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Ubiquitin carboxyl terminal hydrolase L1 negatively regulates TNFα-mediated vascular smooth muscle cell proliferation via suppressing ERK activation

Tomonaga Ichikawa[a], **Jinqing Li**[a], **Xiaoyu Dong**[a], **Jay D. Potts**[a], **Dong-qi Tang**[b], **Dong-Sheng Li**[c], and **Taixing Cui**[a]

[a]Department of Cell Biology and Anatomy, University of South Carolina School of Medicine, Columbia, SC 29208, USA

[b]Department of Pathology, Immunology, and Laboratory Medicine, University of Florida College of Medicine, Gainesville, FL, USA

[c]Regenerative Medicine Research Institute, Yunyang Medical College Tai-he Hospital, 29 S. Renmin Rd, Shiyan, Hubei, China

Abstract

Deubiquitinating enzymes (DUBs) appear to be critical regulators of a multitude of processes such as proliferation, apoptosis, differentiation, and inflammation. We have recently demonstrated that a DUB of ubiquitin carboxyl terminal hydrolyase L1 (UCH-L1) inhibits vascular lesion formation via suppressing inflammatory responses in vasculature. However, the precise underlying mechanism remains to be defined. Herein, we report that a posttranscriptional up-regulation of UCH-L1 provides a negative feedback to tumor necrosis factor alpha (TNFα)-mediated activation of extracellular signal-regulated kinases (ERK) and proliferation in vascular smooth muscle cells (VSMCs). In rat adult VSMCs, adenoviral over-expression of UCH-L1 inhibited TNFα-induced activation of ERK and DNA synthesis. In contrast, over-expression of UCH-L1 did not affect platelet derived growth factor (PDGF)-induced VSMC proliferation and activation of growth stimulating cascades including ERK. TNFα hardly altered UCH-L1 mRNA expression and stability; however, up-regulated UCH-L1 protein expression via increasing UCH-L1 translation. These results uncover a novel mechanism by which UCH-L1 suppresses vascular inflammation.

Keywords

UCH-L1; Vascular smooth muscle cells; TNFα; Inflammation; Proliferation; Posttranscriptional regulation

Introduction

Cardiovascular diseases continue to be a leading cause of disability and mortality in the United States, and the majority of cardiovascular disorders results from complications of

Corresponding author: Dong-Sheng Li, MD, PhD, +(86)-719-880-1418 dsli@yymc.edu.cn, or Taixing Cui, MD, PhD, Tel: 803-253-5837; taixing.cui@uscmed.sc.edu.

vascular diseases [1]. While it is still far from a comprehensive understanding of molecular and cellular mechanisms leading to vascular diseases, a preponderance of evidence supports a notion that inflammation plays a critical role in a wide range of vascular complications and dysfunctions [2–6].

Vascular inflammation has been characterized as a complex process involving endothelial dysfunction, leukocyte recruitment, VSMC activation, and malfunction of inflammatory mediators including both anti-inflammatory and pro-inflammatory cytokines [2–7]. Recently, TNFα, a pro-inflammatory cytokine, has emerged as a key factor in the pathogenesis of vascular diseases [7, 8]. TNFα triggers myriads of pro-inflammatory effects on vascular cells such as VSMC migration and proliferation, thereby contributing to maladaptive vascular modeling. It has been demonstrated that nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) cascades are major component of TNFα signal transduction [9]; however, the precise signaling mechanisms responsible for the pathological TNFα activity in vasculature remain to be defined.

We have recently demonstrated that a DUB of ubiquitin carboxyl terminal hydrolyase L1 (UCH-L1) inhibits vascular lesion formation via suppressing inflammatory responses in vasculature [10]. However, cellular and signaling mechanisms by which UCH-L1 suppresses vascular inflammatory responses remain to be further investigated. In the present study, we explored role of UCH-L1 in the regulation of TNFα-mediated VSMC proliferation *in vitro*. Our results uncovered for the first time that UCH-L1 negatively regulates TNFα-mediated VSMC proliferation via suppressing ERK activity.

