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## Effect of Combined Treatment with Ursolic Acid and Resveratrol on Skin Tumor Promotion by 12-O-tetradecanoylphorbol-13-acetate

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### Abstract

In this study, the effects of combining ursolic acid (UA) + resveratrol (Res), for possible combined inhibitory effects on skin tumor promotion were evaluated. UA, Res and the combination of UA + Res were applied topically prior to TPA treatment on mouse skin to examine their effect on TPA-induced signaling pathways, epidermal hyperproliferation, skin inflammation, inflammatory gene expression and skin tumor promotion. The combination of UA + Res produced a greater inhibition of 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced epidermal hyperproliferation. The combination of UA + Res inhibited TPA-induced signaling pathways, including EGFR, STAT3, Src, Akt, Cox-2, Fas, NF- $\kappa$ B, p38 MAPK, c-Jun, and JNK1/2 while increasing levels of tumor suppressors such as p21 and PDCD4 to a greater extent compared to the groups treated with the individual compounds. UA + Res also induced a dramatic increase of p-AMPK- $\alpha^{\text{Thr172}}$ .

Combined treatment with UA + Res resulted in a greater inhibition of expression of proinflammatory cytokines including IL-1 $\alpha$ , IL-1 $\beta$ , and IL-22. Furthermore, NF- $\kappa$ B, Egr-1, and AP-1 DNA binding activities after TPA treatment were dramatically decreased by the combination of UA + Res. Treatment with UA + Res during skin tumor promotion with TPA produced greater inhibition of tumor multiplicity and tumor size than with either agent alone. Collectively, the greater ability of the combination of UA + Res to inhibit skin tumor promotion was due to the greater inhibitory effects on growth factor and inflammatory signaling, skin inflammation and epidermal hyperproliferation induced by TPA treatment.

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**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

## Keywords

Ursolic acid; resveratrol; combination; chemoprevention; skin tumor promotion

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## Introduction

Ursolic acid (UA) is a natural pentacyclic triterpenoid carboxylic acid found in many plants including *P. frutescens* (Japanese basil), rosemary, apples, elder flowers and many others. UA has been shown to have apoptotic, anti-inflammatory and anti-tumorigenic effects in various cancer models including prostate, ovary, stomach, intestine, and skin (1–3). Further studies have revealed that UA has broad-spectrum anti-carcinogenic effects including prevention of DNA damage, inhibition of EGFR/MAPK signaling, inhibition of angiogenesis, activation of apoptotic pathways, and inhibition of Akt/mTOR, NF- $\kappa$ B, Cox-2, and STAT3 signaling pathways (1, 4). Although several studies have reported that UA inhibited carcinogen and 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation, hyperplasia and tumor promotion in mouse skin (2, 3, 5), its inhibitory mechanism on skin tumor promotion is not fully understood. Recently, several studies reported that UA has an anti-obesity effect and mimics some of the effects of calorie restriction (CR) by modulating Akt/mTOR signaling pathways (6–8). UA has also been shown to activate the LKB1/AMPK pathway for inhibition of adipogenesis (9).

Resveratrol (Res) is a phytoalexin and is present in grapes, berries, peanuts and red wine. Res has been shown to have cardiovascular benefit and anti-diabetic effects in both mice and humans. In addition, Res was shown to inhibit skin tumor promotion and also inhibit the growth of many cancer cell lines, including breast, prostate, colon and liver (5, 10–13). Mechanisms associated with the anti-tumor promoting effects of Res include inhibition NF- $\kappa$ B, AP-1, and Cox-2 (10, 13, 14). Several reports have suggested that Res also mimics some of the effects of CR on life span in worms and other model organisms, especially by inhibiting inflammation and mTOR signaling (15, 16). Res also mimics effects of CR by increasing SirT1 and AMPK activation (17). Boily *et al.* have suggested that the anti-promoting effect of Res on mouse skin is at least partially mediated by SirT1 (18).

Emerging evidence suggests that combinations of phytochemicals may be an effective strategy to achieve a greater chemopreventive effect than with single agents (19–21). Several studies have shown that combinations of natural compounds can produce potential synergistic inhibitory effects in various cancers (e.g., Res + grape seed extract and ellagic acid + grape seed extract) (12, 20–24). Recently, Junco *et al.* reported that Res potentiates the growth inhibitory effect of UA in mouse skin papilloma and carcinoma cell lines (25). Thus, combining agents may provide the most rational and effective approach to cancer chemoprevention. In addition, using combinations of phytochemicals may produce overall effects that more similarly mimic CR.

In the present study, topical treatment with a combination of UA + Res produced a greater inhibitory effect on skin tumor promotion by TPA than with either agent alone. Further mechanistic studies revealed that this combination produced a greater inhibition of multiple growth factor and inflammation signaling pathways as well as greater upregulation of tumor

suppressor genes such as p21 and PDCD4. Interestingly, the combination of UA + Res induced a dramatic increase of p-AMPK- $\alpha^{\text{Thr172}}$  and its downstream target p-Ulk1 $^{\text{Ser555}}$ . Collectively, the current data suggest that combined treatment of UA + Res is a more effective inhibitor of skin tumor promotion than either UA or Res given alone. The mechanism for this greater inhibition appears to be multi-faceted with similarities to changes observed with CR.

## Materials and Methods

### Animals and diets

For all experiments except the labeling retaining cell assay (LRC assay), Female Hsd : ICR (CD-1)mice 6–7 weeks of age were used and purchased from Harlan Laboratories Inc. (Houston, TX). For LRC assays, 10-day old mice were obtained by breeding FVB/N female and male mice (purchased from the National Cancer Institute). Mice were group housed in a 12 hr dark/12 hr light cycle at 24 °C for all experiments. For the short-term experiments, mice were fed a regular chow diet. For tumor experiments, mice received either an overweight control diet (D12450B, 10 Kcal% fat; Research Diets Inc.) or an obesity-inducing diet (D12492, 60 Kcal% fat; Research Diets Inc). All animal experiments were conducted in accordance with both Institutional as well as NIH guidelines under an approved IACUC protocol.

