*Original Research*

# Extracellular signal-regulated kinase plays a proapoptotic role in podocytes after reactive oxygen species treatment and inhibition of integrin–extracellular matrix interaction

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## Abstract

**The effect of reactive oxygen species (ROS) and blocking integrin–extracellular matrix (ECM) interaction on apoptosis in podocytes, and the related signal transduction pathways remain unclear. Primary cultured rat podocytes were exposed to ROS. Integrin–ECM interaction was inhibited with anti-$\beta$1-integrin monoclonal antibody (mAb) or RGDS (Arg–Gly–Asp–Ser). Extracellular signal-regulated kinase (ERK) activation was evaluated with Western blotting. U0126 was used to inhibit ERK activation. Terminal deoxynucleotidyl transferase-mediated dUTP-peroxidase nick end-labeling of DNA (TUNEL) was used to evaluate apoptosis. We found that ROS-treated podocytes exhibited increased apoptosis, and both anti-$\beta$1-integrin mAb and RGDS induce apoptosis. Addition of ROS to either anti-$\beta$1-integrin mAb or RGDS enhanced apoptosis in both conditions. ERK activation was increased by either ROS or blocking integrin–ECM interaction. Preincubation with U0126 decreased apoptosis induced by ROS, anti-$\beta$1-integrin mAb or RGDS, respectively. Our study demonstrated that ROS and blocking integrin–ECM interaction induce podocyte apoptosis, which is mediated by ERK activation.**

**Keywords:** extracellular signal-regulated kinase, integrin, podocytes, reactive oxygen species (ROS), apoptosis

*Experimental Biology and Medicine* 2012; **237**: 777–783. DOI: 10.1258/ebm.2012.011157

## Introduction

Reactive oxygen species (ROS) are responsible for injury in various glomerular diseases,[1,2] and can induce apoptosis in various cell types.[3] ROS were also found to play a major role in foot process effacement and proteinuria in puromycin aminonucleoside (PAN)-treated rats by the xanthine oxidase pathway.[4] We wondered what pathway played a major role in ROS-induced podocyte apoptosis.

Cell interactions with the extracellular matrix (ECM) are necessary for cell survival and proliferation. These interactions are dependent on cell surface receptors. Integrins are a family of membrane receptors that mediate cell interactions with the ECM.[5] Integrin-mediated interactions are involved in the regulation of many cell functions, including migration, proliferation, differentiation, cell cycle regulation and apoptosis.[6] Integrins are transmembrane heterodimeric glycoproteins composed of $\alpha$ and $\beta$ subunits. There have been eight different $\beta$ and 16 $\alpha$ subunits described. The $\beta$1 integrins are expressed in several cell types and mainly bind to laminin, collagen and fibronectin.[7] Various $\beta$1 integrin distributions and modifications have been identified during nephropathies.[8,9] Studies of $\beta$1 distribution in the kidney have found that $\alpha3\beta1$ appears to be the major $\beta$1 integrin expressed by podocytes.[10] Integrin-mediated signals include the extracellular signal-regulated kinase (ERK) family of mitogen-activated protein kinases (MAPK).[11] In our previous reports, we found that $\alpha3\beta1$ integrins and podocytes were decreased in humans and chronic PAN-treated animal models of focal segmental glomerulosclerosis (FSGS).[12] We also found that blocking the integrin–ECM interaction promoted podocyte apoptosis.[13] Thereafter, the decreased podocyte numbers in humans and chronic PAN-treated animal models of FSGS might be due to the combined effects of ROS and decreased integrin–ECM interaction that induced podocyte apoptosis. In this study, we evaluated the effects of ROS and integrin–ECM interaction on podocyte survival. We also evaluated the downstream signaling pathways that control podocyte survival regulated by ROS and integrin–ECM interaction.

