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Advanced Glycation End-Products Activate Extracellular Signal-Regulated Kinase Via the Oxidative Stress-EGF Receptor Pathway in Renal Fibroblasts

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ABSTRACT

Advanced glycation end-products (AGEs), epidermal growth factor receptor (EGFR), reactive oxygen species (ROS), and extracellular signal-regulated kinases (ERK) are implicated in diabetic nephropathy (DN). Therefore, we asked if AGEs-induced ERK protein phosphorylation and mitogenesis are dependent on the receptor for AGEs (RAGE)–ROS–EGFR pathway in normal rat kidney interstitial fibroblast (NRK-49F) cells. We found that AGEs (100 μg/ml) activated EGFR and ERK1/2, which was attenuated by RAGE short-hairpin RNA (shRNA). AGEs also increased RAGE protein and intracellular ROS levels while RAGE shRNA and *N*-acetylcysteine (NAC) attenuated AGEs-induced intracellular ROS. Hydrogen peroxide (5–25 μM) increased RAGE protein level while activating both EGFR and ERK1/2. Low-dose hydrogen peroxide (5 μM) increased whereas high-dose hydrogen peroxide (100 μM) decreased mitogenesis at 3 days. AGEs-activated EGFR and ERK1/2 were attenuated by an anti-oxidant (NAC) and an EGFR inhibitor (Iressa). Moreover, AGEs-induced mitogenesis was attenuated by RAGE shRNA, NAC, Iressa, and an ERK1/2 inhibitor (PD98059). In conclusion, it was found that AGEs-induced mitogenesis is dependent on the RAGE–ROS–EGFR–ERK1/2 pathway whereas AGEs-activated ERK1/2 is dependent on the RAGE–ROS–EGFR pathway in NRK-49F cells. J. Cell. Biochem. 109: 38–48, 2010.

KEY WORDS: RENAL INTERSTITIAL FIBROBLASTS; MITOGENESIS; ADVANCED GLYCATION END-PRODUCTS; OXIDATIVE STRESS; EGFR; ERK

The hallmark of diabetic nephropathy (DN) consists of glomerulopathy and tubulointerstitial disease culminating in renal fibrosis (glomerulosclerosis and tubulointerstitial fibrosis) and end-stage renal disease [Chuang and Guh, 2001; Forbes et al., 2007].

Advanced glycation end-products (AGEs) [Thomas et al., 2005], growth factors, and intracellular signal transducers [Chuang and Guh, 2001; Marrero et al., 2006; Forbes et al., 2007; Valko et al., 2007] (e.g., reactive oxygen species [ROS] and extracellular signal-regulated regulated kinases [ERK]) are three key mechanisms implicated in the development of DN whereas renal interstitial fibroblasts are important in the pathogenesis of diabetic tubulointerstitial fibrosis [Ina et al., 2002]. Thus, we studied the effects of AGEs in normal rat kidney interstitial fibroblast (NRK-49F) cells [Chuang and Guh, 2001].

Abbreviations used: AGEs, advanced glycation end-products; EGFR, epidermal growth factor receptor; ROS, reactive oxygen species; ERK, extracellular signal-regulated regulated kinase; FCS, fetal calf serum; DMEM, Dulbecco's Modified Eagle's Medium; NAC, *N*-acetylcysteine; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide.

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AGEs are formed by non-enzymatic glycosylation whereby reducing sugars such as glucose or reactive intermediates such as methylglyoxal are added to lysine or other amino groups of proteins to form adducts [Tan et al., 2007]. For example, N^{ε}-carboxyethyllysine is one of the major AGEs derived from methylglyoxal [Tan et al., 2007]. AGEs bind to various cell surface receptors to induce biologic effects [Tan et al., 2007]. Among them, the receptor for AGEs (RAGE) is responsible for many of AGEs-induced pathologies, including DN [Tan et al., 2007]. Moreover, RAGE activates ROS, ERK, and other signal transduction pathways upon ligand binding [Tan et al., 2007]. For example, we have shown that AGEs-induced mitogenesis in NRK-49F cells is dependent on the RAGE signaling pathways [Huang et al., 2001]. However, the interaction between the AGEs–RAGE system and the growth factors in DN remains poorly understood.

