereby preventing p65 DNA-binding [3].

Recent attention has been focused on mechanisms by which helenalin exerts anti-tumor effects. Helenalin induces apoptosis at 10–50 μM concentrations in leukemia Jurkat T cells [4,5], a desired outcome of chemotherapeutic treatments. Interestingly, helenalin has also been shown to be an effective inhibitor of telomerase activity in cancer cells, primarily through alkylation and subsequent inactivation of the enzyme [6]. In addition, several sesquiterpene lactones, including helenalin, possess powerful antioxidant activity, causing decreased lipid peroxidation [7,8]. These and other studies provide clear evidence that helenalin has multiple modes of action within the cell, aside from its commonly cited ability to inhibit NFκB DNA-binding. While helenalin is known to have anti-proliferative properties, a mechanistic role in regulating cell cycle progression has yet to be determined.

The F-box protein, S-phase kinase-associated protein (Skp2), has been shown to promote cell cycle progression through targeting specific proteins for ubiquitylation and degradation by the 26S proteasome [9–11]. Skp2 functions

* Corresponding author. Fax: +1 336 334 4129.
E-mail address: ron_morrison@uncg.edu (R.F. Morrison).

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as a variable receptor component of the SCF^{Skp2} (Skp1/Cul1/F-box protein; superscript denotes the F-box protein) E3 ligase where it recruits substrates to the core of the E3 Ligase via its F-box motif, bringing the substrate in close proximity to the ubiquitin-conjugating enzyme of the SCF complex [12]. We have recently demonstrated a functional role for Skp2 in mediating p27 degradation during S and G_2 phase progression of replicating 3T3-L1 preadipocytes during early phases of adipocyte differentiation.[1] Furthermore, periodic Skp2 accumulation during cell cycle progression was found to be regulated by transcriptional mechanisms involving hormonally induced PI3K and MAPK signaling pathways.

In this report, we demonstrate that helenalin blocks periodic Skp2 protein accumulation during early stages of 3T3-L1 adipocyte differentiation. Downstream events, such as degradation of p27, accumulation of cyclin A, and G_1/S transition, were also abolished resulting in complete G_1 arrest. Data presented here demonstrate that helenalin inhibits hormonally induced Skp2 mRNA accumulation and promoter activity, suggesting that helenalin impinges on signaling pathways leading to Skp2 transcription. Finally, we show that helenalin suppresses Akt phosphorylation immediately preceding the effect on Skp2 expression. These data represent the first study of the effect of helenalin on Skp2 and Akt regulation in any cell type and provide a link between cell cycle progression, early signaling events, and adipocyte hyperplasia.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM) was purchased from Cellgro by Mediatech. Calf bovine serum, fetal bovine serum (FBS), and trypsin-EDTA were from Invitrogen Corporation. Propidium iodide and RNase A were purchased from Sigma. Helenalin was purchased from Biomol. The following antibodies were used for immunoblotting: phospho-Akt (Ser473), total Akt, phospho-Erk1/2 (Thr202/Tyr204), and total Erk1/2 (Cell Signaling); Skp2, Skp1, and cyclin A (Santa Cruz Biotechnology); and p27 (Transduction Laboratories). Enhanced chemiluminescence (ECL) reagents were from Perkin-Elmer Life Sciences.

Cell culture and differentiation. Murine 3T3-L1 preadipocytes were propagated in growth medium containing DMEM supplemented with 10% calf bovine serum as described previously [13]. By standard differentiation protocol, preadipocytes were propagated in growth medium until reaching a state of density arrest at 2 days post-confluence. Growth medium was then replaced with differentiation medium comprised of DMEM supplemented with 10% FBS, 0.5 mM 1-methyl-3-isobutylxanthine, 1 μM dexamethasone, and 1.7 μM insulin (MDI). The term "post-MDI" refers to the time elapsed since the addition of MDI to the culture medium. Throughout the study, "time 0" refers to density arrested cells immediately before the addition of MDI to the culture medium. Experiments described herein were conducted within the period of differentiation spanning from density arrest (0 h) through 20 h post-MDI. All experiments were repeated 3–5 times to validate results and ensure reliability.

