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Enhancement of p53 Expression in Keratinocytes by the Bioflavonoid Apigenin Is Associated with RNA-binding Protein HuR

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Abstract

We have reported previously that apigenin, a naturally occurring non-mutagenic flavonoid, increased wild type p53 protein expression in the mouse keratinocyte 308 cell line by a mechanism involving p53 protein stabilization. Here we further demonstrated that the increase in p53 protein level induced by apigenin treatment of 308 keratinocytes was not the result of enhanced transcription, mRNA stabilization or cytoplasmic export of p53 mRNA. Instead, biosynthetic labeling showed that apigenin increased nascent p53 protein synthesis by enhancing p53 translation. The AU-rich element (ARE) within the 3'-untranslated region (UTR) of p53 mRNA was found to be responsible for apigenin's ability to increase p53 translation, as demonstrated in studies wherein the 3'-UTR of p53 mRNA containing the ARE was fused downstream of a luciferase reporter gene. Furthermore, apigenin treatment increased the level of association of the RNA binding protein HuR with endogenous p53 mRNA. Apigenin treatment also augmented HuR translocation into the cytoplasm. Overexpression of HuR enhanced apigenin-induced p53 protein expression in 308 keratinocytes, whereas siRNA-mediated HuR reduction suppressed apigenin-induced p53 protein expression and de novo translation of p53. Moreover, apigenin treatment of cells induced p16 protein expression, which in turn was correlated with cytoplasmic localization of HuR induced by apigenin. Overall, these findings indicate that, in addition to modulating p53 protein stability, one of the mechanisms by which apigenin induces p53 protein expression is enhancement of translation through the RNA binding protein HuR.

Keywords

p53; HuR; AU-rich element; apigenin; keratinocytes

INTRODUCTION

Approximately half of human cancers carry *p53* gene mutations [1], and intensive studies over the past 20 years have shown p53 plays a critical role in guarding against cancer development. Activation of p53 can induce several responses, including DNA repair, senescence, differentiation, inhibition of angiogenesis and alteration of metabolism, but the best known functions of p53 are associated with inducing cell cycle arrest and apoptosis after a variety of mitogenic and stressful stimuli [2,3]. Therefore, a long-term goal of research in our laboratory has been to target cellular responses involving the p53 protein as a strategy for developing chemopreventive agents against ultraviolet (UV)-induced skin

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cancer. Thus far, a number of studies have identified chemopreventive agents that activate p53, hence protecting against UV-induced skin cancers [4–6].

One such candidate agent is apigenin, a naturally occurring flavonoid present in a variety of fruits and leafy vegetables [7]. Apigenin has shown promise as a chemopreventive agent by stimulation of gap junctional and intercellular communication; inhibition of transformation and angiogenesis, inhibition of many enzymes that play important roles in tumor promotion; and induction of cell cycle arrest [8]. Topical application of apigenin to mouse epidermis has been shown to reduce the number and size of tumors in mouse skin induced by both chemical carcinogens [9] and UV exposure [10]. We have reported previously that apigenin stabilizes and activates wild type p53 protein [11], indicating that one important aspect of apigenin's chemopreventive action may be its ability to induce p53 expression.

Although transcriptional regulation of p53 expression has been observed [12], regulation of p53 expression has historically been associated with mechanisms regulated primarily by post-transcriptional modifications of protein stability, p53 subcellular localization, and association with other proteins [13]. In recent years, however, several reports have suggested that p53 mRNA stability and translational regulation also contribute to p53 induction. For example, p53 negatively regulates its own translation by binding to the 5'-untranslated region (UTR) stem-loop structure of mRNA [14]. p53 translation is suppressed by various proteins binding to p53 mRNA. For example, thymidylate synthase suppresses p53 translation by binding to the coding sequence of p53 mRNA [15]. Ribosomal protein L26 and nucleolin bind to the 5'-UTR of p53 mRNA and control p53 translation and induction after DNA damage [16]. Fu and coworkers have demonstrated that a translation suppressor element is located in the 3'-UTR of p53 mRNA [17], and the mRNA stabilizing protein HuR enhances p53 translation in response to UV irradiation by binding to the 3'-UTR of p53 mRNA [18].

HuR is a member of the embryonic lethal abnormal vision family of mRNA-binding proteins, containing RNA recognition motifs which bind with high affinity and specificity to the AU-rich elements (ARE) in a variety of mRNAs [19]. HuR binding to mRNA results in increased stability and enhanced translation, or both [18,20]. Although HuR is localized predominantly in the nucleus, it can shuttle between the nucleus and cytoplasm, and the exact mechanism by which translocation of HuR is regulated remains elusive.