Among the many growth factors implicated in DN [Flyvbjerg, 2000], we were the first to demonstrate the role of the epidermal growth factor (EGF) in DN [Guh et al., 1991], and this finding is corroborated by other studies [Gilbert et al., 1997; Thulesen et al., 1999; Wassef et al., 2004; Benter et al., 2005; Portik-Dobos et al., 2006]. For example, epidermal growth factor receptor (EGFR) has been shown to be activated in experimental DN [Portik-Dobos et al., 2006] while EGFR inhibition attenuates experimental DN [Wassef et al., 2004; Benter et al., 2005]. Moreover, EGF activates the EGFR by autophosphorylating EGFR while activating ERK and other signal transducers in many cells [Singh and Harris, 2005], including renal fibroblasts [Stevens et al., 2007].

Interestingly, AGEs have been shown to phosphorylate EGFR in mesangial cells [Cai et al., 2006]. However, the role of EGFR in AGEs-induced effects in general and in renal fibroblasts in particular remains unknown. Therefore, the objective of this study is to study the role of RAGE and EGFR in AGEs-induced effects in NRK-49F cells. To this end, we asked if AGEs-induced ERK1/2 protein phosphorylation and mitogenesis are dependent on the RAGE–ROS–EGFR pathway in NRK-49F cells.

MATERIALS AND METHODS

REAGENTS

Fetal calf serum (FCS), Dulbecco's Modified Eagle's Medium (DMEM), antibiotics, molecular weight standards, trypsin-EDTA, trypan blue, and other medium additives were obtained from the Invitrogen Corp. (Carlsbad, CA). Anti-EGFR antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-ERK1/2 (p-ERK1/2), ERK1/2 antibodies were obtained from Cell Signaling, Inc. AGEs (N^{ε}-carboxyethyllysine) antibody was obtained from the Cosmo Bio Co., Ltd. (Tokyo, Japan). Protein A/G-coupled agarose beads, p-EGFR antibody, and PD98059 were obtained from the Calbiochem Corp. (San Diego, CA). Methylglyoxal was obtained from the Sigma–Aldrich Co. (St. Louis, MO). Horseradish peroxidase-conjugated donkey anti-goat, goat anti-rabbit, and anti-mouse secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. Enhanced chemiluminescence kits were obtained from the Amersham Corp. (Amersham, UK). Iressa was a gift of AstraZeneca Co. (London, UK). *N*,*N*′-methylenebisacrylamide, acrylamide, sodium dodecyl sulfate, ammonium persulfate, Temed, Tween 20, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), *N*-ethyl-maleimide, and all other chemicals were obtained from the Sigma–Aldrich Co.

CELL CULTURE

NRK-49F cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were grown in culture dishes and maintained in DMEM (5.5 mM D-glucose), supplemented by 100 IU/ml penicillin, 100 μg/ml streptomycin, and 5% FCS in a humidified 5% CO_2 incubator at 37°C. The cells were exposed to serum-free (0.1% FCS) DMEM for 24 h prior to timed exposure to BSA or AGEs in the presence of 5% FCS. In some experiments, cells were pre-treated with *N*-acetylcysteine (NAC) for 1 h. NAC was dissolved in phosphate-buffered saline while the other inhibitors were dissolved in dimethyl sulfoxide.

PREPARATION OF AGEs

Advanced glycation end-products were prepared by incubating 7.2 mg/ml fatty acid-free BSA with 100 mM methylglyoxal in 100 mM phosphate buffer, pH 7.4, for 50 h at 37°C in sterile conditions [Geoffroy et al., 2004]. Control BSA was obtained after the incubation of BSA in the same conditions with the exception of the absence of methylglyoxal. AGEs were eluted on a PD-10 column with DMEM to remove salts and un-reacted carbonyls, and then it was sterilized by filtration and kept at −20°C until used. The content of AGEs was determined spectrofluorometrically with excitation set at 355 nm and emission set at 460 nm [Lin et al., 2008], and was expressed as a percentage of relative fluorescence compared with the control BSA. The average fluorescence for AGEs was 200 arbitrary units compared with 1 arbitrary unit per milligram for BSA.