Immunoblotting. Cell monolayers were washed with PBS and scraped into ice-cold lysis buffer containing 1.0 M Tris, pH 7.4, 150 mM NaCl, 1% Triton X, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, and 10 mM *N*-ethylmaleimide. Lysis buffer was freshly supplemented with phosphatase (20 mM β-glycerophosphate, 10 mM NaFl, and 2 μM sodium vanadate), as well as protease (0.3 μM aprotinin, 21 μM leupeptin, E-64, 1 μM pepstatin, 50 μM phenanthroline, and 0.5 μM phenylmethylsulfonyl fluoride) inhibitors. Cell lysates were clarified by centrifugation (13,000*g*, 10 min, 4 °C) and protein concentration was determined by BCA assay (Pierce). Lysates were resuspended in loading buffer containing 0.25 M Tris, pH 6.8, 4% SDS, 10% glycerol, 10% dithiotreitol, 0.01% bromophenol blue, heated for 5 min at 80 °C, and placed on ice. Lysates were resolved on SDS–PAGE gels and transferred to polyvinylidene fluoride membranes (Millipore). Following transfer, membranes were blocked with 4% milk and probed with indicated primary antibodies overnight at 4 °C and horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Immunoblots were developed with ECL and visualized by autoradiography using CL-XPosure film (Pierce).

Flow cytometry. Cell cycle progression was assessed by flow cytometry. Briefly, cell monolayers were washed with phosphate-buffered saline (PBS), trypsinized, and detached cells diluted in ice-cold PBS. Cells were gently pelleted by centrifugation (300*g*, 5 min, 4 °C), PBS was decanted, and cells were fixed and permeabilized by dropwise addition of 70% ethanol at −20 °C while vortexing. Fixed cells were washed with PBS and incubated in the dark for 30 min with propidium iodide staining solution containing 50 μg/ml propidium iodide (PI) and 100 μg/ml RNase A in PBS. DNA fluorescence was measured with a FACS Calibur Flow Cytometer (Becton–Dickinson) equipped with a 488-nm argon laser. Width (FL2W) and area (FL2A) of PI fluorescence were recorded for at least 10,000 counts. DNA histograms were extracted from FL2W–FL2A dot plots. The percentage of cells in each phase of the cell cycle was analyzed using ModFit software (Verity).

RNA isolation and analysis. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Total RNA (1 μg) was subjected to Reverse-Transcriptase Polymerase Chain Reaction using One-Step RT-PCR kit (Qiagen) according to manufacturer's instruction. Briefly, RT-PCRs were carried out in the presence of the supplied buffer, dNTPs (400 μM), enzyme mix (reverse transcriptases and DNA polymerase), and RNase inhibitor (10 U). Gene-specific primers were used at a final concentration of 0.6 μM and designed as follows: Skp2 forward, 5-GGCAAAGGGAGTGACAAAGA-3; Skp2 reverse, 5-TCAAAGCACCAGGAGAGATT-3 (product size = 590 bp). QuantamRNA Classic II 18S primer/competimers were used in the reactions as internal standards at a final concentration of 0.6 μM.

Plasmids. Promega's pGL3-basic vector was used to create mouse pGL3-Skp2 promoter constructs for luciferase assays. Primers flanking the region of −2473 to +74 were used to amplify the murine Skp2 promoter from 3T3-L1 preadipocyte genomic DNA. The resulting cDNA fragment was subcloned into the *Kpn*I and *Bgl*II restriction sites of the pGL3-basic firefly luciferase vector (Promega). Nucleotide sequence was confirmed by SeqWright DNA Technology Services. This process from genomic DNA to sequenced vector was repeated twice to ensure reliability of results. Progressive 5′ truncations of the 2.4 kb promoter were constructed using existing endonuclease restriction sites as follows: 2.2 kb (−2248 to +74) using *Kpn*I and *Bbv*CI; 1.5 kb (−1573 to +74) using *Kpn*I and *Eco*RI; 1.0 kb (−1073 to +74) using *Kpn*I and *Nsi*I; 0.4 kb (−0.454 to +74) using *Kpn*I and *Mlu*I.