### Two-stage skin carcinogenesis assays

Female ICR mice (n=30/group) 7–8 weeks of age were shaved on the dorsal skin and then 48 hrs later initiated with a single topical application of 25 nmol of 7, 12-dimethylbenz[a]anthracene (DMBA; Sigma-Aldrich) in 0.2 ml acetone or acetone vehicle. Two weeks after initiation, mice were randomized to receive one of the two experimental diets (overweight control and obesity-inducing diet) for 6 weeks before starting treatment with the tumor promoter, TPA. During tumor promotion, mice received 2  $\mu\text{mol}$  of UA (Sabinsa Corporation) and 2  $\mu\text{mol}$  of Res (Orchid Chemicals & Pharmaceuticals Ltd.) 15 min prior to each TPA application. For the combination, Res was given 30 min and UA was given 15 min prior to each 6.8 nmol dose of TPA (LC Laboratories) to allow time for absorption of each compound prior to TPA application. All other aspects of the tumor experiments were as previously described (26–28).

### Short-term treatment protocol

For a number of experiments, mice were treated using a short-term treatment protocol involving 4 applications of TPA. For this protocol, groups of mice (7–8 weeks of age) were shaved on the dorsal skin and then two days later treated twice weekly for two weeks with 0.2 ml acetone vehicle, UA (2  $\mu\text{mol}$ ) or Res (2  $\mu\text{mol}$ ) 15 min prior to each 6.8 nmol of TPA treatment. For the combination, mice received UA (2  $\mu\text{mol}$ ) and Res (2  $\mu\text{mol}$ ) 15 min and 30 min prior to TPA treatment, respectively. Mice were then sacrificed at various times thereafter for collection of epidermal tissue.

### **Label retaining cell (LRC) assay**

For these experiments, 10-day old FVB/N mice were injected with BrdU [50 µg/g body weight (B.W.)] *i.p.* every 12 hrs over 2 days. Seventy days later, mice were shaved on the dorsal skin and then treated with the short-term protocol. Mice were sacrificed 48 hrs after the last treatment and dorsal skin samples were prepared and analyzed as previously described (28).

### **Histological analyses**

For analysis of epidermal thickness and labeling index (LI) as well as the number of dermal inflammatory cells, mice were shaved on the dorsal skin and then treated with the short-term protocol. All procedures for these analyses were as previously described (26–28).

### **Preparation of epidermal protein lysates, cytosolic fractions, nuclear fractions and RNA**

Groups of mice were treated with the short-term protocol and then sacrificed 6 hrs after the last TPA treatment. After sacrifice, epidermal protein lysates were collected as previously described (26). For electrophoretic mobility shift assays (EMSAs), the epidermal cytosolic and nuclear fractions were isolated using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific Inc). The protein lysates and nuclear/cytosolic fractions were used immediately or stored at –80 °C until used. Epidermal RNA samples were isolated as previously described (29, 30) and subjected to quantitative real-time PCR (qRT-PCR) analysis.

### **Western blot analysis**

Western blot analyses were performed as previously described (26). Antibodies used are listed in supplemental Table 1.

### **EMSA**

EMSA was performed using a DNA-protein binding detection kit according to the manufacturer's protocol (Thermo Scientific Inc.). See supplemental Table 2 for the sequences of the NF-κB, Egr-1 and AP-1 oligos used.

### **qRT-PCR analysis**

qRT-PCR analyses were performed as previously described (29, 30). cDNA (150 ng) was mixed with 2× TaqMan gene expression master mix (AB Applied Biosciences), 20× primer sets (IL-1α, IL-1β, IL-22, ribosomal 18 S), and nuclease-free water in a total volume of 10 µl. For qPCR of Cox-2 mRNA, 2× iTaq™ universal SYBR® green supermix (Bio-rad), 1 µM primers (Cox-2, and GAPDH), and nuclease-free water were added to cDNA (150 ng). The mixtures were then subjected to qRT-PCR using ViiA™ 7 real time instrument and analysis software.

### **Statistical analysis**

For comparisons of quantitative protein expression, gene expression, epidermal thickness, labeling index, the number of infiltrated inflammatory cells, transcriptional activities, and tumor multiplicity and tumor size, the Mann-Whitney *U* test was used. A one-tailed Fisher's

exact test and Mantel-Cox test was used for comparisons of tumor incidence and tumor latency, respectively. Significance in all cases was set at  $p = 0.05$ .

## Results

### Effect of UA + Res on skin tumor promotion by TPA

The ability of a combination of UA + Res to inhibit skin tumor promotion by TPA was evaluated in ICR mice maintained on either an overweight control diet or a diet-induced obesity diet (DIO diet). After completion of the tumor experiment, the tumor responses in both diet groups were similar for all groups (see supplemental Figs. 1A and 1B). Therefore, the data for the corresponding treatment groups on each diet were combined as presented in Fig. 1. As shown in Fig. 1, pretreatment with UA or Res alone inhibited tumor multiplicity by 38.6% and 20.8%, respectively. The reduction in tumor multiplicity with UA was statistically significant ( $p < 0.05$ ; Mann-Whitney  $U$  test) compared to the group treated with TPA alone. Pretreatment with the combination of UA + Res resulted in 56% reduction in tumor multiplicity that was significantly lower when compared to both the Res + TPA and UA + TPA groups ( $p < 0.05$ ; Mann-Whitney  $U$  test). Furthermore, the incidence of papillomas in the mice treated with UA + Res was significantly lower than that observed in the TPA and Res + TPA treated groups ( $p < 0.05$ ; Fisher's exact test) but not the UA + TPA group. An effect on tumor latency was also observed as shown in Fig. 1C. In this regard, the percent of tumor-free mice treated with the combination of UA + Res was significantly higher than that of the TPA only and Res + TPA groups over the 23 week observation period ( $p < 0.05$ ; Mantel-Cox test) but not the UA + TPA treated group.

As shown in Fig. 1D, the combination of UA + Res significantly reduced the size of papillomas compared to the TPA only group as well as both the UA + TPA and Res + TPA groups ( $p < 0.05$ ; Mann-Whitney  $U$  test). Thus, the combination was more effective at reducing both the number and size of papillomas when compared to either agent given alone.

Body weight gain for the 23 week experiment for each group is shown in Supplemental Fig. 1C and 1D. As expected, there were significant differences in body weight between untreated mice on the overweight control and DIO diets ( $41.57 \text{ g} \pm 2.49$  and  $56.57 \text{ g} \pm 2.39$ , respectively;  $p < 0.05$ ; Mann-Whitney  $U$  test). No significant differences were observed in body weight between the treated groups in mice on either the control diet or DIO diet. Overall, these data suggest that the combined treatment of UA + Res, at the doses used, had a greater inhibitory effect on skin tumor promotion compared to the groups pretreated with either of the compounds alone and with no apparent toxicity.