In the present study, we investigated the mechanisms by which apigenin induced p53 expression in mouse keratinocytes. We demonstrated that apigenin treatment did not increase p53 transcription, mRNA stability or exportation of more p53 mRNA from nucleus to cytoplasm, but rather enhanced nascent p53 protein synthesis. We further showed that the ARE of p53 mRNA is important for apigenin's effect on translational activity, as demonstrated in studies wherein the ARE-containing 3'-UTR of p53 mRNA was fused downstream of a luciferase reporter gene, resulting in enhanced luciferase expression. The RNA binding protein HuR was observed to bind to endogenous p53 mRNA, and apigenin treatment increased HuR translocation into cytoplasm. Modulation of HuR levels in the mouse 308 keratinocyte cell line affected apigenin-induced p53 protein expression, and apigenin treatment also induced p16 expression, which in turn was correlated with cytoplasmic localization of HuR. In short, the results indicate that, in addition to modulating p53 protein stability, one of the mechanisms by which apigenin induces p53 expression is enhancement of translation by the RNA binding protein HuR.

MATERIALS AND METHODS

Cell Culture and Apigenin Treatment

The 308 mouse keratinocyte cell line was derived from Balb/c mouse skin initiated with dimethylbenz[a]anthracene and contains a wild type p53 gene [21]. The 308 keratinocyte cell line was maintained in Suspension Minimum Essential Medium (United States Biological, Swampscott, MA) supplemented with 8% chelexed (Bio-Rad Laboratories, Hercules, CA) FBS and 0.05 mM Ca^{2+} . Prior to treatment, cells were grown to 80% confluence. Apigenin (Sigma, St. Louis, MO) stock solutions were prepared in dimethyl sulfoxide (DMSO) and added to the existing culture medium to achieve the desired final concentration. The concentration of DMSO in cell cultures was less than 0.1%.

Reporter Gene Construction

Full-length murine p53 cDNA containing the 3'-UTR was purchased from Open Biosystems (Huntsville, AL. Item number: MMM101364179). Various regions of p53 cDNA 3'-UTR were amplified by PCR using primers ending in an *Xba* I recognition sequence. PCR products were ligated into the unique *Xba* I site of the pGL3-control vector (Promega, Madison, WI), located in the 3'-UTR of the firefly luciferase gene, and confirmed by DNA sequencing.

Transient Transfection

Cells were transiently transfected using Lipofectamine PLUS reagent (Invitrogen, Carlsbad, CA). Briefly, cells were plated in 24-well plates and grown to 80–90% confluence, 0.2 μg reporter gene plasmid DNA and 2 ng control *Renilla* luciferase plasmid DNA (Promega) were prepared in 25 μl of serum-free medium and incubated with 4 μl of PLUS reagent at room temperature for 15 min, followed by 1 μl of Lipofectamine in an additional 25 μl serum-free medium. The mixture was incubated for another 15 min and layered onto the cells. After 3 h incubation, normal medium containing 2 $\times$ FBS was added, and cells were incubated overnight for gene expression.

Luciferase Assay

Luciferase activity was determined using Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Cells were rinsed with phosphate buffered saline (PBS) and removed by scraping into 100 μl of passive lysis buffer. The lysate was then transferred into a tube and subjected to 1 or 2 freeze-thaw cycles to accomplish complete lysis of cells. Assays were performed by using a Monolight 3010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Firefly luciferase activity is expressed as relative light units and was normalized to *Renilla* luciferase activity.

Western Blot Analysis and Cell Fractionation

Western blot analysis and the preparation of cytoplasmic and nuclear cell fractions were performed as described previously [22]. Briefly, for polysome separation, cell cytoplasmic fraction lysates were layered onto an ice-cold buffer (20 mM HEPES, pH 7.5, 50 mM KOAc, 5 mM MgOAc, 1 mM DTT, 100 Units/ml RNase OUT, and protease inhibitors) containing 30% sucrose. After ultra-centrifugation at $100,000 \times g$ for 2 h at 4 °C in a Beckman SW 55 Ti rotor, the supernatant was saved as cytosolic fraction. The pellet was resuspended in buffer A containing 0.3 M NaCl, incubated on ice for 1 h, centrifuged at $10,000 \times g$ for 15 min at 4 °C, and the resulting supernatant was saved as polysomal fraction. Antibodies against p53 (FL-393), HuR (3A2), p21 (C-19), PUMA α/β (FL-193), p16 (M-156), Actin (I-19), α -Tubulin (B-7), and hnRNP A0 (G-17) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Bax and GAPDH antibodies were from

Millipore (Billerica, MA), and antibodies against AMPK α , Phospho-AMPK α (Thr172) and Phospho-p53 (Ser15) were from Cell Signaling Technology (Danvers, MA).

Immunofluorescence

After treatment, cells were washed with PBS, fixed in PBS containing 4% paraformaldehyde at room temperature for 30 min, washed with PBS again, and incubated in PBS containing 0.1% Triton X-100 for 10 min. After incubation in blocking buffer (PBS containing 0.1% Tween-20, 3% BSA and 3% goat serum) for 1 h, the slides were incubated with anti-HuR antibody in PBS containing 5% goat serum for 48 h at 4 °C, washed with PBS containing 0.1% Tween-20, and further incubated with Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) for 2 h in dark. After final wash with PBS, the slides were mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA) and visualized with a Zeiss Axiovert 200 (Carl Zeiss MicroImaging Inc., Thornwood, NY) fluorescence microscope.