Luciferase reporter assays. 3T3-L1 preadipocytes (2×10^6 cells) were transiently transfected with 5 μg of firefly reporter plasmid using Amaxa's Nucleofector (program T20; Solution V) and incubated with pre-warmed proliferation medium for no more than 10 min at 37 °C. Cells were then replated at saturation density. Fresh medium was replaced 24 h post-transfection and cultured as described above. Firefly luciferase was measured using the Dual-Glo Luciferase Assay System (Promega).

Results

Helenalin inhibits hormonally induced Skp2 accumulation, p27 degradation, and G_1/S phase transition

We have previously determined that Skp2 protein periodically accumulates during S/G_2 phase progression of

[1] C.A. Auld and R.F. Morrison, manuscript under review.

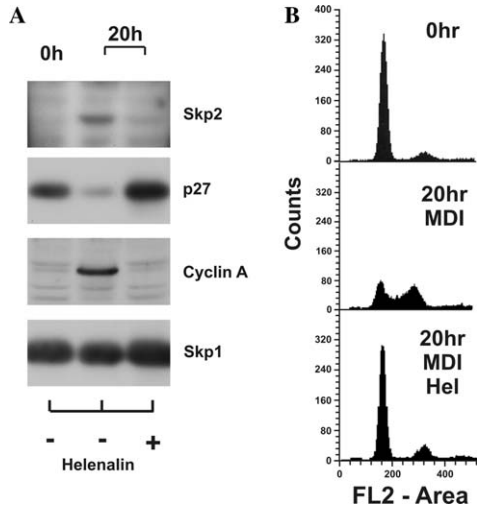

Fig. 1. Helenalin inhibits hormonally induced Skp2 accumulation, p27 degradation, and G_1/S phase transition. (A) Whole cell lysates were collected from density-arrested 3T3-L1 preadipocytes at 0 and 20 h post-MDI in the absence and presence of helenalin (3 μM) and immunoblotted as illustrated. (B) Nuclei were stained under identical conditions and DNA histograms generated with flow cytometric analysis.

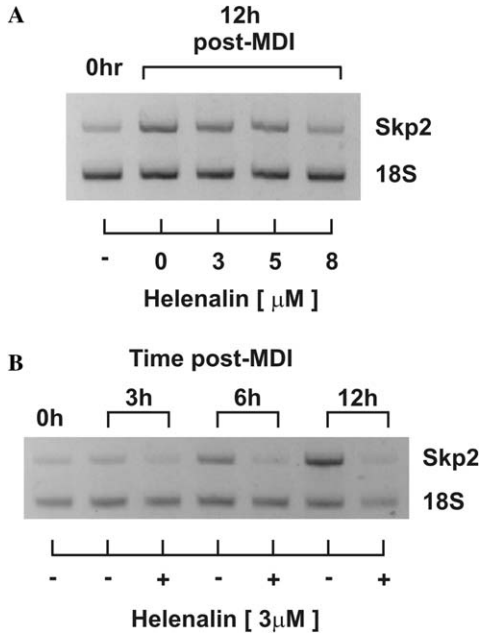

Fig. 2. Helenalin blocks the induction of Skp2 mRNA accumulation in a dose-dependent manner. (A) Total RNA was harvested from density-arrested preadipocytes at 12 h following MDI stimulation supplemented with varying concentrations of helenalin as illustrated. (B) Total RNA was harvested from density-arrested preadipocytes over time following MDI stimulation supplemented with and without helenalin (3 μM). Relative mRNA accumulation was determined by RT-PCR. Abundance of 18S was concomitantly determined for each sample as a reference for loading and consistency.