### Effect of UA + Res Treatment on TPA-induced epidermal hyperproliferation and LRCs

As shown in Figs. 2A and 2B, UA or Res alone significantly reduced BrdU incorporation and epidermal thickness following TPA treatment ( $p < 0.05$ ; Mann-Whitney  $U$  test). However, treatment with the combination of UA + Res produced a greater inhibition of BrdU incorporation and epidermal thickness induced by TPA compared to that observed with either of the compounds given alone with TPA ( $p < 0.05$ ; Mann-Whitney  $U$  test).

As shown in supplemental Fig. 2, LRCs in acetone-treated mice were confined to the hair follicle bulge-region as expected based on previous studies (28, 30). However, after a two-week treatment regimen with TPA, the LRCs can be seen moving up and out of the hair follicle into the interfollicular epidermis (supplemental Fig. 2A). Pretreatment with UA or Res partially inhibited the effect of TPA on proliferation and migration of LRCs. However, treatment with the combination of UA + Res prior to application of TPA produced a greater inhibitory effect on the proliferation and migration of these cells compared to the UA or Res only treated groups (supplemental Fig. 2B;  $p < 0.05$ ; Mann-Whitney *U* test).

### Effect of UA + Res on TPA-induced epidermal signaling pathways

As shown in the Fig. 3A and 3B, UA + Res significantly inhibited TPA-activated p-STAT3<sup>Tyr705</sup>, p-Akt<sup>Thr308</sup>, p-NF- $\kappa$ B p65<sup>Ser536</sup>, p-JNK1/2<sup>Thr183/Tyr185</sup>, p-c-Jun<sup>Ser73</sup>, and p-p38 MAPK<sup>Thr180/Tyr182</sup>, whereas UA or Res alone either produced no significant effects or a moderate inhibition of phosphorylation of these proteins relative to the combination. Cox-2 induction by TPA was not significantly decreased by pretreatment with either UA or Res alone, however, the combination of UA + Res produced a statistically significant inhibition of Cox-2 induction by TPA. The levels of several tumor suppressors were also evaluated (see again Fig. 3A and 3B). The combination of UA + Res significantly reversed the effect of TPA on PDCD4 and p21 levels while pretreatment with either compound alone had no effect. In contrast, none of the treatments reversed the effects of TPA treatment on p27 levels.

The phosphorylation of both EGFR<sup>Tyr1086</sup> and Src<sup>Tyr416</sup> was also significantly inhibited by the combination of UA + Res at the doses and time points examined whereas neither UA nor Res alone significantly inhibited phosphorylation of these proteins. On the other hand, the increased level of Fas induced by TPA was decreased by treatment with Res alone and the combination of UA + Res but not with UA. Again, the combination was the most effective at inhibiting the increase in Fas seen following treatment with TPA.

As shown in Fig. 4A and 4B, treatment with TPA alone produced a ~2.5-fold increase in p-AMPK- $\alpha$ <sup>Thr172</sup> compared to the acetone treated control group. Both UA and Res when given with TPA further increased p-AMPK- $\alpha$ <sup>Thr172</sup>, while the combination of UA + Res together with TPA produced an even greater activation of AMPK- $\alpha$  that was significantly greater than with either UA or Res given alone ( $p < 0.05$ ). The level of SirT1 was not changed by treatment with TPA or pretreatment with any of the compounds given together with TPA, including the combination of UA + Res (again see Fig. 4A and 4B). TPA treatment reduced the level of p-LKB1<sup>Ser428</sup> compared to the acetone group, however, neither UA nor Res had any further effect. In contrast, the level of p-LKB1<sup>Ser428</sup> was further decreased when the combination of UA + Res was given before TPA treatment. TPA treatment led to activation of mTORC1 signaling as previously reported (26, 28, 31), however, the levels of p-mTORC1<sup>Ser2448</sup> and its downstream target, p-S6-ribosomal protein<sup>Ser240/244</sup>, were not affected by treatment with UA, Res or the combination of UA + Res. Notably, the level of p-Ulk1<sup>Ser555</sup> was significantly increased when the combination of UA + Res was given together with TPA. The combination of UA + Res when given together with TPA significantly increased the level of LC3IIB compared to the acetone, TPA and UA

+ TPA groups while the levels of ATG5 and Beclin1 that were reduced by TPA treatment were not significantly altered further by any of the treatments.

### **Effect of UA + Res on TPA-induced inflammation and inflammatory gene expression**

As shown in Fig. 5A, the levels of IL-1 $\alpha$ , IL-1 $\beta$ , IL-22 and Cox-2 mRNA were increased following treatment with TPA (given twice weekly for two weeks) and significantly decreased in the UA + Res pretreated group ( $p < 0.05$ ; Mann-Whitney  $U$  test). With the exception of UA pretreatment on Cox-2 mRNA, neither UA nor Res pretreatment significantly reduced the mRNA levels of these inflammatory genes. As shown in Supplemental Fig. 3 and Figs. 5B–C, pretreatment with UA and Res decreased the number of mast cells in the dermis seen following TPA treatment, however, an additional decrease in the number of dermal mast cells was observed after treatment with UA + Res + TPA ( $p < 0.05$ ; Mann-Whitney  $U$  test). UA alone and UA + Res also produced a significant decrease in the number of CD45<sup>+</sup> cells in dermis ( $p < 0.05$ ). Again, the combination of UA + Res produced the greatest reduction in the numbers of both mast cells and CD45<sup>+</sup> cells.

### **Effect of UA + Res on NF- $\kappa$ B, Egr-1, and AP-1 DNA binding activities induced by TPA**

Treatment with TPA significantly increased the amount of NF- $\kappa$ B, Egr-1 and AP-1 bound to their consensus DNA binding oligos (supplemental Figs. 4A–C and supplemental Figs. 5A–C). The TPA-induced increase in DNA binding activity of all three transcription factors was significantly reduced by pretreatment with the combination of UA + Res. Pretreatment with UA or Res alone significantly reduced binding of NF- $\kappa$ B and UA pretreatment significantly reduced binding of AP-1. Thus, the combination of UA + Res was highly effective at inhibiting the activation of all three of these transcription factors by TPA. Additionally, the nuclear translocation of NF- $\kappa$ B, Egr-1 and AP-1 induced by TPA was also significantly inhibited by UA + Res to a greater extent than pretreatment with either UA or Res (supplemental Figs. 4G–I and supplemental Figs. 5D–F).