TRANSIENT TRANSFECTION OF ShRNA PLASMIDS

Transient transfection was performed as described in our previous study [Guh et al., 2003]. RAGE short-hairpin RNA (shRNA) and luciferase shRNA in pLKO.1 plasmids were purchased from the RNAi Consortium at the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). Luciferase shRNA was used as a negative control. Briefly, cells (10^5 cells/well) were plated onto 6-well plates and grown overnight. Transient transfection of the shRNA plasmids was performed by adding 200 ng of RAGE or luciferase shRNA plasmids with 3 μl LipofectAMINE (Invitrogen) into cells according to the manufacturer's instructions.

MEASUREMENT OF INTRACELLULAR ROS

Intracellular ROS was measured by using a fluorescent probe 5,6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCF-DA, Molecular Probes, Inc., Eugene, OR) [Fukami et al., 2004]. Briefly, the cells were plated (5×10^3 cells/well) in 96-well plates. The cells were starved (0.1% FCS) before adding 5% FCS-containing BSA or AGEs for 1–60 min, and then the cells were loaded with 20 μmol/L CM-H_2DCF-DA and incubated for 15 min at 37°C. The cells were washed with PBS and then 30 μl lysis buffer (0.1% Triton X-100, 0.5 mM EDTA in PBS) was added. The cell lysates were centrifuged for 5 min and lysis buffer (95 μl) was added to the supernatant (5 μl). Fluorescence (excitation 495 nm, emission 530 nm) was measured by a spectrofluorometer (FLUOstar galaxy,

BMG Labtech GmbH, Offenburg, Germany) and analyzed by using the FLUOstar galaxy software (ReTiSoft Inc., Toronto, Canada). Intracellular ROS was also measured by using the GSH-Glo Glutathione Assay kit according to the manufacturer's instructions (Promega Corp., Madison, WI). This kit is based on the conversion of a luciferin derivative into luciferin in the presence of glutathione, catalyzed by glutathione *S*-transferase. The signal generated in a coupled reaction with firefly luciferase is proportional to the amount of glutathione present in the sample. Each experimental datum point represents the mean of quadruplicate wells from five independent experiments.

IMMUNOBLOTTING

Immunoblotting was performed as described in our previous studies [Guh et al., 2001]. Briefly, cells (10^5 cells/well) were plated onto 6-well plates and grown overnight. The cells were exposed to serum-free (0.1% FCS) DMEM for 24 h prior to timed exposure to BSA or AGEs in the presence of 5% FCS. The cells were lysed in lysis buffer (50 mM Tris–HCl pH 7.4, 1% NP 40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Na_3VO_4, and 1 mM NaF) for 2 h at 4°C. The total cell lysates (20 μg protein) were harvested, resolved by 8% sodium dodecyl sulfate–poylacrylamide gel, and then transferred to 0.45 μm Protran membranes (Schieicher and Schuell, Keene, NH). The membranes were blocked in a blocking solution and were subsequently probed by primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Protein bands were detected by using the enhanced chemiluminescence system (Amersham Corp.).

MITOGENESIS

Mitogenesis was assessed by ^{3}H-thymidine incorporation as described in our previous study [Huang et al., 1999]. Briefly, 8×10^3 cells were seeded in 24-well microplates, and were maintained in 5% FCS for 1 day. After fasting (0.5% FCS) for 1 day, fresh medium containing BSA or AGEs (with 5% FCS-containing DMEM) was added at various times. ^{3}H-thymidine (4 μCi) (Amersham, Arlington Heights, IL) was added during the last 5 h. The cells were washed and protein was precipitated, cell lysate was harvested to 2 ml of scintillation fluid and counted on a β-counter (Beckman, Palo Alto, CA). The results were plotted as cpm per well. Each experimental datum point represents the mean of quadruplicate wells from five independent experiments.

MEASUREMENT OF APOPTOSIS AND NECROSIS

Cell death was measured by apoptosis and necrosis. Attached cells were harvested by trypsinization and floating cells were collected by centrifugation. The cells were pooled and apoptosis was measured by using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA) by flow cytometry according to the manufacturer's instructions. Early apoptosis is characterized by the binding of annexin V to phosphatidylserine, which is redistributed from the inner to the outer plasma membrane leaflet [Brumatti et al., 2008]. Late apoptosis is characterized by the cellular uptake of propidium iodide [Brumatti et al., 2008]. In this study, apoptosis was defined as either early or late apoptosis.