Material and methods

Cell culture and adenoviral infection

Vascular smooth muscle cells (VSMCs) were isolated from thoracic aorta of adult Spague-Dawley rats as previously described [11], and cultured in low glucose (1 g/L) Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum. Sub-confluent rat aortic smooth muscle cells (RASMCs) were infected with adenovirus of control beta-galactoside (Ad-βGal) and human UCH-L1 (Ad-hUCH-L1) (Welgen Inc.) in serum free DMEM for 48 hours.

[^{3}H]thymidine uptake

RASMCs were cultured in serum free DMEM for 24 hours to induce a quiescent status, and then stimulated with or without TNFα (Sigma-Aldrich) for 40 hours. [^{3}H]thymidine (MP Biomedicals) was added to the media (final concentration 1 μCi/ml) during the last 24 hours. [^{3}H]thymidine uptake was measured by a Beckman LS6000 scintillation counter (Beckman Coulter, Inc.) as previously described [11]. [^{3}H]thymidine incorporation was normalized by the amount of cellular protein counted.

Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real time (Q-PCR)

Total RNA purification, RT reaction, and Q-PCR were performed as described previously [12]. Expression levels of target genes were normalized by concurrent measurement of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. Primers that were used for Q-PCR are as follows: Forward primer (5'-CCCCGAGATGCTGAACAAAGT-3') and reverse primer (5'-ATGGTCTGCTTCATGAAGTA-3') were used for PCR amplification of rat UCH-L1 (NM_017237). Forward primer (5'-ACCACAGTCCATGCCATCAC-3') and reverse primer (5'-

TCCACCACCCTGTTGCTGTA-3') were used for PCR amplification of rat GAPDH (NM_017008).

UCH-L1 mRNA stability assessment

Quiescent RASMCs were pretreated with or without actinomysin D, an inhibitor of gene transcription, for 1 hour, and then stimulated with or without TNFα (5 ng/ml) as indicated. As actinomysin D at a dose of 1 μg/ml has been demonstrated not to inhibit GAPDH but other genes' transcription in RASMCs [13], we applied actinomysin D (1 μg/ml) in the present study. UCH-L1 mRNA expression was quantified by Q-PCR as described above. Expression UCH-L1 mRNA levels in RASMCs treated with vehicle alone was considered as 100%.

Western blot

Cell lysates were subjected to immunoblot analysis as previously describe [11] using antibodies of phosphor-ERK (Cat# 9101, Cell Signaling Technology), anti-UCH-L1 (AB1761, Millipore) and anti-GAPDH (FL-335, sc-25778, Santa Cruz Biotechnology). Densitometric analysis was performed using an image scanner (EPSON GT-8000) and NIH image software.

Statistical analysis

Data are shown as mean ± s.d.. Means were compared by ANOVA, followed by Bonferroni test for multiple comparisons. Differences were considered significant at $p<0.05$.

Results and discussion

Over-expression of UCH-L1 inhibits TNFα-mediated VSMC proliferation

We have observed that UCH-L1 is up-regulated in injured arteries and local gene delivery of UCH-L1 inhibited vascular lesion formation with suppression of inflammatory responses in vasculature [10]. To establish a direct link between the up-regulation of UCH-L1 expression and the inhibition of inflammatory vascular remodeling, we first explored an effect of over-expression of UCH-L1 on TNFα-mediated VSMC proliferation, a key feature of vascular diseases including atherosclerosis, restenosis and hypertension [2, 6, 14, 15]. Because of the controversial reports on TNFα-mediated rat VSMC proliferation [16, 17], we carefully determined experimental conditions that TNFα stimulates RASMC proliferation. We established that TNFα dose-dependently stimulated RASMC proliferation with a maximum effect at a dose of 5 ng/ml (Fig. 1A). In addition, up-regulation of UCH-L1 expression was achieved by adenoviral over-expression of hUCH-L1. Adenovirus of hUCH-L1 at 20 MOI led to a substantial increase in UCH-L1 protein expression without any observable cytotoxic effects in RASMCs (Fig. 1B). Thus, we used TNFα at a dose of 5 ng/ml and adenovirus of control or hUCH-L1 at doses up to 20 MOI for the subsequent studies. Importantly, adenoviral over-expression of UCH-L1 dose-dependently inhibited TNFα-mediated RASMC proliferation (Fig. 1C). These results provide direct evidence that UCH-L1 suppresses vascular lesion formation via inhibiting proinflammatory cytokine TNFα-mediated VSMC proliferation.