## **Discussion**

In the present study, topical application of UA + Res followed by TPA treatment inhibited skin tumor promotion to a greater extent when compared to the groups treated with either Res + TPA or UA + TPA alone. Further analyses revealed that the greater ability of the combination to inhibit skin tumor promotion correlated with a greater ability to inhibit epidermal proliferation induced by TPA. In addition, combined treatment with UA + Res produced greater inhibitory effects on TPA-induced epidermal signaling pathways including EGFR, STAT3, Fas, Src, Akt, Cox-2, NF- $\kappa$ B, p38 MAPK, and JNK1/2. Notably, treatment with the combination also increased the levels of the tumor suppressor proteins p21 and PDCD4 compared to the groups treated with the individual compounds. Both UA and Res treatment followed by TPA increased AMPK- $\alpha$  activation, however, the combination of UA + Res together with TPA produced an even greater activation of AMPK- $\alpha$ . Further studies revealed that the activation of NF- $\kappa$ B, Egr-1, and AP-1 (DNA binding activity and nuclear translocation) were significantly inhibited by the combination of UA + Res compared to the groups treated with either UA or Res alone. The combination also produced greater effect on TPA-induced inflammation and inflammatory gene expression. Overall, the current data

indicate that combined treatment with UA + Res led to a greater inhibitory effect on skin tumor promotion than either compound alone via effects on multiple events and pathways critical to the process of skin tumor promotion.

As noted in the Introduction, UA was previously shown to have inhibitory effects on TPA-induced skin inflammation as well as skin carcinogenesis (2, 5). Res was also shown to be as an effective inhibitor on skin tumor promotion (5, 10, 13, 32) along with inhibitory effects on breast, colorectal, hepatic, pancreatic, and prostate cancers (11). Recently, several studies have shown potential combinatorial chemopreventive effects with these agents in preclinical models of cancer. For example, melatonin was shown to potentiate the inhibitory effect of UA on proliferation and apoptosis in colon cancer cells by modulating multiple signaling pathways including caspases, PARP, NF- $\kappa$ B and Cox-2 (33). Kowalczyk *et al.* (5) tested a combination of 2% calcium D-glucarate (CG) given in the diet, with either 2.5  $\mu$ mol of Res or 1  $\mu$ mol of UA applied topically in two-stage skin carcinogenesis model. In this study, UA applied alone and in combination with CG showed inhibitory effects on skin tumor incidence and multiplicity.

Combinations of Res with other phytochemicals have been shown to have a greater inhibitory effect in several tumor models. For example, combined dietary administration of Res, quercetin and catechin (combinations at 0.5, 5 or 25 mg/kg) reduced primary tumor growth of breast cancer xenografts in a nude mouse model (34). Res + black tea polyphenol inhibited mouse skin tumor growth by modulating MAPKs and p53 (32). In other studies, Res + curcumin produced a better chemopreventive effect by maintaining adequate zinc and regulating p21 and Cox-2 level during lung carcinogenesis (35). Genistein + Res also reduced the most severe grade of prostate cancer in the SV-40 tag rat (23).

As shown in the current study, the combination of UA + Res was a more effective inhibitor of skin tumor promotion by TPA in ICR mice than either agent alone at the dose used and this was true for both tumor multiplicity and tumor size (see again Fig. 1). Although we did not design the current studies to analyze the development of squamous cell carcinomas (SCCs), papillomas are considered premalignant tumors and previous studies have shown that reductions in numbers of papillomas leads to reductions in SCCs (27, 36, 37). As noted above, previous studies have shown that UA broadly inhibited a number of signaling pathways including EGFR, MAPK, Akt/mTOR, NF- $\kappa$ B, Cox-2 and STAT3 in a variety of cell types, including mouse epidermis *in vivo* (1, 4). Furthermore, mechanisms associated with the anti-tumor promoting effects of Res include modulation of NF- $\kappa$ B, Cox-2, mTORC1, and SirT1 (10, 13, 17, 18). In our current study, we evaluated a number of oncogenic signaling molecules including EGFR, Src, STAT3, Fas, NF- $\kappa$ B, Akt, p38, JNK1/2, c-Jun, and mTOR as well as the tumor suppressors p27, p21, PDCD4, and AMPK. As shown in Figs. 3 and 4 we found that the combination of UA + Res was more effective at altering these pathways during tumor promotion than either UA or Res given alone. In particular, the combination was significantly more effective at inhibiting TPA-induced activation (phosphorylation) of EGFR, Src and p38 MAPK and at altering the levels of Fas (decrease) and p-AMPK- $\alpha^{\text{Thr172}}$  and p21 (increase) compared to either UA or Res given alone. In addition, the combination of UA + Res produced the greatest inhibition of NF- $\kappa$ B, Egr-1 and AP-1 DNA binding activities and nuclear translocation (supplemental Figs. 4 and



5). All three of these transcription factors are known to be upregulated during skin tumor promotion and skin carcinogenesis (38, 39). Thus, the combination of UA + Res produced a more global and robust inhibition of epidermal signaling pathways compared to either UA or Res given alone.

TPA-induced epidermal hyperproliferation is required for its tumor promoting activity (37, 39, 40). Previous studies have shown that topical treatment of both 1  $\mu\text{mol}$  of UA and 2  $\mu\text{mol}$  of Res reduced TPA-induced BrdU incorporation in SENCAR mouse skin (5). In the current study, we observed that the combination of UA + Res together with TPA at a dose of 2  $\mu\text{mol}$  each inhibited epidermal hyperproliferation to a greater extent than with either compound alone at the same dose. The greater inhibition of TPA-induced epidermal hyperproliferation with the combination was likely due to the greater effects observed on the multiple signaling pathways noted above. Skin inflammation is also known to be an important component of the process of skin tumor promotion by TPA involving the production of pro-inflammatory cytokines and infiltration of inflammatory cells (39, 41, 42). Again, as seen with the analyses of epidermal proliferation, the combination produced greater inhibition of inflammation. In this regard, the combination of UA + Res inhibited to a greater extent the increased expression of IL-1 $\alpha$ , IL-1 $\beta$ , IL-22 and Cox-2 seen following treatment with TPA. In addition, as shown in Fig. 5B, the number of mast cells was decreased by either UA or Res treatment followed by TPA, however, the combination gave rise to an even greater inhibitory effect. The number of lymphocytes, monocytes and leukocytes (CD45<sup>+</sup> cells) in the dermis were also significantly inhibited by the combination of UA + Res compared to the individual compounds alone. The greater inhibition of inflammation by the combination of UA + Res was likely due to the greater inhibition of inflammatory signaling pathways and to a greater reduction in NF- $\kappa$ B DNA binding activity.