Briefly, cells (10^5 cells/well) were plated onto 6-well plates and grown overnight. After fasting (0.5% FCS) for 1 day, fresh medium containing BSA, AGEs, or hydrogen peroxide (with 5% FCS-containing DMEM) was added for 3 days. The cells were washed twice with cold PBS and then re-suspend cells in 1× binding buffer at a concentration of 1×10^6 cells/ml. FITC Annexin V (5 μl) and propidium iodide (5 μl) were added and gently vortexed, incubated for 15 min at 25°C in the dark and flow cytometry was performed within 1 h.

Necrosis was measured by the percentage of lactate dehydrogenenase (LDH) release into the media as compared to the total cell lysates [Kim et al., 2009] by using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega Corp.) according to the manufacturer's instructions. Briefly, the cells (10^5 cells/well) were plated onto 6-well plates and grown overnight. The cells were fasted (0.5% FCS) for 1 day and BSA, AGEs, or hydrogen peroxide (with 5% FCS-containing DMEM) was added for three more days before the measurement of absorbance at 490 nm. For the apoptosis and necrosis assays, each experimental datum point represents the mean of quadruplicate wells from five independent experiments.

STATISTICS

The results were expressed as the mean ± standard errors of the mean (SEM). Unpaired Student's *t*-tests were used to compare the two groups. A one-way analysis of variance followed by unpaired Student's *t*-test was used to compare among more than three groups. A *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

TIME- AND DOSE-DEPENDENT EFFECTS OF AGEs ON EGFR PROTEIN PHOSPHORYLATION IN NRK-49F CELLS

The diabetic milieu is characterized by a plethora of growth factors and cytokines [Flyvbjerg, 2000; Chuang and Guh, 2001; Forbes et al., 2007]. Thus, any given cell must integrate all these sources of information to respond to any given stimulus such as AGEs [Rodland et al., 2008]. An important example of information integration is the ability of multiple stimuli to activate the EGFR signaling pathway [Higashiyama et al., 2008; Rodland et al., 2008]. Activation of EGFR by its natural ligands or transactivation of EGFR by other agents is characterized by EGFR protein phosphorylation [Melenhorst et al., 2008; Rodland et al., 2008]. Thus, to determine the effects of AGEs on EGFR activation, phosphorylation of EGFR protein was measured by immunoblotting. We found that the AGEs time (5 min–1 h)-dependently (Fig. 1A) and dose (100–300 μg/ml)-dependently (Fig. 1B) increased EGFR protein phosphorylation. Note that FCS or the culture media does not contain EGF [Zheng et al., 2006].

TIME- AND DOSE-DEPENDENT EFFECTS OF AGEs ON ERK1/2 PROTEIN PHOSPHORYLATION

AGEs have been shown to activate (phosphorylate) ERK1/2 protein [Tan et al., 2007]. Thus, to determine the effects of AGEs on ERK1/2 activation, the ERK1/2 protein phosphorylation was measured by immunoblotting. As shown in Figure 1C, the AGEs (100 μg/ml) only transiently increased ERK1/2 protein phosphorylation at 15 min, which is later than the onset of EGFR phosphorylation. Moreover, AGEs dose-dependently (50–300 μg/ml, maximal at 200 μg/ml)