## Materials and methods

### Primary podocyte cell culture

The method used was the same as in our previous report.[13] Briefly, isolation of glomeruli from Sprague-Dawley rats of 150–200 g body weight was performed by sieving methods.[14] Isolated glomeruli were plated in plastic culture flasks that contained RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 $\mu$g/mL streptomycin for a period of five days. Cultures were kept at 37°C in a humidified 5% $CO_2$ atmosphere. Outgrowing epithelial cells were trypsinized and passed through sieves of 25 $\mu$m pore size to remove the remaining glomerular cores. Cells were plated on type IV collagen-coated plastic dishes. Proliferating cells in confluent cultures were subcultured after detachment with 0.15% trypsin in phosphate-buffered saline (PBS). For the following studies, cells were subcultured on chamber slides coated with type IV collagen. Experiments were performed with subconfluent cells of the secondary and fourth passage. The identity of the cells was confirmed by identification of rabbit polyclonal anti-Wilms' tumor protein WT-1 (1:400, WT-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and synaptopodin (1:1000, polyclonal rabbit antibody SE-19; Sigma, St Louis, MO, USA).[14,15]

### Cell injury with ROS

Cells were planted in collagen IV-coated six-well plates with 10% FCS-containing RPMI 1640 medium for 2–3 days. Cells were then cultured in serum-free RPMI 1640 medium for one day. Thereafter, cells were washed twice with PBS and then incubated for one hour at 37°C in serum-free RPMI 1640 containing xanthine (0, 0.01, 0.05, 0.1, 0.25, 0.5 and 1 mmol/L, respectively), xanthine oxidase (0.05 U/mL) and ferric chloride (0.2 $\mu$mol/L) (Sigma Chemical Co., St Louis, MO, USA) as a source of ROS. The reaction protocol generates superoxide radicals and hydroxyl radicals that are important mediators of cell injury.[16,17] The reaction was stopped by washing cells with PBS and reincubated with serum-free RPMI 1640 for three hours. Control cells were incubated in serum-free RPMI 1640 in the absence of ROS for one hour. Cell viability was assessed by trypan blue exclusion (Sigma Chemical Co.).[18] Cells from a single well were counted and approximately 400 were considered as $N = 1$. The percentage of live cells was calculated by dividing the number of unstained cells by the total number of cells per field. All experiments were performed on $N = 10$.

### Terminal deoxynucleotidyl transferase-mediated dUTP-peroxidase nick end labeling for detection of apoptosis

DNA fragmentation associated with apoptosis was detected with terminal deoxynucleotidyl transferase-mediated dUTP-peroxidase nick end-labeling (TUNEL).[19] The method was the same as in our previous report.[13] In brief, the chamber slide cells were fixed by air dry followed by treatment with 4% paraformaldehyde solution in PBS (pH 7.4) for one hour at room temperature. After blocking endogenous peroxidase activity by incubation with 0.3% $H_2O_2$ in methanol for one hour, the slides were rinsed with PBS and incubated in a permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for two minutes on ice (4°C). The slides were rinsed with PBS and incubated with 50 $\mu$L of TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and dUTP (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) in a humidified chamber for 60 min at 37°C. The reaction was terminated by rinses with PBS and followed by peroxidase-mediated color development. At least 400 cells were counted for each slide and the number of TUNEL-positive cells was expressed as a percentage of the total cells. All experiments were performed in triplicate. Negative control was performed by using Label Solution (without terminal transferase) instead of TUNEL reaction mixture. Positive control was performed by using DNase I to induce DNA strand breaks.[13]

### Western blot analysis

The method used was the same as in our previous report.[13] For total protein extraction, cells were washed in PBS and then lyzed in 1 mL of 1% Nonidet P-40, 25 mmol/L Tris-HCl, 150 mmol/L NaCl and 10 mmol/L ethylenediaminetetraacetic acid at pH 8.0, containing a one in 50 dilution of protease inhibitor cocktail for 30 min on ice. Samples were centrifuged at 14,000 $g$ for five minutes to obtain pellet cell debris. Protein estimation was performed by a bicinchoninic acid protein assay kit according to the manufacturer's instructions. Samples (20 $\mu$g) were mixed with a sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis sample buffer, boiled for five minutes and electrophoresis was performed on 10% SDS polyacrylamide gel. Gels were blotted for one hour on Hybond-ECL nitrocellulose membranes (Amersham International, Buckinghamshire, UK). Non-specific background was blocked by washing with PBS containing 5% skimmed milk powder, 1% FCS and 0.02% Tween 20 for 30 min followed by incubation with the primary antibody overnight.