In conclusion, the current study shows for the first time the efficacy of a combination of UA + Res for inhibition of tumor promotion in mouse skin. This combination of UA + Res produced a greater inhibition of skin tumor promotion by TPA compared to UA or Res alone. In addition, the combination targeted multiple TPA-induced signaling pathways involved in both epidermal proliferation and inflammation and produced effects on a number of these pathways greater than either compound alone. For the current experiments we choose to apply the compounds via the topical route. An important goal for future studies will be to examine the efficacy of this and other combinations, when given in the diet. Combining phytochemicals such as UA and Res appears to produce a CR mimetic type of effect by targeting multiple signaling pathways and should be explored further for potential cancer chemopreventive efficacy.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

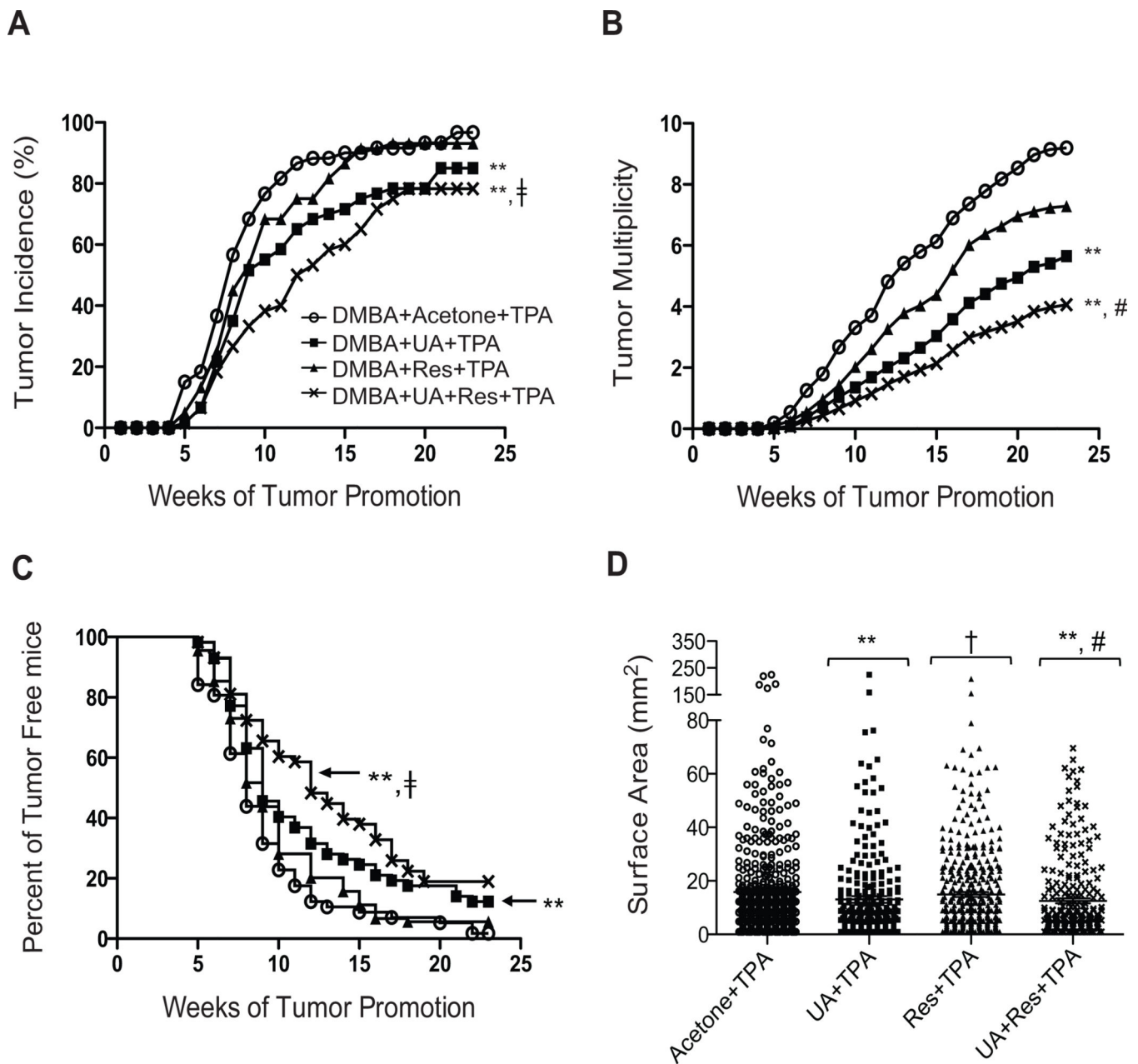
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**Figure 1.** Effect of UA + Res on skin tumor promotion in ICR mice. Panel A, Incidence of tumors (percentage of mice with papillomas). The percentage of mice with papillomas in the group treated with UA + Res +TPA was significantly lower than TPA (\*\*) or Res + TPA (‡) treated groups ( $p < 0.05$ ; Fisher's exact test). Panel B, tumor multiplicity (average number of papillomas per mouse). Both the UA and the UA + Res pretreated groups had significantly reduced tumor multiplicity compared to the TPA-treated group (\*\*,  $p < 0.05$ ). The tumor multiplicity in the UA + Res + TPA-treated (#,  $p < 0.05$ ; Mann-Whitney  $U$  test) was also significantly lower than both UA + TPA and Res + TPA-treated group. Panel C, tumor latency (tumor free survival). Significant differences were observed between the UA + TPA

and UA + Res + TPA compared to the TPA only group (\*\*,  $p < 0.05$ ; Mantel-Cox test). Percent of tumor free mice in the combination group (‡,  $p < 0.05$ ; Mantel-Cox test) was greater than the Res + TPA group. Panel D, tumor size. The surface area of papillomas was measured at the 23<sup>rd</sup> week. \*\*,  $p < 0.05$  when compared to TPA group; †,  $p < 0.05$  when compared to UA + TPA group; and #,  $p < 0.05$  when compared to both the Res + TPA and UA + TPA groups. The Mann-Whitney  $U$  test was used for all statistical comparisons of tumor size.