Over-expression of UCH-L1 suppresses TNFα-induced ERK activation in VSMCs

It has been well documented that TNFα activates MAPKs including ERK, c-Jun N-terminal kinases (JNK) and p38, phosphoinositide 3-kinase (PI3-K), as well as NF-κB, contributing to VSMC growth [18, 19]. As we have demonstrated that over-expression of UCH-L1 suppresses NF-κB p65 transcriptional activity in VSMCs [10], it is conceivable that UCH-L1 inhibits VSMC proliferation via at least partly suppressing NF-κB pathway. However, it

still remains unclear whether UCH-L1 regulates the TNFα-mediated activation of other signaling cascades leading to VSMC proliferation. In our pilot experiments, we observed that TNFα (5 ng/ml) induced phosphorylation of ERK without any detectable phosphorylation of JNK, p38, and Akt, a downstream kinase of PI3-K in RASMCs (data not shown). Interestingly, over-expression of UCH-L1 attenuated TNFα-induced activation of ERK in RASMCs (Fig. 2), suggesting that suppression of ERK activation also contributes to the growth inhibitory effect of UCH-L1 in TNFα-inflamed VSMCs.

To study a specificity of the UCH-L1-mediated growth inhibitory effect in VSMCs, we examined the effect of UCH-L1 over-expression on the PDGF-mediated RASMC proliferation. Notably, adenoviral over-expression of UCH-L1 hardly affected the PDGF-induced activation of ERK, JNK, Akt, and signal transducer and activator of transcription 3 (STAT3), as well as DNA synthesis in RASMCs (Fig. 3). Moreover, PDGF did not regulate UCH-L1 expression at either mRNA or protein levels (data not shown). These results demonstrate a unique growth inhibitory role of UCH-L1 preferentially in pro-inflammatory cytokines such as TNFα-inflamed VSMCs.

Up-regulation of UCH-L1 in TNFα-inflamed VSMCs via a posttranscriptional regulation

To gain mechanistic insight into the anti-inflammatory role of UCH-L1 in VSMCs, we characterized expression profile of UCH-L1 in TNFα-inflamed RASMCs. Consistent with our previous observation that TNFα did not regulate UCH-L1 mRNA expression in human VSMCs [10], TNFα stimulation for 48 hours had no effect on UCH-L1 mRNA expression in RASMCs (Fig. 4A). While actinomycin D, an inhibitor of gene transcription, suppressed UCH-L1 mRNA expression, TNFα did not affect actinomysin D-induced suppression of UCH-L1 mRNA in RASMCs (Fig. 4A). These results demonstrate that TNFα could not regulate either UCH-L1 transcription or its mRNA stability. However, Western blot analysis with a long time exposure revealed that TNFα stimulation slightly increased UCH-L1 protein expression (Fig. 1B). Immunochemistry with biotin-labeled secondary antibodies to enhance UCH-L1 staining signal confirmed that TNFα did up-regulate UCH-L1 protein expression in RASMCs (data not shown). These results suggest that TNFα up-regulates UCH-L1 protein via a posttranscriptional regulation in VSMCs. To explore the underlying mechanism, we determined effect of MG-132, a proteasome inhibitor, on UCH-L1 protein expression in RASMCs. We observed that MG-132 at concentration over 10 μM exhibited cytotoxic effects in RASMCs (data not shown). MG-132 (0–5 μM) alone dose-dependently up-regulated UCH-L1 protein expression in RASMCs (data not shown). Therefore, we treated the cells with MG-132 at a non-cytotoxic dose of 5 μM. As shown in Fig. 4C, MG-132 treatment for 24 hours significantly increased basal UCH-L1 protein levels, and the MG-132-induced up-regulation of UCH-L1 protein expression was further enhanced by TNFα in RASMCs. These results suggest that TNFα up-regulates UCH-L1 protein levels by inhibiting UCH-L1 degradation and/or increasing UCH-L1 translation. To clarify this issue, we used cycloheximide, an inhibitor of protein synthesis. Presumably, TNFα is able to up-regulate UCH-L1 protein levels in RASMCs that UCH-L1 gene translation is blocked by cycloheximine if TNFα inhibits UCH-L1 degradation. While TNFα dramatically enhanced UCH-L1 protein expression in vehicle treated RASMCs over-expressed with UCH-L1, it could not up-regulate UCH-L1 protein expression in cycloheximide treated cells (Fig. 4C). We used cycloheximide (5 μg/ml) that has been established not to suppress house keeping GAPDH protein synthesis but significantly suppress other protein synthesis in RASMCs [20]. These results indicate that TNFα up-regulates UCH-L1 via a translational regulation. Cycloheximide alone up to 24 hours did not change the level of UCH-L1 protein expression in RASMCs (Fig. 4C), indicating that the turnover of UCH-L1 protein is slow in VSMCs. Taken together, we demonstrate that TNFα upregulates UCH-L1 protein expression by enhancing UCH-L1 translation rather than inhibiting its degradation in VSMCs. Because