The following primary antibodies were used: anti-phospho-ERK (1:3000; Cell Signaling Technology, Beverly, MA, USA) and anti-total ERK1/2 (1:3000; Santa Cruz Biotechnology). After washing, the membrane was incubated with peroxidase-conjugated secondary antibody for one hour at room temperature. Protein bands were made visible with an ECL detection kit (Amersham International) to produce a chemiluminescence signal that was captured on X-ray film. Equality of loading was ensured by testing for actin using anti-$\beta$-actin (Sigma Chemical Co.). Films were scanned using a Bio-Rad model GS-700 imaging densitometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and densitometric analysis was performed using Molecular Analyst Version 2.1 (Bio-Rad Laboratories, Inc.). Three experiments were performed.

## Experimental design

### Influence of ROS on apoptosis

Cells were planted in collagen IV-coated six-well plates with 10% FCS-contained RPMI 1640 medium for 2–3 days. Cells were then cultured in serum-free RPMI 1640 medium for one day. Cells that were treated with or without by ROS for one hour were followed by recovery in serum-free RPMI 1640 for three hours. They were then analyzed for apoptosis using TUNEL ($N = 3$).

### Influence of blocking integrin–ECM interaction on apoptosis

Cells were planted in collagen IV-coated six-well plates with 10% FCS-contained RPMI 1640 medium for 2–3 days. Cells were then cultured in serum-free RPMI 1640 medium for one day. Cells, with or without ROS treatment, were exposed to anti-$\beta$1-integrin monoclonal antibody (mAb) (10 $\mu$g/mL) (mouse anti-human integrin beta1 [CD29] adhesion-blocking monoclonal antibody, catalog number: MAB2253; Chemicon International, Inc., Temecula, CA, USA) or normal mouse IgG1 isotype control antibody (10 $\mu$g/mL; Santa Cruz Biotechology) for one hour.[20,21] The integrin reception sequence tetrapeptide RGDS (Arg–Gly–Asp–Ser; 250 $\mu$g/mL) (Sigma Chemical Co.) was used to competitively inhibit integrin–ECM interaction for one hour.[20,21] Cells were then rinsed with PBS to stop the reaction, followed by recovery in RPMI 1640 for three hours. Apoptosis was then detected using the TUNEL method ($N = 3$).

### Influence of ROS and blocking integrin–ECM interaction on ERK activation

Cells were planted on collagen type IV-coated plastic dishes with serum-free RPMI 1640 medium for 24 h, and then cells were incubated with ROS or anti-$\beta$1-integrin mAb (10 $\mu$g/mL) for 4–6 h. Cells were washed by PBS to stop the reaction. Cell lysates were assessed for ERK activation by Western blotting.

### Confirming the capacity of U0126 to inhibit activation of ERK

ERK activation is known to require phosphorylation by MAPK/ERK kinase (MEK). The MEK inhibitor, U0126 (Calbiochem, San Diego, CA, USA), was used to inhibit ERK activation.[22,23] For confirming the ability of U0126 to depress the activation of ERK (p-ERK/ERK), cells were incubated with U0126 10 $\mu$mol/L (Calbiochem) for 0, 1, 2, 4, 6 and 12 h. Cells were washed by PBS to stop the reaction. Cell lysates were assessed for ERK activation by Western blotting.

### Influence of ERK activation on apoptosis

U0126 at a concentration of 10 $\mu$mol/L was added to cells during the preincubation period of 30 min.[22,23] Dimethyl sulfoxide with an equivalent volume was used as the control. Thereafter, cells were incubated with ROS, anti-integrin $\beta$1 mAb or RGDS for one hour. Cells were rinsed with PBS to stop the reaction. Cells were then incubated for one hour in RPMI 1640 containing U0126. Apoptosis was detected using the TUNEL methods ($N = 3$).

### Statistical analysis

Results are expressed as means $\pm$ SD. One-way analysis of variance was used to test the result of trypan blue exclusion. Statistical comparisons were made using the unpaired *t*-test. *P* values less than 0.05 were considered to be statistically significant.

## Results

### Cell injury with ROS

Cell viability was assessed by trypan blue exclusion. Cell viability was as follows: $93 \pm 4\%$ (xanthine 0 mmol/L), $97 \pm 1\%$ (xanthine 0.01 mmol/L), $87 \pm 3\%$ (xanthine 0.05 mmol/L), $86 \pm 1\%$ (xanthine 0.1 mmol/L), $81 \pm 3\%$ (xanthine 0.25 mmol/L), $85 \pm 3\%$ (xanthine 0.25 mmol/L) and $62 \pm 18\%$ (xanthine 1.0 mmol/L), respectively (Figure 1). Based on these results, an xanthine concentration of 0.05 mmol/L led to the least amount of cell death and was selected to induce cell injury.