Real-time PCR Assays

Total RNA was isolated at various times after treatment using TRIzol reagent (Invitrogen) and treated with a DNA-free kit (Ambion, Austin, TX) to eliminate genomic DNA contamination. RNA was reverse-transcribed using SuperScript III First-Strand Synthesis System with random hexamer primers (Invitrogen). After first-strand synthesis, for quantification of p53 mRNA, real-time PCR was performed using TaqMan Gene Express Assay (Applied Biosystems, Foster City, CA. Assay ID: Mm01731287_m1) specific for the p53 gene. Fluorescence was detected with an ABI Prism 7900HT real-time PCR system and normalized to ribosomal RNA as measured using TaqMan eukaryotic 18S rRNA endogenous control (Applied Biosystems). For quantification of luciferase reporter gene mRNA, real-time PCR was performed using luciferase gene-specific primers and the double-stranded DNA-binding dye SYBR green I as described [23], and normalized to GAPDH content.

Immunoprecipitation of mRNP Complex and RT-PCR

Cytoplasmic extracts were pre-incubated with normal mouse IgG and protein A/G PLUS-agarose beads (Santa Cruz Biotechnology), then the suspensions were mixed with 15 μ g of HuR or normal IgG antibody. After incubation on ice for 2 h, 30 μ l Protein A/G PLUS-agarose beads were added to each sample, followed by mixture for a further 1 h at 4 °C. Samples were centrifuged at 2,000 $\times$ g for 2 min at 4 °C, the pelleted beads were washed 4 times with cold wash buffer supplemented with protease and RNase inhibitors, and RNA was isolated using TRIzol reagent. DNase I treatment and reverse transcription were performed as described above. The cDNA was used as template for PCR with p53 specific primers (forward: 5'-AGAGACCGCCGTACAGAAGA-3' and reverse: 5'-CTGTAGCATGGGCATCCTTT-3') and β -Actin specific primers (forward: 5'-TATGGAATCCTGTGGCATCC-3' and reverse: 5'-GTACTTGCGCTCAGGAGGAG-3'). These primers produce amplicons that span different introns, allowing the discrimination of cDNA- and genomic DNA-related amplification. PCR products were visualized by electrophoresis in an ethidium bromide-stained 1.2% agarose gel.

RNA Interference Experiment

A pool of 3 target-specific siRNA duplex targeting mouse HuR mRNA, p16 mRNA and non-targeting control siRNA were purchased from Santa Cruz Biotechnology. The transfection of siRNA into the mouse 308 keratinocyte cell line was performed by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For p16 knockdown, cells were harvested 24 h after transfection. For HuR knockdown, cells were harvested at 96 h after transfection.

Analysis of Nascent p53 Protein

For metabolic labeling, mouse 308 keratinocyte cells were incubated for 1 h in DMEM medium without methionine and cysteine (Sigma) before apigenin (50 μ M) treatment. At 4 h post-treatment with apigenin, each sample was labeled with 500 μ Ci L-[³⁵S]methionine and L-[³⁵S]cysteine (GE Healthcare, Piscataway, NJ) for 15 min, cells were harvested in lysis buffer as whole-cell extracts and immunoprecipitations (IP) were carried out overnight at 4 °C using anti-HuR antibody or normal mouse IgG. Following extensive washes in washing buffer (50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA and 0.5% NP-40), the immunoprecipitated materials were resolved by SDS-PAGE and assessed by autoradiography.

RESULTS

Apigenin Increases the Level of p53 Protein in the 308 Keratinocyte Cell Line

We investigated the effect of apigenin treatment on wild type p53 protein levels in the mouse 308 keratinocyte cell line. The p53 protein levels were examined in cells treated with a range of apigenin concentrations (0–70 μ M), and Western blot analysis demonstrated that apigenin induced p53 protein expression dramatically, with a peak at 50 μ M (Figure 1A). To further determine the time of maximal p53 protein induction, cells were treated with 50 μ M apigenin and harvested at different time points. The increase in p53 protein expression occurred rapidly after treatment, reached maximal increase at 8 h after treatment, and then began to decline after 12 h of apigenin treatment (Figure 1B).

To assess whether the elevated p53 protein has function to activate transcription of downstream genes, we also examined protein levels of p21, Bax and PUMA by Western blot analysis. The p21 gene is involved in cell cycle regulation, and Bax and PUMA are genes responsible for induction of apoptosis [24]. All three genes are directly transcriptionally activated by p53. As shown in Figure 1C, treatment of cells with apigenin resulted in a moderate, time-dependent increase in p21 protein levels beginning at 8 h, while PUMA and Bax protein levels were induced substantially by apigenin treatment at 18 h and 24 h, respectively. Overall, these results demonstrated that apigenin not only increased p53 protein level but also functionally activated p53 and its downstream transcriptional targets.