UCH-L1 protein is quite stable, the up-regulated UCH-L1 might provide a powerful negative feed back on TNFα-mediated pro-inflammatory signaling in VSMCs.

Overall, our data demonstrate that TNFα up-regulates UCH-L1 via a translational regulation to inhibit ERK activity, thereby providing a negative feedback to control its growth promoting signaling in VSMCs. Recently, UCH-L1 has been shown to inhibit α_2-adrenergic receptor (AR) agonist-mediated activation of ERK via a direct association with α_{2A}-AR receptor, implicating a role of UCH-L1 in certain tumor suppression and neuro-protection [21]. In contrast, other studies have documented that UCH-L1 up-regulates β-catenin/TCF via a positive feedback mechanism or exerts anti-apoptotic and growth stimulating effects, supporting an oncogenic potential of UCH-L1 [22, 23]. These results suggest that UCH-L1 appears to be a multifunctional protein and exerts cell type and/or tissue specific actions. To further address precise mechanism by which UCH-L1 suppresses TNFα-mediated activation of ERK will provide novel insight into the understanding of TNFα-mediated inflammatory responses in VSMCs, facilitating development of new therapeutic approaches for the treatment of vascular diseases.

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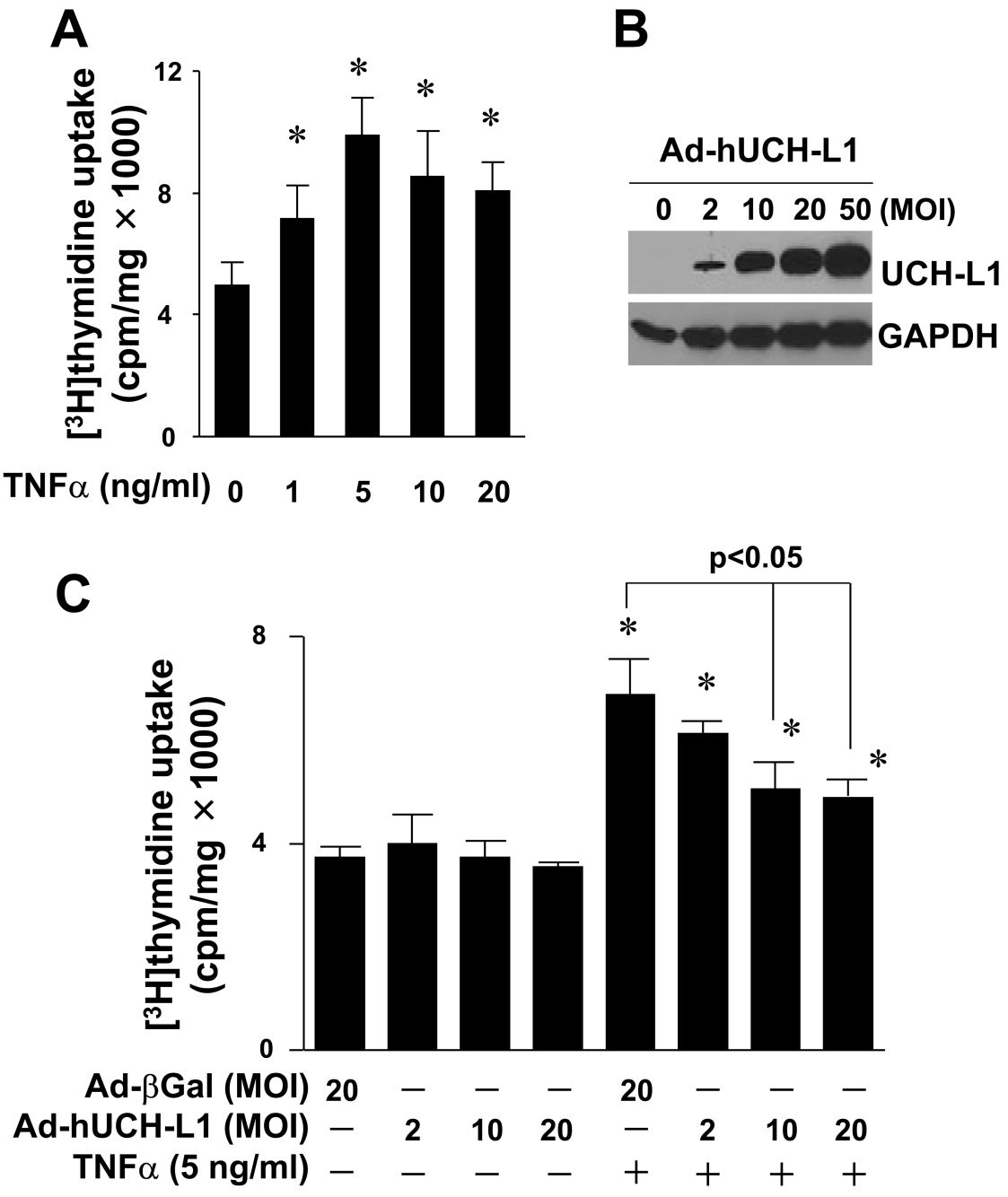