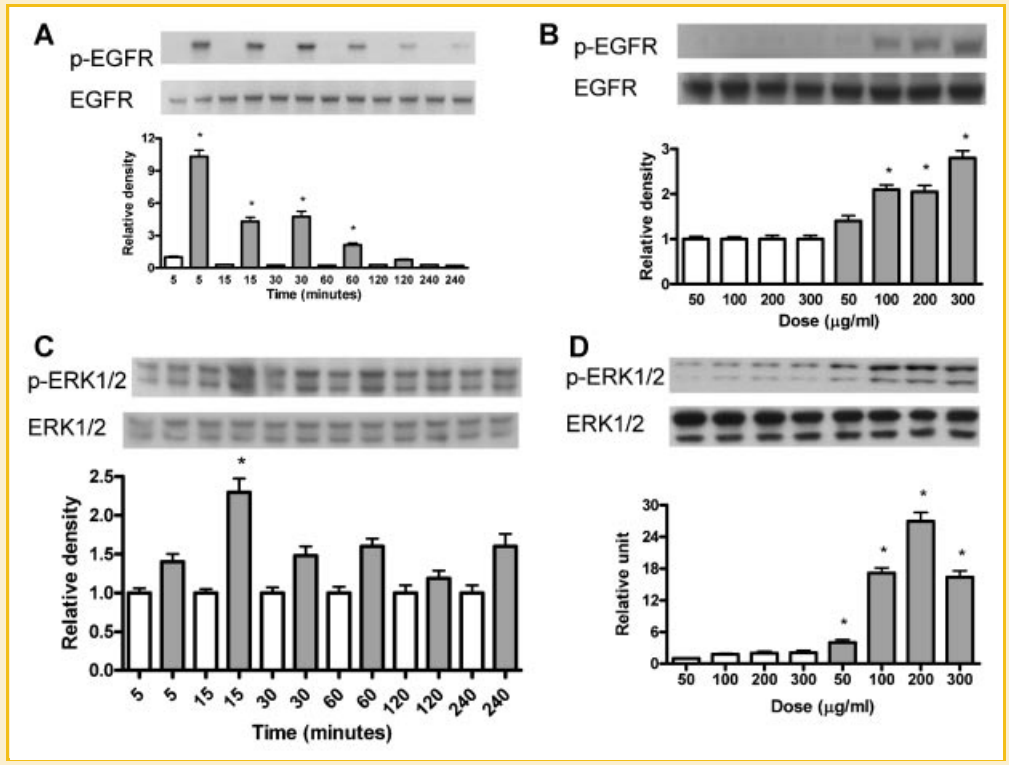

Fig. 1. Effects of AGEs on phosphorylation of EGFR and ERK1/2 protein in NRK-49F cells. Cells (8×10^3/well) were treated with control BSA (100 μg/ml, empty bars) or AGEs (100 μg/ml, gray bars) for 5 min–4 h (A and C). Dose-dependent (50–300 μg/ml) effects of AGEs were studied at 5 min (B) or 15 min (D). Phosphorylation of EGFR (p-EGFR, A and B) and ERK1/2 protein (p-ERK1/2, C and D) was measured by immunoblotting. Relative density was expressed as the ratio between p-EGFR and EGFR or between p-ERK1/2 and ERK1/2. The results were expressed as the mean ± SEM of five independent experiments. $^*P < 0.05$ versus BSA.

increased ERK1/2 protein phosphorylation (Fig. 1D). Because EGFR, which has tyrosine kinase activity, activates ERK1/2 upon ligand binding or transactivation [Singh and Harris, 2005; Melenhorst et al., 2008], our finding that AGEs phosphorylate ERK1/2 later than EGFR is compatible with the notion that AGEs-induced ERK1/2 protein phosphorylation is downstream of EGFR transactivation.

ROLE OF RAGE IN AGEs-INDUCED PHOSPHORYLATION OF EGFR AND ERK1/2 PROTEIN

To determine the role of RAGE in AGEs-induced phosphorylation of EGFR and ERK1/2 protein, we inhibited RAGE protein expression by shRNA and measured phosphorylation of EGFR and ERK1/2 protein by immunoblotting. We found that RAGE shRNA (but not the control luciferase shRNA) attenuated AGEs-increased RAGE protein expression at 4 h (Fig. 2A). Moreover, RAGE shRNA (but not luciferase shRNA) attenuated AGEs-induced EGFR protein phosphorylation at 5 min (Fig. 2B) and attenuated AGEs-induced ERK1/2 protein phosphorylation at 15 min (Fig. 2C).

ROLE OF EGFR IN AGEs-INDUCED MITOGENESIS AND PHOSPHORYLATION OF ERK1/2 PROTEIN

As shown in Figure 7, Iressa (1 μM) attenuated AGEs-induced mitogenesis at 3 days. To determine the role of EGFR in AGEs-induced phosphorylation of ERK1/2 protein, phosphorylation of ERK1/2 protein was measured by immunoblotting. As shown in Figure 3A, AGEs (100 μg/ml)-induced EGFR protein phosphorylation was attenuated by Iressa (1 μM) at 5 min. Moreover, AGEs-induced ERK1/2 protein phosphorylation was attenuated by Iressa (1 μM) at 15 min (Fig. 3B). Thus, AGEs-induced mitogenesis and ERK1/2 activation are dependent on EGFR.