3T3-L1 preadipocyte differentiation. To examine the effect of helenalin on Skp2 accumulation, density-arrested 3T3-L1 preadipocytes were stimulated with MDI in the presence or absence of helenalin (3 μM). Whole cell lysates were collected at 0 h (density arrest) and 20 h post-MDI and immunoblotted as indicated in Fig. 1A. We demonstrate that helenalin completely suppressed hormonally induced Skp2 protein accumulation during cell cycle progression. A functional effect of Skp2 suppression was demonstrated by a total rescue of phase-specific degradation of p27, a principal target of the SCF^{Skp2} E3 Ligase. We also observed a dramatic inhibition of cyclin A accumulation, suggesting that entry into S phase was prohibited. This notion was confirmed as helenalin treatment resulted in complete and absolute G_1 arrest in hormonally stimulated preadipocytes as assessed by flow cytometric analysis of propidium iodide DNA staining (Fig. 1B). No significant apoptotic (sub-2n) or polyploidy (>4n) cells were noted. These suppressive effects were specific to cell cycle progression as helenalin treatment did not alter protein levels of Skp1, a constitutively expressed member of the SCF E3 ligase. It is important to note that these observations occurred with helenalin being administered concomitantly with MDI stimulation. As no pretreatment was necessary for complete cell cycle arrest, these data suggest that helenalin likely targeted events beyond immediate early signaling, but prior to G_1/S phase transition when p27 is degraded allowing cyclin A to accumulate.

Dose-dependent suppression of Skp2 mRNA accumulation by helenalin

We have previously observed that periodic Skp2 expression is regulated at the level of transcription with maximal mRNA and protein accumulation occurring at 12 and 20 h post-MDI, respectively. To determine if the observed effect of helenalin on Skp2 protein levels shown above was reflected by changes in mRNA accumulation, total RNA was collected at 0 and 12 h post-MDI in the absence or presence of varying concentrations of helenalin. Relative Skp2 and 18S mRNA levels were assessed by RT-PCR and illustrated in Fig. 2A. We demonstrate that helenalin co-treatment with MDI stimulation decreased Skp2 mRNA in a dose-dependent manner, suggesting that helenalin may suppress signaling pathways that impinge upon Skp2 promoter activity. As the IC_{50} for Jurkat leukemia T cells has been reported to be 6 μM [4] with 10–50 μM showing signs of cytotoxicity [4,5], all subsequent experiments were performed with 3 μM helenalin. No obvious signs of toxicity (i.e., changes in cell morphology, appearance of apoptotic nuclei, or reduced 18S mRNA levels) were noted for preadipocytes over the range of helenalin concentrations used in this experiment.

Helenalin blocks early induction of Skp2 mRNA accumulation

To further elucidate the mechanism regarding helenalin and Skp2 expression, total RNA was collected over time from density-arrested preadipocytes stimulated with MDI in the absence or presence of helenalin (3 μM). Relative mRNA levels, assessed by RT-PCR, are illustrated in

Fig. 2B. In the absence of helenalin, Skp2 mRNA levels increased significantly with time following induction of differentiation. The data are consistent with previous observations showing periodic Skp2 mRNA accumulation where low levels observed in density-arrested preadipocytes measurably increased by 4 h post-MDI to achieve maximal accumulation by 12 h and returned to basal levels during G_2/M phases of cell cycle progression. Helenalin completely abolished early Skp2 mRNA accumulation at 6 h post-MDI further refining the time frame by which this phytochemical functions to repress cell cycle progression.