### ROS and blocking integrin–ECM interaction induce apoptosis

To evaluate the effect of ROS and blocking integrin-ECM interaction on podocyte apoptosis, cells were treated with ROS or inhibited integrin–ECM interaction by anti-$\beta$1-integrin mAb or RGDS. Apoptosis was detected by the TUNEL method (Figure 2). We found that ROS-treated cells exhibited increased apoptosis (ROS: $13.42 \pm 8.16\%$

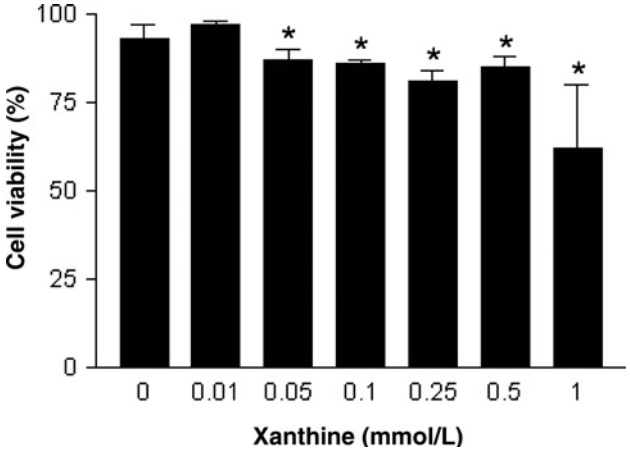


**Figure 1** Cell viability by trypan blue exclusion. After exposure of podocytes to xanthine (0, 0.01, 0.05, 0.1, 0.25, 0.5 and 1 mmol/L, respectively), xanthine oxidase (0.05 U/mL) and ferric chloride (0.2 $\mu$mol/L) as a source of reactive oxygen species, cell death was detected by trypan blue analysis. The 0.05 mmol/L concentration of xanthine caused the minimal necrosis of podocytes. $P < 0.05$ compared with 0 mmol/L xanthine groups

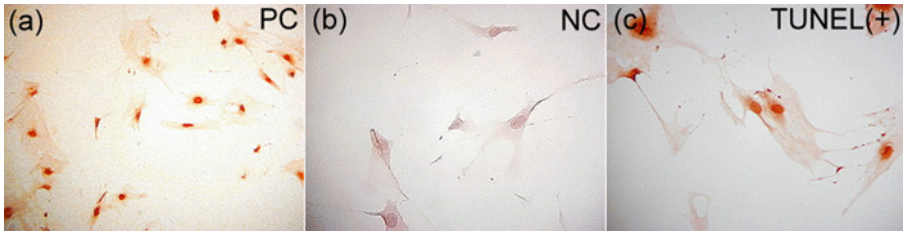


**Figure 2** TUNEL assay for detection of apoptosis. (a) Positive control by DNase I to induce DNA strands breaks in the TUNEL method. Almost all cells had brown color nuclei (TUNEL-positive) (original magnification ×100). (b) and (c) Cells underwent ROS injury. Then the TUNEL method was performed to detect apoptosis. (b) Negative control. Negative control was performed by using Label Solution (without terminal transferase) instead of TUNEL reaction mixture. Nuclei were not brown color. These were TUNEL-negative, no apoptosis (original magnification ×200). (c) Nuclei were of brown color. These were TUNEL-positive, representing apoptosis (original magnification ×200). ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-peroxidase nick end-labeling; PC, positive control; NC, negative control; TUNEL(+), TUNEL positive.