Elevated Level of p53 Protein Is Not Due to p53 Transcription, mRNA Stabilization or Subcellular Distribution

To investigate the mechanism responsible for the induction of p53 protein by apigenin, we first examined the steady-state levels of p53 mRNA by real-time reverse transcriptase (RT)-PCR assay. Apigenin was added to the existing culture media to avoid adding fresh serum-containing medium that could stimulate serum-related effects on mRNA transcription. Total RNA was purified from cells that had been treated with apigenin and harvested at different times post-treatment. As shown in Figure 2A, steady-state p53 mRNA levels decreased slightly after 2 h of treatment and decreased significantly after 4 h of treatment. Since the steady-state level of mRNA is determined by both rates of synthesis and degradation, we further investigated whether mRNA stabilization/destabilization could play a role in apigenin-mediated p53 protein expression. To this end, cells were pre-treated with medium containing apigenin or DMSO as vehicle control for 2 h or 4 h, and subsequent transcription was stopped by adding actinomycin D. RNA was isolated at different time points after the addition of actinomycin D and subjected to TaqMan real-time PCR analysis. As shown in Figure 2B at 2 h and Figure S1 at 4 h, apigenin treatment did not change the stability of p53 mRNA, demonstrating that the increase in p53 protein level was not due to either an elevation in p53 mRNA level or stabilization. However, it is possible that apigenin could affect the export of the p53 mRNA from nucleus to cytoplasm. To investigate this

possibility, we also measured p53 mRNA abundance in different cellular fractions. Although the abundance of p53 mRNA was diverse in different cellular fractions, apigenin treatment did not change their distribution (Figure 2C). Together, these results suggested that the observed increase in p53 protein expression by apigenin treatment was regulated by either translational or post-translational events.

Apigenin Enhances p53 Translation

In view of the fact that a wide body of evidence from numerous laboratories confirms that p53 expression is primarily regulated through modulation of steady-state level of p53 protein, and protein phosphorylation contributes to p53 protein stability, we next investigated phospho-p53 levels after apigenin treatment by using phospho-p53 specific antibody (Ser-15). As shown in Figure 3A, phosphorylation of p53 protein occurred rapidly after apigenin treatment, and was sustained for at least 12 h. However, we observed that total p53 protein level reached a peak at 8 h after treatment and then diminished to a level at 12 h which is significantly lower than that at 8 h. This result raised the possibility that phosphorylated p53 may only represent a small portion of the elevated total p53 protein induced by apigenin, and there may be other mechanism(s) responsible for apigenin-induced p53 expression.

Considering there is increasing evidence that translational regulation is involved in p53 expression, we next employed metabolic labeling to measure nascent p53 protein synthesis in apigenin-treated cells. A brief (15 min) incubation of 308 cells with ^{35}S -labelled amino acids immediately followed by IP using anti-p53 antibody revealed that the level of nascent p53 was increased by 4 h after apigenin treatment (Figure 3B, upper panel), indicating that apigenin-induced p53 expression was regulated, at least in part, by increased mRNA translation. In the lower panel of Figure 3B, whole-cell extracts (WCE) were analyzed to demonstrate that an equal amount of [^{35}S]methionine/cysteine was incorporated into cells, confirming that protein translation in general was not affected by apigenin treatment.

The ARE of p53 mRNA Is Involved in Its Translational Up-regulation

The murine p53 mRNA 3'-UTR has an ARE-containing region that includes both a typical AUUUA sequence and one AU-rich region (Figure 4A). In view of the growing evidence that the ARE within the 3'-UTR of mRNAs can affect translation [18,23], we next examined whether the presence of the ARE mediated the effect of apigenin treatment. Transient transfections were performed with reporter gene constructs containing the luciferase gene fused at its 3'-end to the full-length p53 3'-UTR (nucleotides [nt] 1302-1743), the ARE-containing region (nt 1605-1743), or the ARE-containing region deleted from the full-length 3'-UTR (nt 1302-1624) (Figure 4A). As illustrated in Figure 4B, the presence of the full-length 3'-UTR and the ARE-containing region was found to significantly enhance the reporter activity at 24 h after apigenin treatment, whereas the reporter constructs with the ARE-containing region deleted from the full-length 3'-UTR appeared to be unresponsive to apigenin.

Changes in luciferase activity could be due to either alterations in mRNA abundance or rates of translation. To distinguish between these two possibilities, we further measured the steady-state levels of luciferase mRNA at 24 h after apigenin treatment using real-time RT-PCR assay. As shown in Figure 4C, apigenin treatment actually reduced luciferase mRNA. Comparison of the results from luciferase activity experiments (Figure 4B) and measurements of luciferase mRNA (Figure 4C) were consistent with the hypothesis that elevation of p53 protein level in apigenin-treated cells was due to enhancement of mRNA translation.