Induction of Skp2 promoter activity abolished with helenalin

Next, we determined if helenalin's effect on Skp2 mRNA accumulation was reflected by changes in promoter activity. For this, a 2.4 kb fragment (−2473 to +74) of the murine Skp2 promoter was amplified from 3T3-L1 genomic DNA, subcloned into a pGL3 luciferase vector, and commercially sequenced for verification. This reporter construct was transferred to a single pool of subconfluent preadipocytes by nucleofection (Amaxa). Cells were replated at saturation density and stimulated with MDI in the absence or presence of helenalin (3 μM). Lysates were harvested at 12 h post-MDI and changes in promoter activity assessed by relative luciferase activity. As shown in Fig. 3A, Skp2 promoter activity increased ~2.5-fold by 12 h following hormonal stimulation. Consistent with changes in mRNA levels, helenalin completely abolished the induction in Skp2 promoter activity, suggesting that helenalin impinges upon signaling pathways that couple hormonal stimulation with Skp2 transcription. It is important to note that helenalin abolished only that portion of Skp2 promoter activity that increased following MDI stimulation, and not basal activity, further suggesting helenalin targeted signaling events leading to Skp2 transcription and

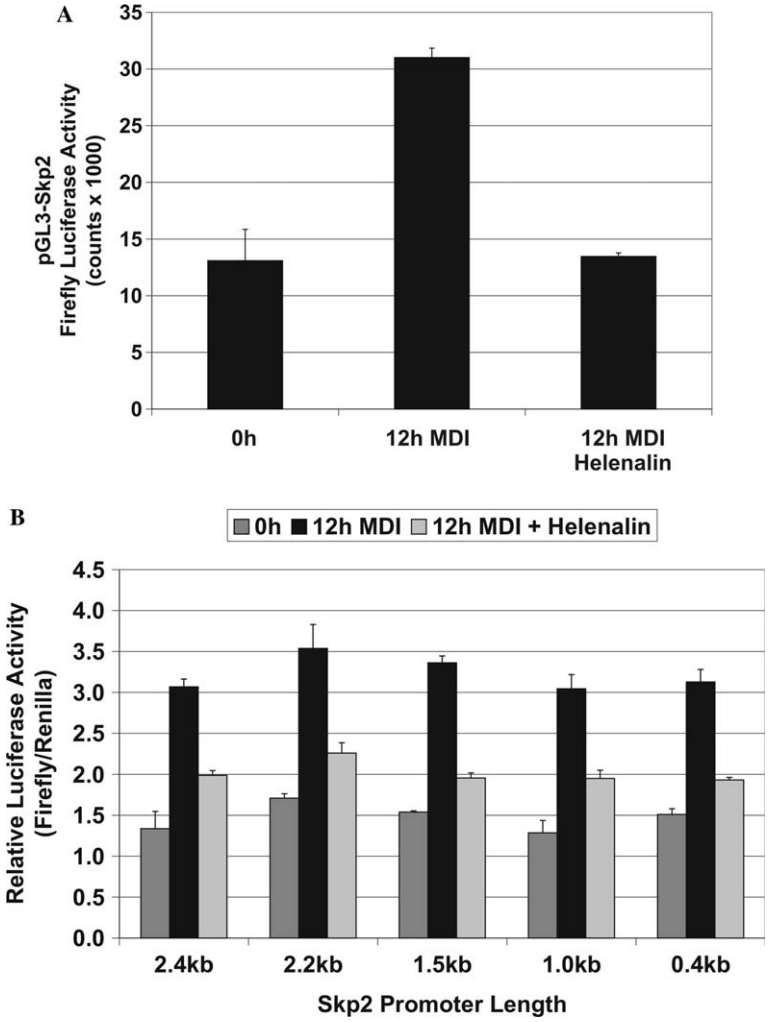

Fig. 3. Helenalin blocks the induction of Skp2 promoter activity. (A) Pooled subconfluent preadipocytes were transfected with 5 μg of a pGL3-Skp2 (~2.4 kb) promoter luciferase vector and plated at saturation density. At 2 days post-confluence, cells were stimulated with MDI supplemented with or without helenalin (3 μM). Relative luciferase activity was determined from cell lysates harvested at 0 and 12 h post-MDI. (B) Subconfluent preadipocytes were cotransfected with 5 μg pGL3-Skp2 promoter deletion constructs as illustrated and 20 ng *Renilla*-SV40 control luciferase vector and treated as discussed in (A). Normalized firefly/*Renilla* luciferase activity was measured 12 h post-stimulation.

not adversely through an unknown repressor element located elsewhere in the Skp2 promoter. Skp2 promoter deletion studies support this notion as the regulatory region responsive to hormonal stimulation was identified as the region essential for helenalin inhibitory activity (Fig. 3B).