Fig. 1.

Effect of UCH-L1 over-expression on TNFα-induced VSMC proliferation. (A) TNFα-induced proliferation of rat aortic smooth muscle cells (RASMCs). Cell proliferation was assessed by measuring [^{3}H]thymidine update as described in "*Methods*". * $p<0.05$ vs TNFα (−), n=4. (B) Adenoviral over-expression of UCH-L1 in RASMCs. Results are representative of three independent Western blot analysis of UCH-L1 in RASMCs infected with or without adenovirus of UCH-L1. (C) Effect of over-expression of UCH-L1 on TNFα-induced RASMC proliferation. Cells infected with Ad-UCH-L1 or Ad-βGal were stimulated with or without TNFα (5 ng/ml) as indicated for 40 hours. * $p<0.05$ vs TNFα (−), n=4.

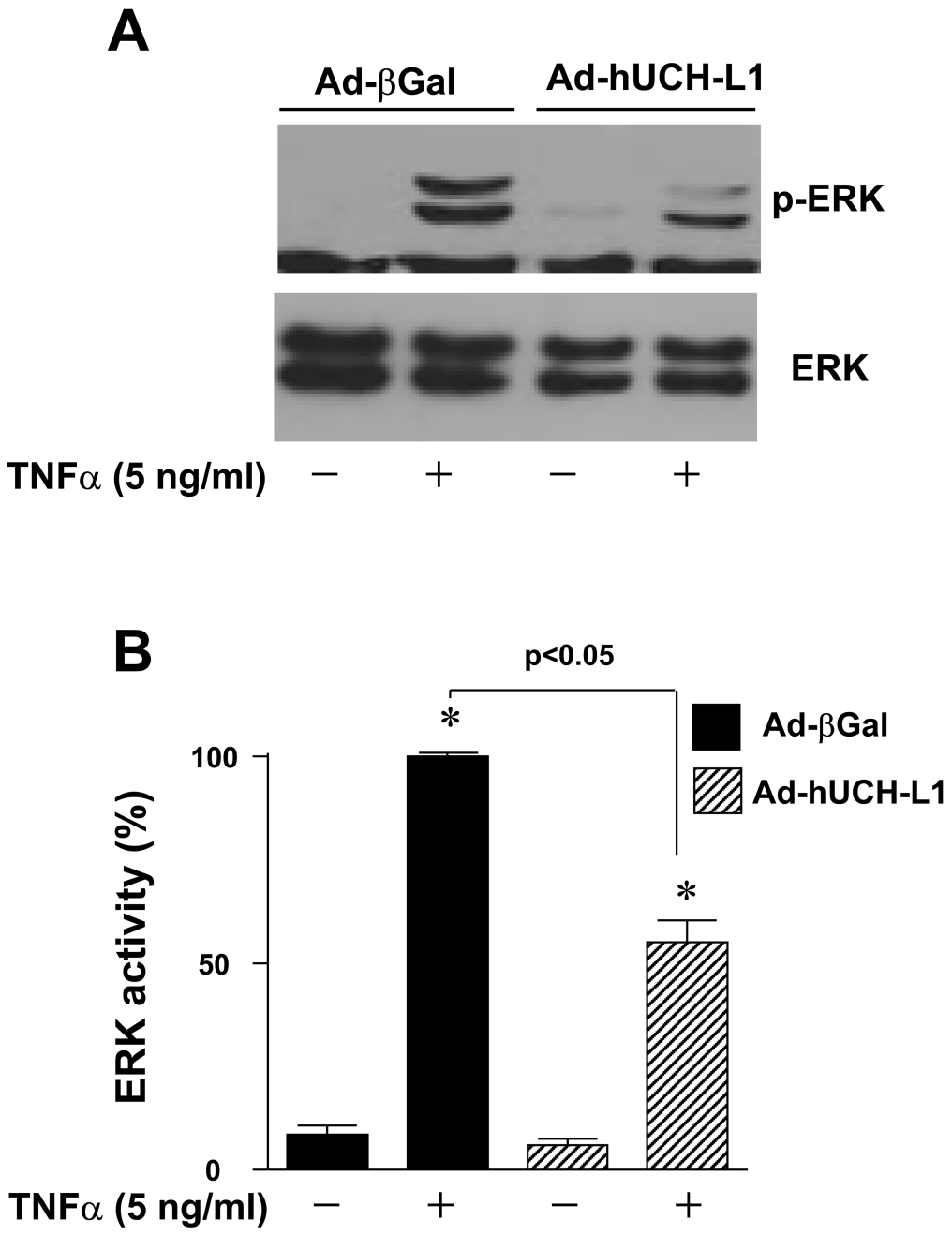

Fig. 2.

Effect of UCH-L1 expression on ERK activity in VSMCs. Cells infected with Ad-βGal (20 MOI) or Ad-hUCH-L1 (20 MOI) were treated with TNFα (5 ng/ml) for 10 minutes, and then subjected to Western blot analysis for ERK phosphorylation. (A) Representatives of ERK phosphorlyation from 4 separate experiments. (B) Densitometric analysis of ERK phosphorylation. The density of ERK phosphorylation induced by TNFα in RASMCs infected with Ad-βGal was set as the maximal increase (100%). *p<0.05 vs Ad-βGal (TNFα −); #p<0.05 vs Ad-βGal (TNFα +), n=4.