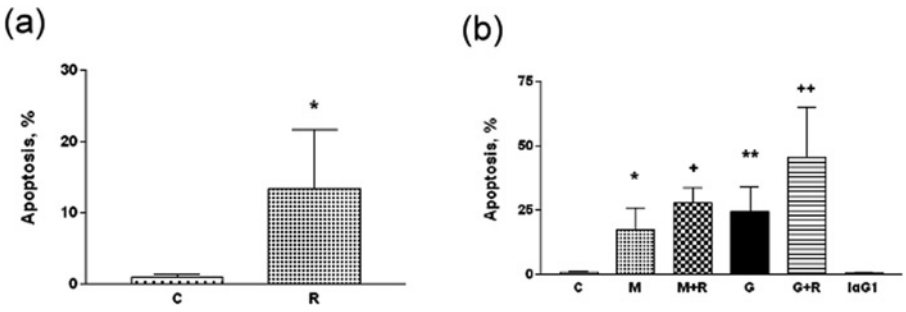


**Figure 3** ROS and blocking integrin function induced apoptosis. (a) Podocytes underwent ROS injury for one hour. Then the TUNEL method was performed to detect apoptosis. Apoptosis increased more significantly in ROS-injured cells than in uninjured control groups. (b) Anti-$\beta$1 integrin mAb and normal mouse IgG1 isotype control antibody, or RGDS, which inhibit competitively integrin–ECM interaction, were used in the incubation of podocytes without or with ROS injury for one hour. Thereafter, the TUNEL method was performed to detect apoptosis. mAb and RGDS greatly increased apoptosis compared with the control groups. mAb and ROS together caused more apoptosis than mAb alone. RGDS and ROS together also caused significantly increased apoptosis compared with RGDS alone. Apoptosis in the control groups and IgG1 groups were the same ($N = 3$). ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-peroxidase nick end-labeling; mAb, monoclonal antibody; RGDS, Arg–Gly–Asp–Ser; C, control; M, anti-$\beta$1 integrin mAb; R, ROS; G, RGDS; M + R, mAb and ROS together; G + R, RGDS and ROS together. $^{*}P < 0.05$ compared with control, $^{**}P < 0.05$ compared with control, $^{+}P < 0.05$ compared with mAb, $^{++}P < 0.05$ compared with RGDS

versus control: $0.91 \pm 0.46\%$, $P < 0.05$) (Figure 3a). We documented that ROS induced podocyte apoptosis.

Blocking integrin–ECM interaction by anti-$\beta$1-integrin mAb or RGDS in the absence of ROS treatment also caused cells to undergo apoptosis (anti-$\beta$1-integrin mAb: $17.40 \pm 8.65\%$ versus control: $0.83 \pm 0.32\%$, $P < 0.05$; RGDS: $24.19 \pm 9.85\%$ versus control: $0.83 \pm 0.32\%$, $P < 0.05$) (Figure 3b). The mouse IgG1 control antibody could not induce cell apoptosis. The addition of anti-$\beta$1-integrin mAb to ROS-treated podocytes enhanced apoptosis (anti-$\beta$1-integrin mAb + ROS: $27.61 \pm 6.08\%$ versus anti-$\beta$1-integrin mAb: $17.40 \pm 6.64\%$, $P < 0.05$) (Figure 3b). The addition of RGDS to ROS-treated podocytes also caused more apoptosis than RGDS alone (RGDS + ROS: $45.36 \pm 19.49\%$ versus RGDS: $24.19 \pm 9.85\%$, $P < 0.05$) (Figure 3b). Therefore, from the above findings, the blocking integrin–ECM interaction induces podocyte apoptosis. ROS treatment and blocking integrin–ECM interaction lead to more severe apoptosis.

## ROS and blocking integrin–ECM interaction lead to ERK activation

To evaluate the effect of ROS and blocking integrin–ECM interaction on ERK activation, cells were incubated with ROS or anti-$\beta$1-integrin mAb (10 $\mu$g/mL) for 4–6 h. Cell lysates were assessed for ERK activation by Western blotting. Western blot showed that ERK activation increased at one, two, four and six hours after ROS treatment. The peak activation was at four hours (Figure 4a). When blocking integrin–ECM interaction by anti-$\beta$1 integrin mAb, ERK activation increased gradually at two and four hours (Figure 4b). From the above results, we documented that ROS or blocking integrin–ECM interaction induced ERK activation.

## Confirming the ability of U0126 to inhibit activation of ERK and decrease apoptosis

To evaluate the effect of U0126 on ERK activation and apoptosis, cells were incubated with U0126 10 $\mu$mol/L. Cell lysates were assessed for ERK activation by Western blotting. Apoptosis was detected by the TUNEL method. The Western blot showed that ERK activation was depressed at one, two and four hours, and returned to its normal level after six hours receiving U0126 treatment (Figure 5a). From the above data, ERK activation can be depressed by U0126.