Helenalin suppression of PI3K, but not MAPK-signaling pathways

We have previously determined that PI3K and to a lesser extent MAPK pathways are required for Skp2 expression during the proliferative phase of early adipocyte differentiation. To determine the influence of helenalin on the activation of these pathways, whole cell lysates were collected over time from density-arrested preadipocytes stimulated with MDI in the absence and presence of helenalin (3 μM) and immunoblotted with phosphospecific antibodies to Akt and Erk1/2, principal downstream targets of PI3K and MAPK activation, respectively. In the presence or absence of helenalin, hormonal stimulation leads to maximal Erk phosphorylation within 15 min post-MDI, which was sustained to a lesser extent through 6 h and returned to basal levels by 12 h post-MDI (Fig. 4). No difference in Erk phosphorylation throughout the time course due to helenalin treatment was noted. Hormonal stimulation in the absence of helenalin also leads to an increase in Akt phosphorylation that was marked by 15 min, maximal by 3 h, and sustained to a lesser extend through 12 h post-MDI. Early Akt activation (i.e., 15 min post-MDI) occurred in a similar fashion in the absence or presence of helenalin. By 3 h, however, Akt phosphorylation in preadipocytes treated with helenalin was markedly less than in cells stimulated with MDI alone. The level of Akt phosphorylation continued to decline reaching near basal levels by 6 h post-MDI. Helenalin did not alter total protein accumulation of either Akt or Erk. Collectively, these data narrow the time frame in which helenalin treatment leads to complete G_1 arrest to less than 6 h post-MDI and demonstrate an effect on Akt signaling that precedes the suppression of Skp2 mRNA, which accumulates during G_1 phase progression of replicating preadipocytes.

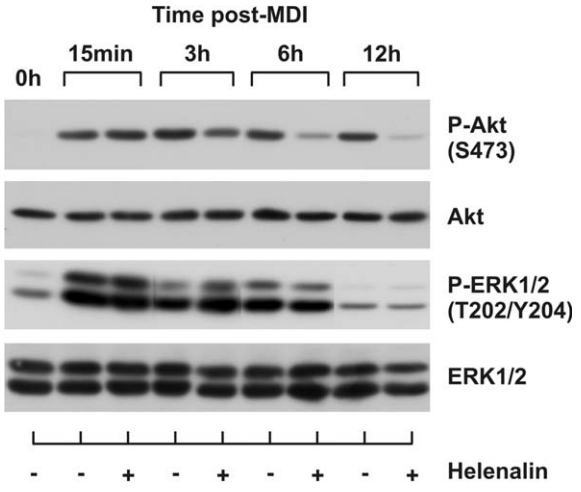

Fig. 4. Helenalin suppresses Akt phosphorylation. Whole cell lysates were collected from density-arrested 3T3-L1 preadipocytes over time following MDI stimulation in the absence and presence of helenalin (3 μM) and immunoblotted as illustrated.