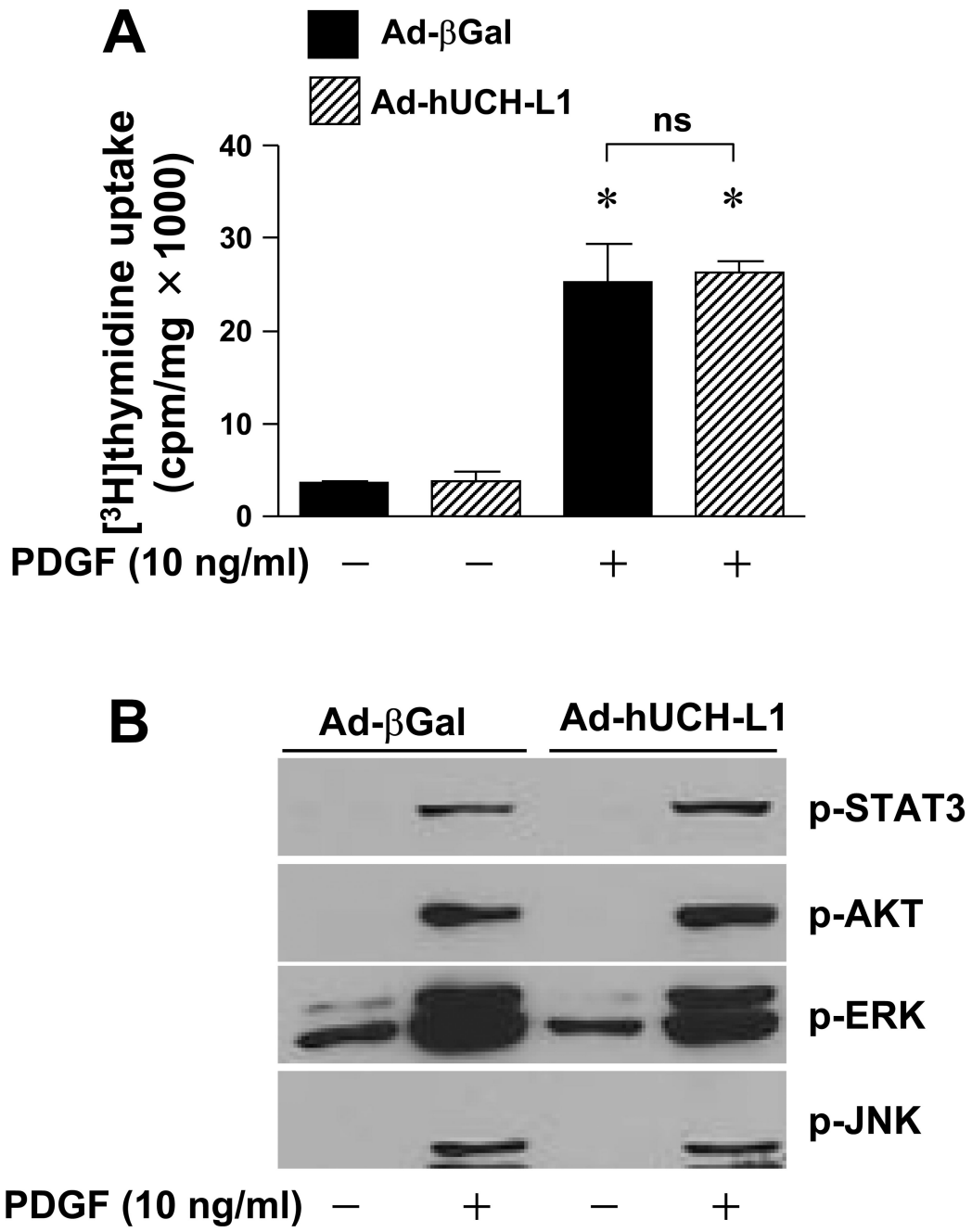

Fig. 3.

Effect of UCH-L1 on PDGF-induced proliferation and activation of ERK in VSMCs. (A) PDGF-induced RASMC proliferation. Cells infected with Ad-βGal (20 MOI) or Ad-hUCH-L1 (20 MOI) were treated with or with out PGDF (10 ng/ml) for 40 hours. Cell proliferation was assessed by measuring [^{3}H]thymidine update as described in "*Methods*". * $p<0.05$ vs PDGF (−), n=4. (B) Infected cells were treated with PDGF (10 ng/ml) for 10 minutes, and then subjected to Western blot analysis as indicated. Results are representative of three independent experiments.