U0126 decreases apoptosis induced by ROS treatment and blocking integrin–ECM interaction (ROS + U0126: $4.1 \pm 0.6\%$ versus ROS: $11.37 \pm 1.2\%$, $P < 0.05$; anti-$\beta$1 integrin mAb + U0126: $4.46 \pm 1.6\%$ versus anti-$\beta$1 integrin

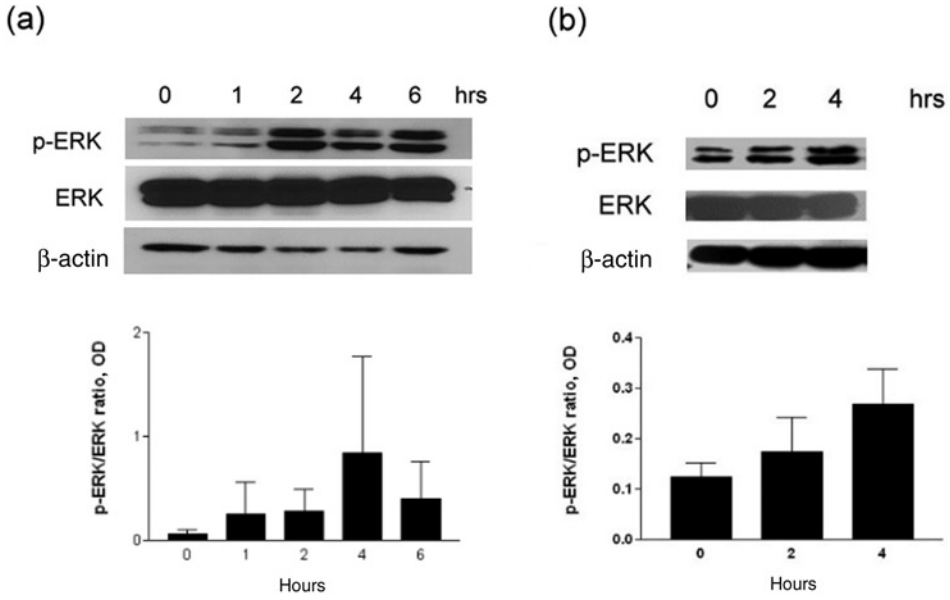


**Figure 4** ROS and blocking integrin function increased ERK activation. Cell lysates were assessed for ERK activation by Western blotting. $\beta$-actin was used as a loading control. (a) Cells were treated with ROS for one, two, four and six hours, respectively. ERK activation (p-ERK/ERK) was increased at one, two, four and six hours after ROS treatment. The maximal increasing ERK activation was at four hours after ROS treatment, and then the activation decreased. (b) Cells were treated with anti-$\beta$1 integrin mAb for two and four hours, respectively. ERK activation (p-ERK/ERK) was increased at two and four hours after blocking integrin $\alpha 3\beta 1$ function. The upper panel is a representative of autoradiography. The lower panel is the mean $\pm$ SD calculated by a densitometer of the ratio in three independent experiments. ROS, reactive oxygen species; ERK, extracellular signal-regulated kinase; mAb, monoclonal antibody