Discussion

Positive energy balance can cause preadipocytes to undergo a transition from quiescence to proliferation during the development of hyperplastic obesity [14,15]. The cyclin-dependent kinase inhibitors (CKIs), p27 and p21, govern this transition as potent inhibitors of the G_1/S phase transition. Recent studies underscore the importance of these CKIs in adipose tissue development as mice defective in p27/p21 gene expression were shown to have a disproportionate increase in adipose tissue mass due to adipocyte hyperplasia, not hypertrophy [16]. Using 3T3-L1 preadipocyte differentiation as a model of adipocyte hyperplasia [17,18], we have previously determined that p27 activity is regulated, in part, through targeted proteolysis involving ubiquitylation by the SCF^{Skp2} E3 ligase and degradation by the 26S proteasome.[1] Although Skp2 knockout mice are reportedly smaller than wild-type littermates [19], the effect of Skp2 gene ablation on adipose tissue mass and development has yet to be reported.

The data presented here, for the first time, demonstrate that the phytochemical, helenalin dramatically inhibits periodic Skp2 protein accumulation, p27 degradation, and S phase progression resulting in G_1 arrest. It is also shown that helenalin completely blocks the induction of Skp2 mRNA and prevents Skp2 promoter activity during early stages of adipocyte differentiation. While the mechanism involving Skp2 regulation is still unclear, helenalin was shown to decrease the extent of Akt phosphorylation immediately preceding the complete suppression of Skp2 mRNA accumulation during early G_1 phase progression of clonal expansion. While other effects are yet to be determined, the events presented here are consistent with the notion that helenalin inhibits preadipocyte replication, at least in part, through sequential Akt, Skp2, and p27 regulation.

Helenalin is commercially available and used in cellular studies as a potent, specific inhibitor of NFκB p65 DNA-binding activity. Although Akt has been shown to be upstream of NFκB, the reverse scenario has yet to be demonstrated. The rapid effect on Akt phosphorylation, however, would suggest that helenalin is working directly through upstream kinases in the PI3K pathway leading to Akt phosphorylation. Although NFκB activity has not been examined during cell cycle progression of preadipocytes, it has been shown to increase during late stages of adipogenesis [20]. Obesity is often regarded as an inflammatory state as adipocytes secrete pro-inflammatory cytokines such as TNFα [21,22] and IL-6 [23], and NFκB has been implicated in the release of these cytokines from

adipocytes [24]. As helenalin is often reported as an anti-inflammatory agent, it would not be unexpected that this phytochemical would also inhibit NFκB activity during late stages of adipocyte differentiation. Additional studies are needed and underway to discern whether NFκB activity is involved in the helenalin effect of cell cycle progression during early stages of adipogenesis reported here.

While the cytotoxic effect of helenalin on tumor cells has been well documented, the mechanism of action is still unclear. Data presented here suggest that helenalin impedes an early growth factor mediated PI3K signaling pathway during preadipocyte replication. The PI3K/Akt pathway is known to activate pro-mitotic survival signals and the ability of this pathway to inhibit apoptosis is dependent on Akt phosphorylation and subsequent activation [25]. Other studies have also shown that Akt blocks apoptosis by inhibiting caspase activity [25]. In this regard, the broad-spectrum caspase inhibitor zVAD-fmk is able to abolish helenalin's ability to induce apoptosis in human leukemia Jurkat T cells [5]. Interestingly, the sesquiterpene lactone, parthenolide, has been shown to cause a dramatic increase in Akt phosphorylation following 2–3 h of exposure [26]. Longer periods of exposure to parthenolide, however, resulted in a decrease in Akt phosphorylation, similar to helenalin's early effect on Akt phosphorylation in 3T3-L1 preadipocytes reported here.

Signs of apoptosis have been reported 16 h following helenalin treatment of Jurkat T leukemia cells [5]. In our report, preadipocytes were treated with helenalin for 12 h or less and at a concentration that does not promote apoptosis in other cells. To this point, healthy PHA-stimulated PBMC cells have been shown to be resistant to the apoptotic effects of helenalin, at concentrations up to 50 μM [5], suggesting a cell-type specific and dose-dependent effect of helenalin on apoptotic signaling. Studies addressing whether or not helenalin inhibits cell cycle progression of replicating preadipocytes during early stages of adipocyte differentiation through mechanisms involving apoptosis are currently underway.

Acknowledgments

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