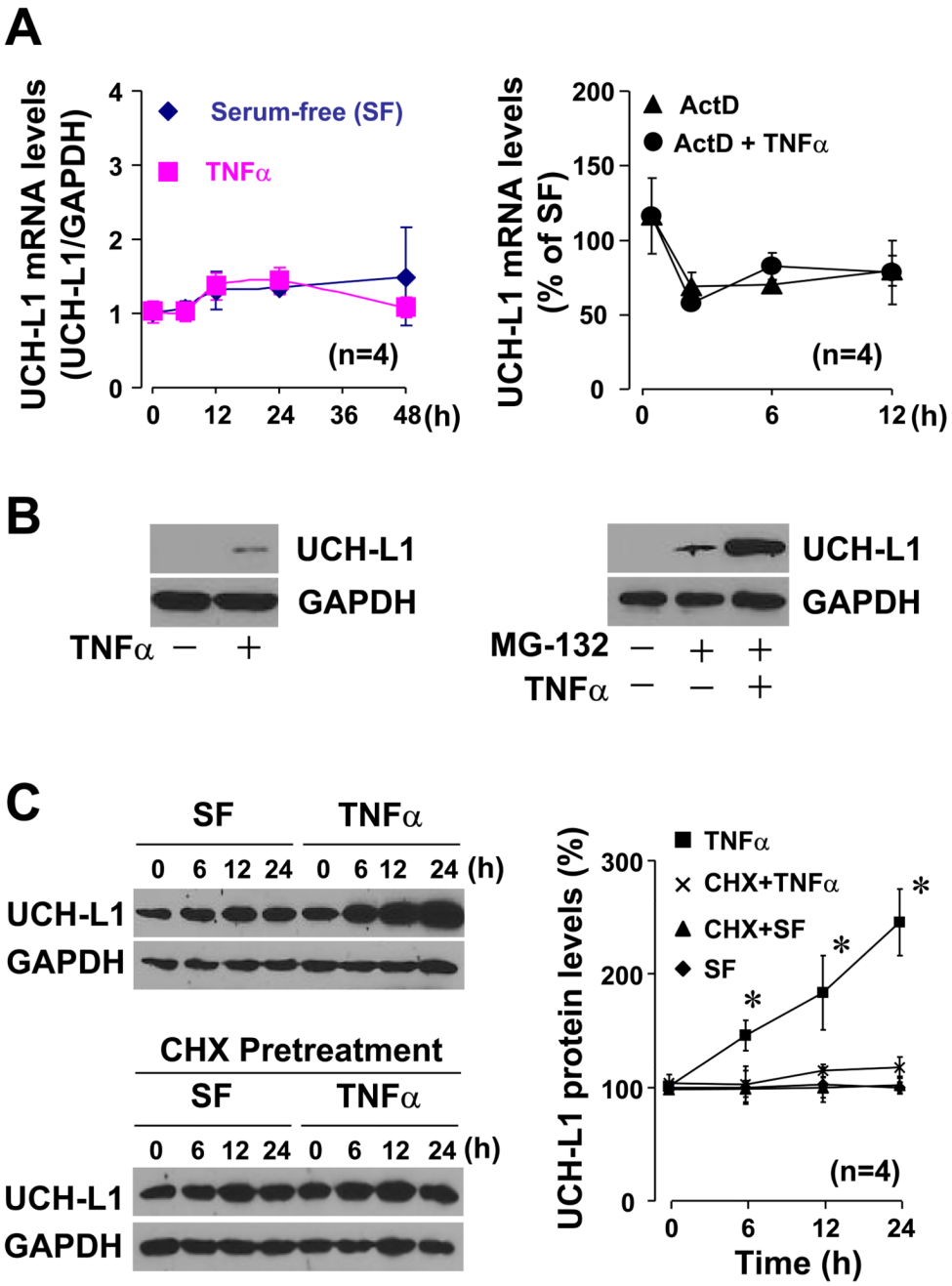

Fig. 4.

UCH-L1 expression in TNFα-inflamed VSMCs. (A) Effect of TNFα on UCH-L1 mRNA expression (Left panel) and mRNA stability (Right panel) in RASMCs. Left; Quiescent RASMCs were treated with or with TNFα (5 ng/ml) as indicated, and then subjected to Q-PCR analysis for UCH-L1 mRNA expression. Right, Quiescent RASMCs were pretreated with or without actinomysin D (ActD, 1 μg/ml) for 1 hour. The cells pretreated with ActD were future stimulated with or with TNFα (5 ng/ml) as indicated while the cells without pretreatment of ActD were cultured with vehicle by the end points as indicated. The expression levels of UCH-L1 mRNA in the cells treated with vehicle alone were set as 100%. UCH-L1 mRNA expression was quantified by Q-PCR. (B) Western blot analysis of

UCH-L1 protein expression in RASMCs. Left panel; cells were treated with or with TNFa (5 ng/ml) for 24 hours. Right panel; cells were pretreated with or without MG-132 (5 μM) for 2 hours, and then stimulated with or without TNFα (5 ng/ml) for additional 24 hours. Results are representative of three separated experiments. (C) Effect of TNFα on UCH-L1 protein synthesis in RASMCs. Quiescent RASMCs infected with Ad-hUCH-L1 (20 MOI) were treated TNFα (5 ng/ml) and/ or cycloheximide (CHX, 5 μg/ml) as indicated. CHX was pretreated for 1 hour. Left panel is representative of 4 separated experiments. Right panel is densitometric analysis of UCH-L1 protein expression. *$p<0.05$ vs SF (0), n=4.