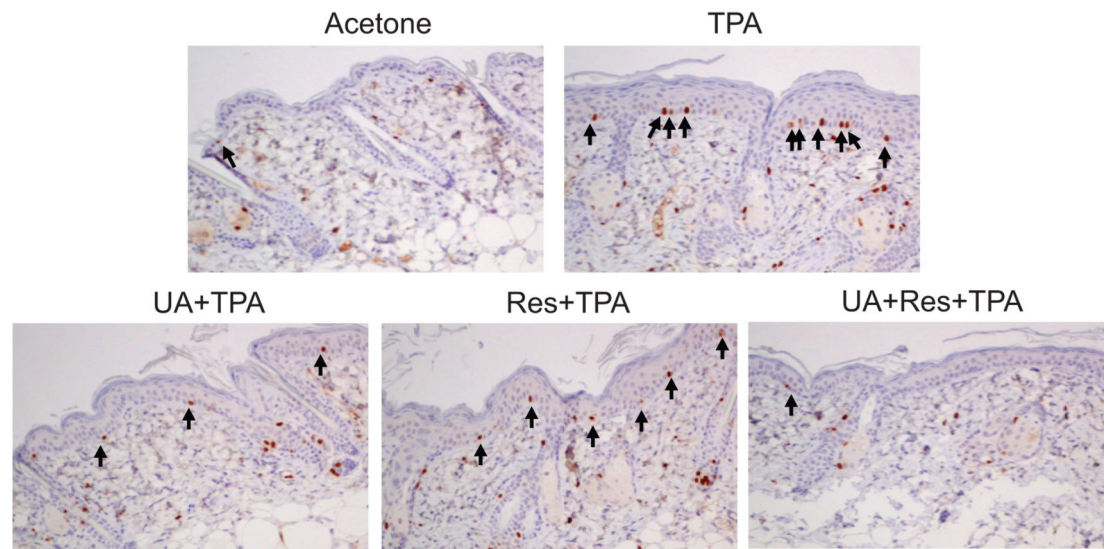
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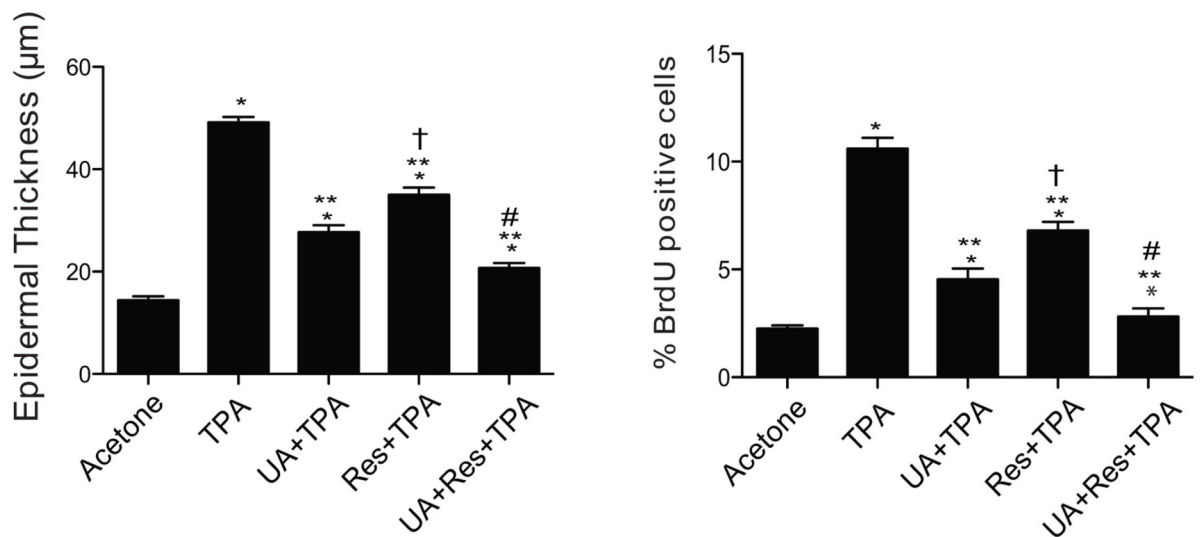
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**A**



**B**



**Figure 2.** Effect of UA + Res on TPA-induced epidermal hyperproliferation in ICR mice. Female ICR mice at 7–8 weeks of age maintained on standard chow diet were treated topically with the short-term protocol. Dorsal skin was collected at 48 hrs after the last treatment for histological evaluation. Sections were stained with H&E and for BrdU incorporation. Panel A, representative sections of BrdU stained skin. Arrows indicate BrdU-positive cells. Magnification,  $\times 20$  Panel B, quantitative analysis of the effects of UA, Res or UA + Res on TPA-induced epidermal thickness and labeling index (% BrdU positive cells). The values in panel B represent the means  $\pm$  SEM. \*,  $p < 0.05$  when compared to the acetone treated group; \*\*,  $p < 0.05$  when compared to the TPA treated group; †,  $p < 0.05$  when compared to the UA +