Apigenin-dependent Binding of HuR to Endogenous p53 mRNA

Post-transcriptional regulation mediated by AREs is primarily governed by RNA binding proteins (RBPs) that bind to mRNAs containing ARE and ensure their proper processing, export, as well as subcytoplasmic transit, stability and translation rate [25]. Among these RBPs, HuR has been confirmed to influence p53 translation [18]. To investigate the endogenous association between HuR protein and p53 mRNA in mouse 308 keratinocytes, we used an IP assay followed by measurement of p53 mRNA by RT-PCR. As shown in Figure 5, HuR co-immunoprecipitated with p53 mRNA in an apigenin-dependent manner when using anti-HuR antibody. Control samples immunoprecipitated with normal IgG showed only weak amplification and no amplification was observed in the absence of antibody (data not shown). Based on the size of all amplicons, there is no genomic contamination because the PCR product would be much larger if the intron sequence had been amplified as well. The levels of the housekeeping β -Actin mRNA were also examined as a sample input control.

Subcellular Distribution of HuR after Apigenin Treatment

Numerous reports have demonstrated that cytoplasmic localization is associated with HuR's functional activity [18,26]. Therefore, we examined the subcellular localization of HuR after apigenin treatment. Western blot analysis using whole cell lysates demonstrated that there was no change in total HuR expression during apigenin treatment (Figure 6A). When subcellular fractions were further analyzed, we observed predominant HuR in the nuclei but a higher level of cytoplasmic HuR in apigenin-treated cells compared to the level of HuR in cytoplasmic fraction from control cells (Figure 6A). Verification that nuclear proteins did not leak into the cytoplasmic fractions during the fractionation process was obtained by immunoblotting the same membranes to detect the nuclear marker hnRNP A0 protein. hnRNP A0 protein was found only in the nuclear fraction, while the cytoplasmic marker α -Tubulin was detected only in the cytoplasmic fraction (Figure 6A). Immunofluorescence was also performed and the result confirmed the increased cytoplasmic localization of HuR after apigenin treatment (Figure 6B). To further monitor the cytoplasmic distribution of HuR, cytosolic and polysomal fractions were separated by ultra-centrifugation of the cytoplasmic fraction through a cushion of 30% sucrose. As shown in Figure 6C, HuR was almost exclusively found associated with the polysomal fraction, and apigenin treatment increased the polysomal level of HuR protein. Accordingly, we found that apigenin treatment increased the abundance of p53 mRNA in the polysomal fraction (Figure 6D), which is consistent with the elevation in nascent p53 protein translation (Figure 3B).

Modulation of HuR Levels Affects Apigenin-induced p53 Expression

Direct analysis of HuR's role in apigenin-induced p53 expression was carried out by employing two methods to modify the HuR expression level in cells. First, we overexpressed HuR in the 308 keratinocyte cell line by transfecting cells with a HuR-expression plasmid. Our findings shown in Figure 7A demonstrated that overexpression of HuR resulted in an increase in apigenin-induced p53 expression, and quantitative analysis showed the enhancement expressed as relative levels of p53 protein was significant with a P value < 0.05 (Figure 7B). As a complimentary approach, we employed specific reduction of HuR expression in 308 keratinocyte cells by using siRNA technology. As shown in Figure 7C, depletion of $\sim 70\%$ of HuR protein was achieved by transfection of HuR siRNA duplex into 308 cells, with no effect on Actin levels. Transfection using a control non-targeting siRNA duplex did not change either HuR or Actin expression. We observed that reduction of HuR protein attenuated the apigenin-induced p53 expression, and quantitative analysis expressed as relative levels of p53 protein also showed the change was significant with a P value < 0.001 (Figure 7D). Furthermore, HuR knockdown reduced apigenin-induced nascent p53 synthesis (Figure 7E, upper panel). Whole-cell extracts (WCE) were also analyzed to

demonstrate that an equal amount of [³⁵S]methionine/cysteine was incorporated into cells, confirming that protein translation in general was not affected by apigenin treatment (Figure 7E, lower panel). Overall, these findings demonstrated that HuR enhanced p53 expression induced by apigenin.