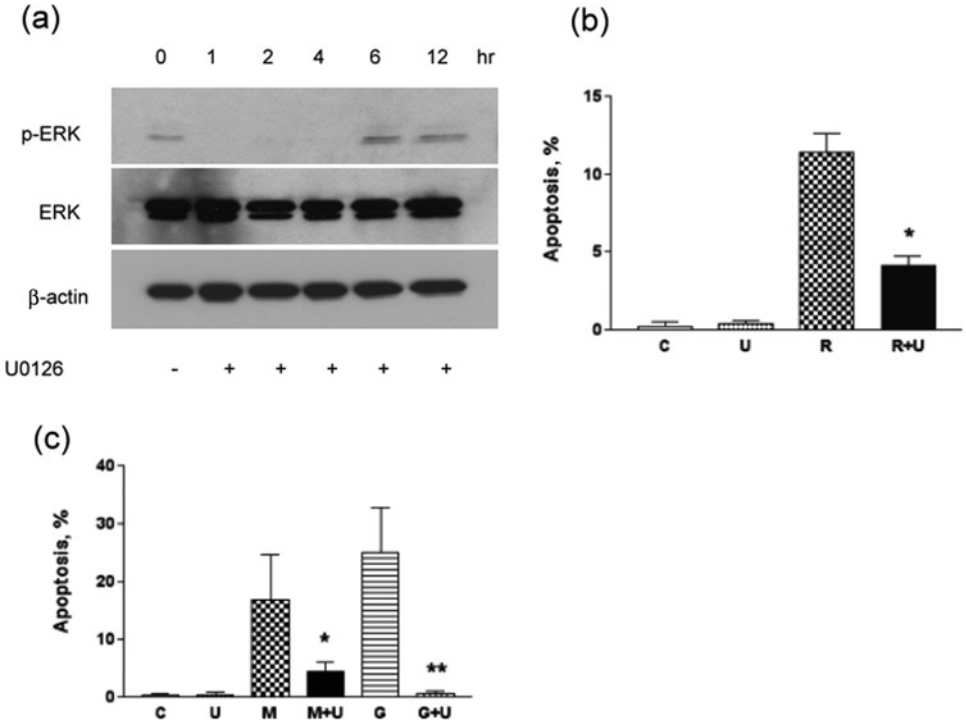


**Figure 5** Confirming the inhibiting effects of U0126 on activation of ERK (p-ERK/ERK), and effects of depressed ERK activation by U0126 on ROS and blocking integrin function-induced apoptosis. (a) Cells were incubated with U0126 (10 $\mu$mol/L) for 0, 1, 2, 4, 6 and 12 h. The reaction was stopped by PBS. Then, cell lysates were assessed for ERK activation by Western blotting. $\beta$-actin was used as a loading control. Activation of ERK was decreased at one, two and four hours, and then returned to normal level after six hours of U0126 treatment. (b and c) Cells were preincubated with U0126 for 30 min. Then cells were treated without and with ROS-mediated injury, or anti-$\beta$1 integrin mAb and RGDS for one hour. The reaction was stopped by PBS. TUNEL method was utilized to detected apoptosis. (b) Apoptosis in the U0126 alone and control groups showed no difference. Apoptosis was significantly decreased in U0126 and ROS together groups compared with ROS alone groups. $^{*}P < 0.05$ compared with ROS alone groups. (c) Apoptosis in U0126 alone and control groups showed no difference. Apoptosis was greatly decreased in mAb and U0126 together groups than in mAb alone groups. Apoptosis was also significantly decreased in RGDS and U0126 together groups than in RGDS alone groups. $^{*}P < 0.05$ compared with mAb groups, $^{**}P < 0.05$ compared with RGDS groups. Three independent experiments were performed. ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; RGDS, Arg–Gly–Asp–Ser; ROS, reactive oxygen species; C, DMSO control; U, U0126; R, ROS; R + U, ROS and U0126 together; M, anti-$\beta$1 integrin mAb; M + U, anti-$\beta$1 integrin mAb and U0126 together; G, RGDS; G + U, RGDS and U0126 together

mAb: $16.73 \pm 7.86\%$, $P < 0.05$; RGDS + U0126: $0.68 \pm 0.37\%$ versus RGDS: $25.05 \pm 7.68\%$, $P < 0.05$) (Figures 5b and c). This documented that up-regulated ERK activation can induce apoptosis.

## Discussion

ROS have been implicated in the pathophysiology of variable renal diseases.[1,2] Resident glomerular cells have been shown to be capable of generating ROS.[1] ROS in general have been shown to act as common mediators of apoptosis.[3,24] However, besides the proapoptotic effect, ROS are also mitogenic and antiapoptotic.[24] It was found that ROS production via the xanthine oxidase pathway might play a major role in PAN-induced nephrosis.[4,25] We reported previously that podocytopenia was present in human and chronic PNA-treated animal models of FSGS.[12] In this study, ROS that were produced by xanthine, xanthine oxidase and ferric chloride led to podocyte apoptosis. It was demonstrated that podocytes lost from the glomeruli after PAN injection were partially due to detached podocytes in Bowman's space and urine, and partially due to TUNEL-positive apoptotic podocytes in the glomeruli.[26] Therefore, podocytopenia might be partially due to ROS-induced apoptosis in our previous report of human and chronic PNA-treated animal models of FSGS.