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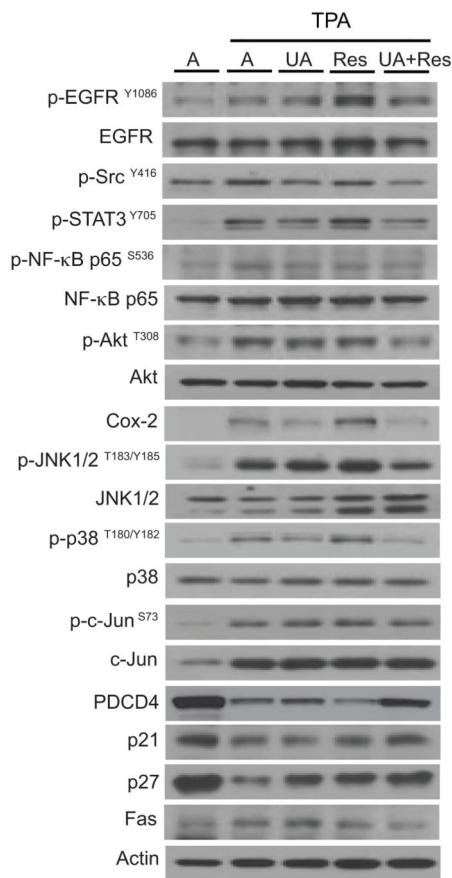
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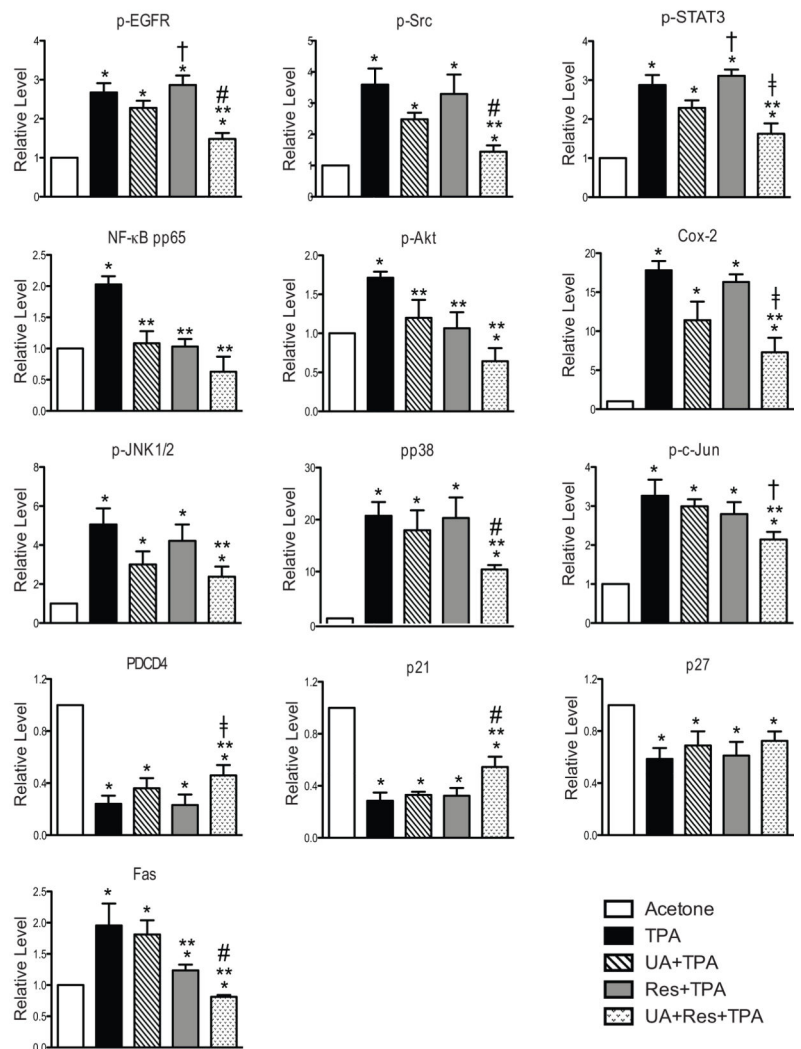
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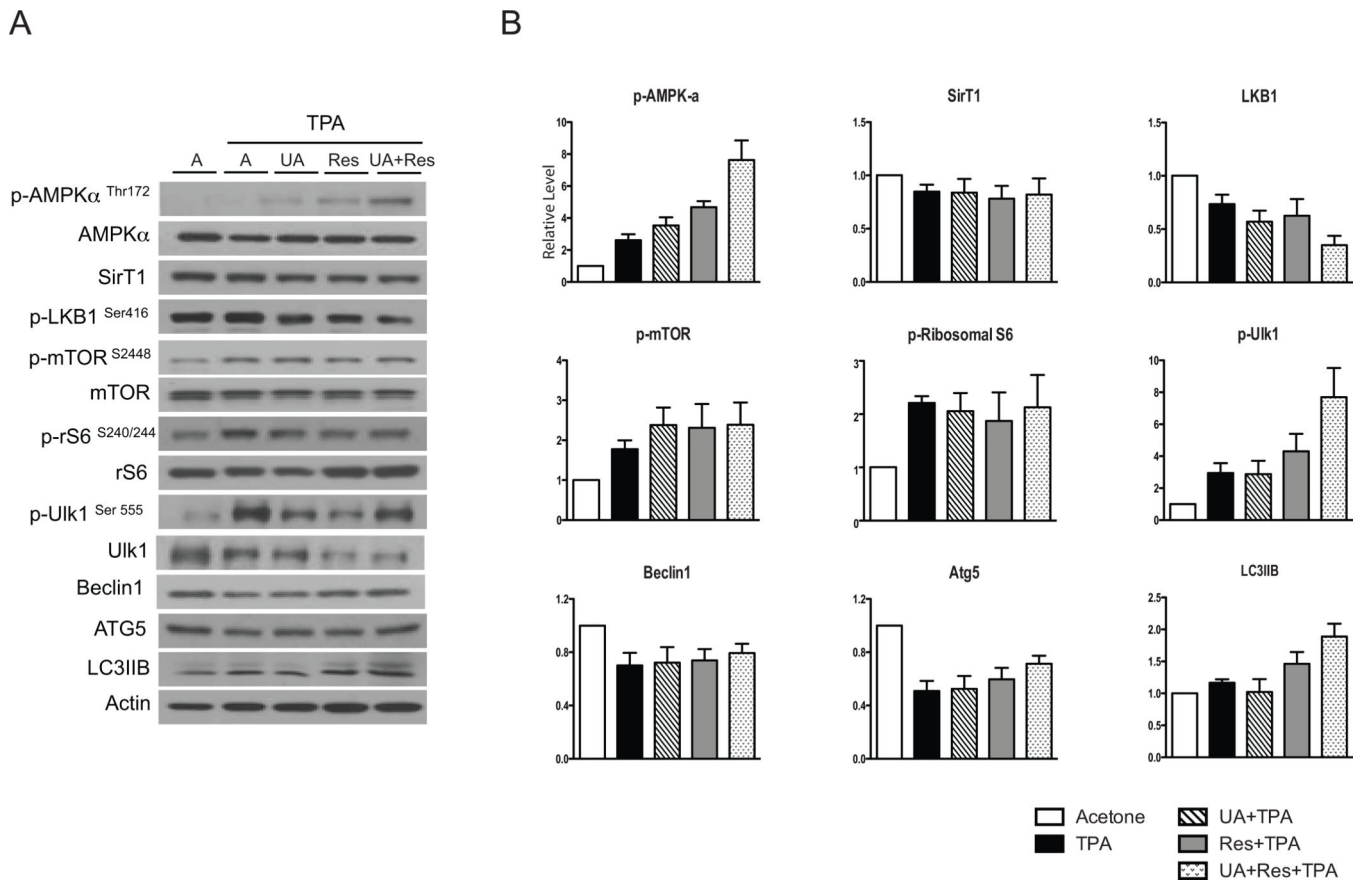
**A**