p16 Knockdown Inhibits Apigenin-induced Cytoplasmic HuR Translocation

A recent report by Shukla and Gupta demonstrated that apigenin given by gavage to nude mice significantly increased levels of cyclin dependent kinase inhibitors including p16 in prostate tumor xenografts [27]. In addition, Al-Mohanna and coworkers observed that p16 controlled the UV-dependent cytoplasmic accumulation of HuR [28]. Taken together these results raised the possibility that apigenin could regulate cytoplasmic localization of HuR through modulating p16 levels in mouse 308 keratinocyte cells. To address this possibility, p16 protein levels were examined in 308 keratinocytes treated with apigenin, and western blot analysis demonstrated that apigenin induced p16 protein expression starting from 4 h after treatment (Figure 8A), which is consistent with the time point at which we observed increased cytoplasmic localization of HuR by apigenin (Figure 6A). To further determine whether p16 is directly involved in regulating HuR's cytoplasmic translocation, we knocked down p16 expression in 308 cells by siRNA. As shown in Figure 8B, p16 specific siRNA reduced p16 protein expression by greater than 70% in 308 keratinocytes, compared to control siRNA. When cytoplasmic and nuclear fractions were prepared from siRNA transfected cells, we found p16 knockdown dramatically reduced the ability of apigenin treatment to induce HuR's cytoplasmic localization (compare Figure 8C, lane 2 and lane 4), indicating that one mechanism by which apigenin increases cytoplasmic localization of HuR is possibly through increasing p16 protein expression.

DISCUSSION

In an effort to develop apigenin as a potential chemopreventive agent, we found previously that apigenin increased p53 expression in the mouse keratinocyte 308 cell line by enhancing p53 protein stabilization [11]. In the present study, we further demonstrated that apigenin treatment increased p53 protein levels in a dose- and time-dependent manner. The increase in p53 protein level in apigenin-treated cells was not due to increased p53 transcription, because the steady-state p53 mRNA levels significantly decreased after apigenin treatment, and apigenin treatment did not affect p53 mRNA stability or cytoplasmic export p53 mRNA, suggesting that the observed increase in p53 protein expression by apigenin treatment is the result of translational or post-translational regulation. In considering mechanisms of post-translational regulation, it is well known that a major mechanism by which p53 protein is stabilized is through protein phosphorylation at serine residues in the N-terminus of p53, thereby inhibiting interaction with the ubiquitin ligase MDM2 [13]. We observed that apigenin treatment increased phospho-p53 levels, however, protein phosphorylation may not be the sole explanation for the elevated total p53 protein levels, since the levels of phospho-p53 were sustained for at least 12 h whereas the level of total p53 protein was reduced dramatically at that time (Figure 3A). Instead, we found apigenin treatment increased nascent p53 protein synthesis, indicating that apigenin can enhance de novo p53 translation (Figure 3B).

In view of our recent study demonstrating that apigenin prevents UVB-induced cyclooxygenase-2 expression through mRNA stabilization and translational inhibition by RNA binding proteins HuR and TIAR [22], and the growing evidence that HuR is involved in regulation of mRNA translation [18,29], we thus explored whether HuR is involved in the enhancement of p53 translation by apigenin. We found that apigenin augmented both the translocation of HuR to cytoplasm and the association of HuR with endogenous p53 mRNA. Since treatment of cells with apigenin resulted in a significant decrease in steady-state p53

mRNA levels, we hypothesize that the increase in HuR-bound p53 mRNA after apigenin treatment is due to the markedly increased levels of cytoplasmic HuR. In testing this hypothesis, we observed that knockdown of HuR expression resulted in significant reduction in apigenin's ability to induce p53 protein expression (both total p53 protein level and nascent p53 synthesis), confirming that HuR plays a critical role in apigenin's induction of p53 protein expression.

As an RNA binding protein, HuR works through binding to the AREs within 3'-UTR of target mRNAs. In comparison with the human p53 mRNA 3'-UTR, which is ~ 1200 nucleotides long and contains an Alu-like repetitive sequence element located immediately upstream of the poly(A) site [30], the mouse p53 3'-UTR is shorter (only ~ 450 nucleotides), lacks overall sequence similarity and the Alu-like sequence is missing [31]. However, both mouse and human p53 mRNAs contain an ARE sequence at the distal end of their 3'-UTR. In our hands, a construct containing the full-length 3'-UTR and the ARE-containing region fused to the 3' end of a luciferase reporter vector was found to significantly enhance reporter gene activity after apigenin treatment. Meanwhile, the reporter gene was unresponsive to apigenin when the ARE-containing region was deleted from the full-length 3'-UTR, indicating the importance of the ARE in regulation of p53 expression induced by apigenin, and this result is consistent with a previous report that the presence of the p53 3'-UTR significantly enhanced the translation of human p53 mRNA [18]. However, in contrast to the study by Fu and coworkers with human p53, we did not observe the translational repression relative to 3'-UTR [17]. Whether this result was due to different stimuli or lack of the Alu-like sequence in the murine 3'-UTR remains to be elucidated.

In addition to increasing HuR translocation into cytoplasm, we have previously reported that apigenin treatment increased the levels of RNA binding protein TIAR, a silencer of translation, in cytoplasm [22]. Therefore, in the present report we also examined the role of TIAR in regulation of p53 expression, and found that there was a slight but statistically insignificant increase in p53 protein levels induced by apigenin when TIAR expression in cells was inhibited by knockdown with TIAR-specific shRNA (Figure S2). These results indicate that the induction of p53 expression by apigenin is specifically mediated by HuR, and TIAR is unlikely to be involved in apigenin-induced p53 expression.