Integrins have been reported to have either prosurvival or proapoptotic roles in the regulation of cell survival.[27,28] It was reported that in the presence of proapoptotic stimuli, active $\alpha M\beta 2$ resulted in enhanced apoptosis.[27] But, Manohar *et al*.[22] also showed that integrin $\alpha 3\beta 1$-mediated adhesion to laminin-5 promotes keratinocyte cell survival. In this study, we found that blocked integrin $\alpha 3\beta 1$–ECM interaction by anti-integrin $\beta 1$ mAb or RGDS caused podocyte apoptosis. Apoptosis of ROS-treated podocytes was further exacerbated with anti-$\beta 1$ integrin mAb. We have found previously that podocyte apoptosis induced by blocking integrin–ECM interaction is through the Bax and cytocmrome *c* pathways.[13] These observations indicate that integrins have either antiapoptotic or proapoptotic effects that may be due to cell type specificity and integrin subunits. However, integrin $\alpha 3\beta 1$ is antiapoptotic for podocytes. Kojima *et al*.[29] reported that total $\alpha 3$ integrin expression of the glomerular wall was decreased in PAN nephrosis rats. But the total $\alpha 3$ integrin expression of the glomerular wall was not decreased in PAN nephrosis rats treated with phosphatidyl choline-bound superoxide dismutase. They concluded that down-regulated expression of $\alpha 3\beta 1$ integrin in podocytes attributed to ROS and could be linked to an increase in proteinuria in PAN nephrosis rats.[29] In our previous report, we found that $\alpha 3\beta 1$ integrin expression of podocytes and podocyte numbers were decreased in humans and chronic PAN-treated animal models of focal segmental glomerulosclerosis.[12] The immunostaining score of both $\alpha 3$- and $\beta 1$-integrin subunits was negatively correlated with the degree of glomerular sclerosing score and the amount of daily protein loss, and was positively correlated with the number of podocytes.[12] From above reports, we can consider that ROS-induced by PAN causes a decrease in $\alpha 3\beta 1$-integrin expression, which increases ROS-induced apoptosis.

ERK has been centered on multiple signal transduction pathways. Integrins can regulate ERK activation.[11,27] Recently, it has been found that ERK is activated in response to ROS.[24,30] ERK activation has either prosurvival or proapoptotic effects.[24,31] It was found that $H_2O_2$-induced apoptosis of cardiac myocytes is enhanced by MEK inhibition.[32] Activation of the MEK/ERK signaling pathway can promote keratinocyte cell survival.[22] According to these statements, ERK activation has a prosurvival function. In this study, we found that ROS induced ERK activation. This ERK activation increased initially at one hour after ROS treatment, reaching a peak at four hours and then decreased at six hours. Blocking integrin–ECM interaction also increased ERK activation. To further evaluate the relationship between ERK and apoptosis, we used U0126 to inhibit ERK activation. We found that inhibition of ERK activation by U0126 decreased apoptosis induced by ROS and blocking integrin–ECM interaction. This demonstrated that apoptosis was associated with ERK activation in podocytes after ROS and blocking integrin–ECM interaction. A report showed that inhibition of ERK activation abolished the stretch-induced apoptosis of ureteric bud cells.[33] It was also found that estradiol induced apoptosis of vascular smooth muscle cells by ERK activation.[34] In rat myocytes, blockade of ERK activation inhibited S100B-induced, advanced glycation end product-mediated myocyte apoptosis.[35] It was considered that ERK activation-inducing apoptosis may be dependent on the p53 pathway.[35,36] According to the above statements and our results, ERK activation promotes apoptosis.

Therefore, ERK activation may be either an antiapoptotic or proapoptotic effect that depends on the kinds of insults and cell-specific types. In this study, ERK activation that was induced by ROS and blocking integrin–ECM interaction was a proapoptotic effect in podocytes. Further study is needed to evaluate whether ERK activation contributes to p53-dependent apoptosis in podocytes.

In summary, the present study shows that ROS and blocking integrin–ECM interaction induce podocyte apoptosis via ERK activation.

**Author contributions:** All authors participated in designing the study, analyzing the results and writing the manuscript. C-AC and T-SC contributed equally to this work.

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*(Received May 5, 2011, Accepted March 15, 2012)*