**B**

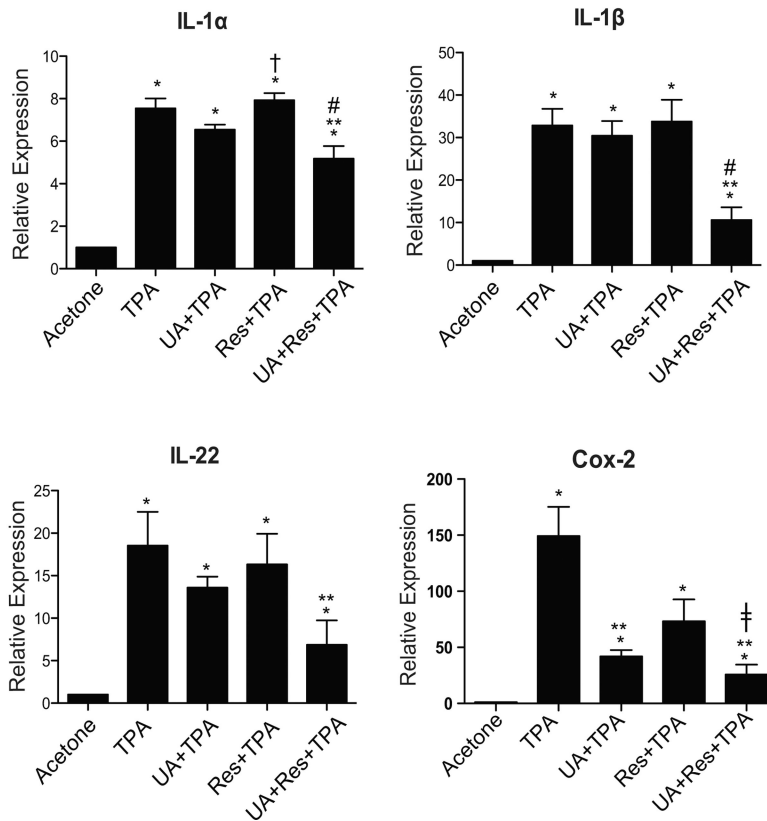


**Figure 3.** Effect of UA + Res on TPA-induced signaling pathways in epidermis of female ICR mice. Western blot analyses were performed using pooled epidermal protein lysates from mice (n=4–5/group) receiving treatment with the short-term protocol. Panel A, representative Western blot analyses of multiple signaling pathways. Panel B, quantitative evaluation of Western blot data. Values represent means ± SEM from at least 3 independent experiments. \*,  $p < 0.05$  when compared to the acetone treated group; \*\*,  $p < 0.05$  when compared to the TPA treated group; †,  $p < 0.05$  when compared to the UA + TPA treated group; ‡,  $p < 0.05$  when compared to the Res + TPA treated group; and #,  $p < 0.05$  when compared to the UA + TPA and Res + TPA treated groups. The Mann-Whitney *U* test was used for all statistical comparisons.

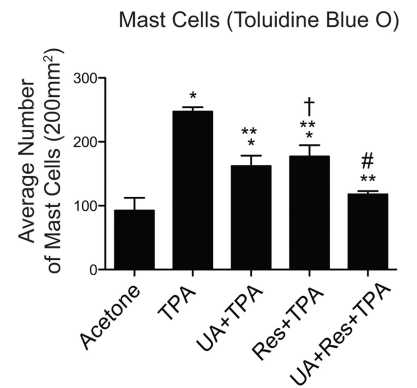


**Figure 4.** Effect of UA + Res on TPA-induced AMPK and mTORC1 signaling pathways in epidermis of female ICR mice. Western blot analyses were performed using pooled epidermal protein lysates from mice (n=4-5/group) that received multiple treatments with short-term protocol. Panel A, representative Western blot analyses. Panel B, quantitative evaluation of the effect of UA, Res or UA + Res on TPA-induced AMPK- $\alpha$  signaling pathway. Values represent means  $\pm$  SEM from at least 3 independent experiments. \*,  $p < 0.05$  when compared to acetone group; \*\*,  $p < 0.05$  when compared to TPA group; †,  $p < 0.05$  when compared to UA + TPA group; ‡,  $p < 0.05$  when compared to the Res + TPA treated group; and #,  $p < 0.05$  when compared to UA + TPA and Res + TPA group. The Mann-Whitney  $U$  test was used for statistical comparisons.

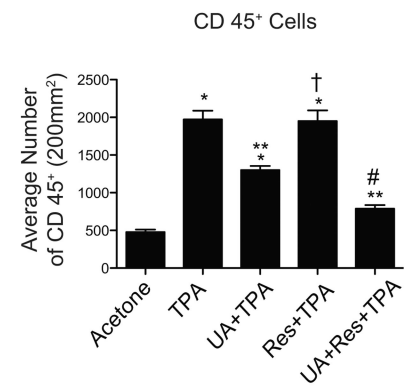
**A**



**B**



**C**



**Figure 5.** Effect of UA + Res on TPA-induced inflammatory gene expression and inflammatory cell infiltration. Epidermal RNA samples were prepared from groups of female ICR mice (n=4–5/group) treated using the short-term protocol. RNA samples were then subjected to qRT-PCR analysis as described in Materials and Methods. Panel A, qRT-PCR analysis of IL-1 $\alpha$ , IL-1 $\beta$ , IL-22, and Cox-2. mRNA levels of IL-1 $\alpha$ , IL-1 $\beta$ , and IL-22 were normalized to 18S and the mRNA level of Cox-2 was normalized to GAPDH. Panel B, quantitative evaluation of the effect of UA, Res and UA + Res on the number of mast cells in the dermis 48 hrs after the last TPA treatment. Positive cells were counted per 200 mm<sup>2</sup>. Panel C, quantitative analysis of the effect of UA, Res and UA + Res on the number of CD45 positive cells in the dermis 48 hrs after the last TPA treat. Positive cells were counted per 200 mm<sup>2</sup>. The graphs in all cases represent means  $\pm$  SEM of at least 3 independent experiments. \*,  $p < 0.05$  when compared to acetone group; \*\*,  $p < 0.05$  when compared to TPA group; †,  $p < 0.05$  when compared to UA + TPA group; ‡,  $p < 0.05$  when compared to Res + TPA group; and #,  $p < 0.05$  when compared to UA + TPA and Res + TPA group. The Mann-Whitney *U* test was used for statistical comparisons.