At this point in time, the mechanism by which HuR shuttles between the nucleus and cytoplasm is not completely understood. For example, studies have indicated that the pp32 and APRIL proteins are involved in HuR nuclear export [32], and several groups have reported that Transportin and Importin $\alpha 1$ are involved in HuR nuclear import [32,33], and β -catenin has been reported to induce the cytoplasmic localization of HuR [34]. More recently, HuR has been shown to be phosphorylated and phosphorylation influences HuR shuttling and HuR-RNA interactions [35,36].

AMP-activated protein kinase (AMPK), an enzyme involved in response to metabolic stresses, has also been reported to regulate HuR's cytoplasmic translocation, although its exact role in different cells is contradictory. For example, AMPK activation reduced cytoplasmic HuR levels in human colorectal carcinoma RKO cells [37]. However, when human hepatocytes were exposed to 5-aminoimidazole-4-carboxamide riboside (AICAR), the most widely used pharmacologic activator of AMPK, the level of cytoplasmic HuR was increased [38]. When we investigated AMPK activity in apigenin-treated 308 keratinocytes, we found that apigenin increased AMPK activity dramatically (Figure S3), but AICAR treatment had no effect on the levels of cytoplasmic HuR (Figure S4), indicating AMPK may not be involved in apigenin-induced HuR translocation. Instead, we found that apigenin treatment of cells induced p16 expression, which in turn was correlated with cytoplasmic localization of HuR induced by apigenin. However, the mechanism by which p16, a cyclin-

dependent kinase inhibitor, regulates cytoplasmic HuR translocation still remains to be elucidated.

In conclusion, we have demonstrated that apigenin treatment induces p53 protein expression in the 308 mouse keratinocyte cell line by increasing nascent p53 protein synthesis, indicating enhancement of p53 translation. We further showed that apigenin's effects were mediated, at least in part, through the RNA binding protein HuR, leading to enhanced expression of p53 which may play an important role in the chemopreventive effect of apigenin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

UV	ultraviolet
UTR	untranslated region
ARE	AU-rich element
nt	nucleotide
RBP	RNA binding protein
IP	Immunoprecipitation
WCE	whole-cell extract
TIAR	T-cell-restricted intracellular antigen 1-related protein
AMPK	AMP-activated protein kinase
AICAR	5-aminoimidazole-4-carboxamide riboside
DAPI	diamidinophenylindole
DMSO	dimethyl sulfoxide

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Figure 1. Apigenin-induced expression of p53 and its downstream target genes in 308 mouse keratinocytes

(A) Western blot analysis showing dose-dependent induction of p53 protein at 8 h after apigenin treatment. (B) Time-dependent induction of p53 protein expression by apigenin. Cells were treated with 50 μ M apigenin, harvested at the indicated time points, and analyzed by Western blot. (C) Apigenin-mediated expression of p21, Bax and PUMA. Cells were treated with 50 μ M apigenin, harvested at the indicated time points, and subjected to Western blot analysis.

Figure 2. Effects of apigenin on p53 mRNA

(A) Real-time PCR analysis of steady-state p53 mRNA levels. Cells were treated with 50 μ M apigenin, and total RNA was harvested at the indicated times. TaqMan real-time quantitative PCR was carried out as described in Materials and Methods (* = significantly different from 0 h, $P < 0.001$). (B) Apigenin treatment does not change the stability of p53 mRNA. Cells were pre-treated with medium containing 50 μ M apigenin or DMSO vehicle for 2 h, 10 μ M actinomycin D was added and incubated for 0, 3, 6, 9 and 12 h. Total RNA was isolated, reverse transcribed and analyzed for p53 mRNA using TaqMan real-time PCR. Amount of p53 mRNA is expressed as a percentage of levels measured at the 0 h time point. (C) Total, cytoplasmic, and nuclear fractions were prepared for real-time PCR analysis of p53 mRNA at 2 h after apigenin (50 μ M) treatment. All the graphs represent the results of three independent experiments.

Figure 3. Apigenin induces p53 protein phosphorylation and enhances nascent p53 protein synthesis

(A) Cells were exposed to 50 μ M apigenin, harvested at the indicated time points, and Western blot analysis was carried out to detect the levels of phosphorylated p53 protein (Ser-15) and total p53 protein. (B) Control cells (C) and cells that had been treated with 50 μ M apigenin for 4 h (A) were incubated with L-[35 S]methionine and L-[35 S]cysteine for 15 min, followed by IP using either normal IgG or anti-p53 antibody. Samples were resolved by SDS-PAGE and the level of nascent p53 protein was measured by autoradiography (upper panel). Analysis of whole-cell extracts (WCE) showed that equal amount of [35 S]methionine/cysteine was incorporated into the cells and that protein translation in general was not affected by apigenin treatment (lower panel).

Figure 4. The ARE of p53 mRNA is important for translation efficiency

(A) Structure of luciferase reporter gene constructs. Various regions of the 3'-UTR (gray bars) of p53 were fused to reporter gene luciferase (black bars) to generate the constructs containing the full-length 3'-UTR (nt 1302-1743), the ARE-containing region (nt 1605-1743), the ARE-containing region deleted from the full-length 3'-UTR (nt 1302-1624) or luciferase control (pGL3c) without the 3'-UTR. The locations of AUUUA sequence and AU-rich region are indicated by asterisks and white bar, respectively. (B) Effects of various regions of the 3'-UTR of the p53 gene on luciferase activity when fused to the 3' end of the reporter gene. Cells were co-transfected with the different reporter gene constructs and Renilla luciferase plasmid. At 24 h after transfection, cells were treated with 50 μ M apigenin for another 8 or 24 h. Relative luciferase activity for each construct was represented as the ratio between apigenin-treated versus untreated culture. All values were normalized to Renilla luciferase activity and results are the means $\pm$ S.E. for three independent experiments, each performed in duplicate (* = significantly different from pGL3c, $P < 0.001$). (C) Effects of various regions from the 3'-UTR of the p53 gene on the luciferase reporter gene message levels. Cells were transfected as described in (B) and treated with apigenin for 24 h. Total RNA was extracted and assayed for luciferase and GAPDH mRNAs by using real-time PCR as described in Materials and Methods. Luciferase message levels were normalized to GAPDH mRNA and expressed relative to untreated cells (* = significantly different from pGL3c, $P < 0.001$).

Figure 5. Apigenin-dependent binding of HuR to endogenous p53 mRNA

Cytoplasmic extracts were prepared from control and apigenin-treated cells, then protein-RNA complexes were immunoprecipitated using either anti-HuR antibody or normal IgG, followed by RT-PCR analysis to detect endogenous p53 mRNA and β -Actin mRNA (M, DNA marker; C, control; A, 50 μ M apigenin for 4 h).

Figure 6. Effect of apigenin on the subcellular localization of HuR

(A) Whole cell, cytoplasmic and nuclear lysates were prepared and subjected to Western blot analysis for HuR. Expression of the cytoplasmic marker α -Tubulin and the nuclear

marker hnRNP A0 were also detected (C, control; A, 50 μ M apigenin for 4 h). (B) The subcellular localization of HuR was monitored by immunofluorescence. The slides were also stained with diamidinophenylindole (DAPI) for visualization of nuclei. (C) Western blot showing subcellular localization of HuR in cytosol and polysomal fractions (C, control; A, 50 μ M apigenin for 4 h). (D) RNA was extracted from polysomal fraction (untreated control or 50 μ M apigenin for 4 h), and the levels of p53 mRNA was measured by real-time RT-PCR (*, $P < 0.01$).

Figure 7. Level of expression of HuR affects apigenin-induced p53 protein expression
 (A) Representative Western blot of HuR and p53 expression levels in 308 cells that had been transfected either with an empty control plasmid or an HuR-expression plasmid (C, control; A, 50 μ M apigenin for 8 h). (B) Quantitative analysis of the effect of HuR over-expression on apigenin-induced p53 expression. p53 protein bands were scanned by densitometry and normalized to their corresponding Actin bands, graph represents the results of three

independent experiments (*, $P < 0.05$). (C) Representative Western blot of HuR and p53 expression levels in 308 cells that had been transfected with either HuR siRNA duplex or control non-targeting siRNA duplex, and exposed to 50 μM apigenin for another 8 h. (D) The effect of HuR knockdown on apigenin-induced p53 expression was measured as described in (B) (*, $P < 0.001$). (E) Cells were transfected with siRNA, treated with 50 μM apigenin for 4 h, and followed by a brief incubation (15 min) with L-[^{35}S]methionine and L-[^{35}S]cysteine. Upper panel: cell lysates were immunoprecipitated by using either normal IgG or anti-p53 antibody, then resolved by SDS-PAGE and the level of nascent p53 protein was measured by autoradiography; lower panel: analysis of whole-cell extracts (WCE) showed that equal amount of [^{35}S]methionine/cysteine was incorporated into the cells and that protein translation in general was not affected by apigenin treatment.

Figure 8. Knockdown of p16 expression correlates with reduction in apigenin-induced cytoplasmic distribution of HuR

(A) Time-dependent induction of p16 protein expression by apigenin. Cells were treated with 50 μ M apigenin, harvested at the indicated time points, and analyzed by Western blot. (B) Cells were transfected with either p16 siRNA duplex or control non-targeting siRNA duplex, and whole cell lysates were used to examine p16 protein level. (C) After p16 knockdown by siRNA, cytoplasmic and nuclear lysates were separated and subjected to Western blot analysis for HuR, α -Tubulin and hnRNP A0 (C, control; A, 50 μ M apigenin for 4 h).