TIME-DEPENDENT EFFECTS OF AGEs ON INTRACELLULAR ROS LEVEL

Upon ligand binding, generation of intracellular ROS is a major mechanism of many of the RAGE-induced effects [Tan et al., 2007]. To determine time-dependent effects of AGEs on intracellular ROS generation, ROS level was detected by using the fluorescent probe CM-H_2DCF-DA and the GSH-Glo Glutathione Assay at 1–60 min. Note that DCF-DA is the most commonly used biomarker for methylglyoxal-induced ROS [Kalapos, 2008], albeit with some controversies [Wardman, 2007]. Thus, glutathione, indispensable for the metabolic degradation of methylglyoxal [Vander Jagt et al., 2001; Kalapos, 2008], has been recommended as a supplement test to detect methylglyoxal-induced ROS [Kalapos, 2008]. We found that AGEs (100 μg/ml) time-dependently (5–60 min) increased intracellular ROS level measured by CM-H_2DCF-DA (Fig. 4A) or the GSH-Glo Glutathione Assay (Fig. 4B). To determine the role of

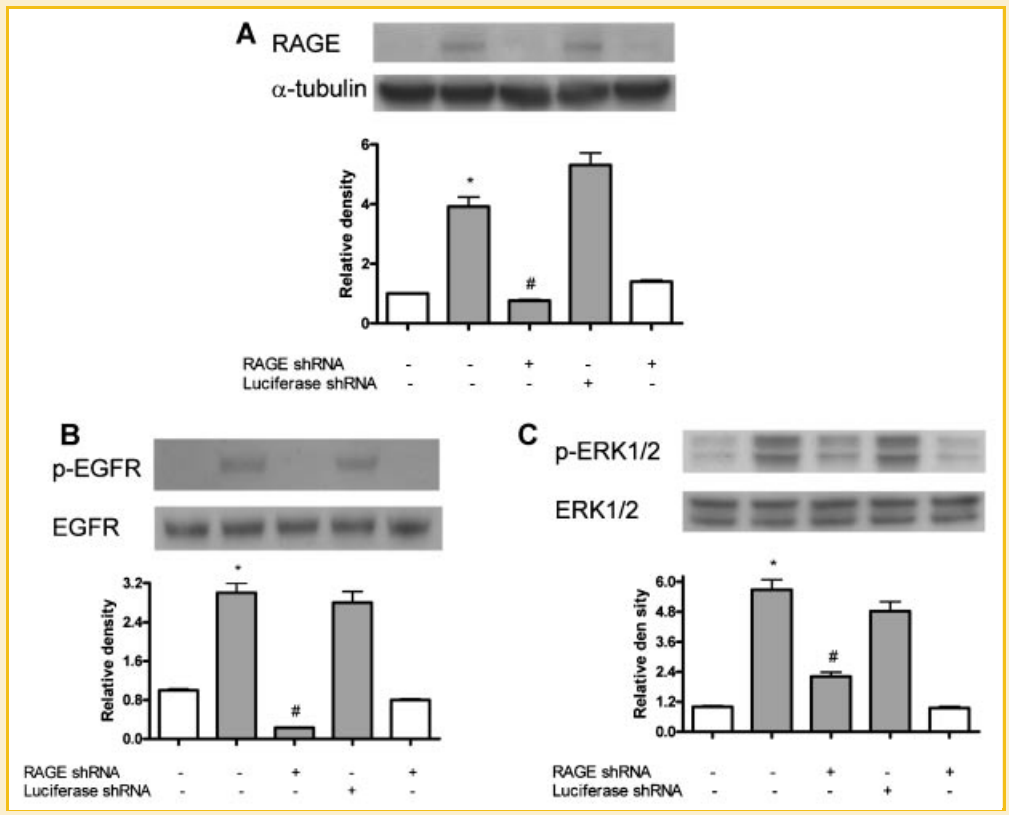

Fig. 2. Role of RAGE in AGEs-induced phosphorylation of EGFR and ERK1/2 protein in NRK-49F cells. Cells (8×10^3/well) were treated with control BSA (100 μg/ml, empty bars) or AGEs (100 μg/ml, gray bars) for 4 h (A), 5 min (B), and 15 min (C). Expression of RAGE protein (A) and phosphorylation of EGFR (p-EGFR, B) and ERK1/2 protein (p-ERK1/2, C) were measured by immunoblotting. Transient transfection of the RAGE shRNA or the control (luciferase) shRNA plasmids was performed as described in Materials and Methods Section. Relative density was expressed as the ratio between p-EGFR and EGFR or between p-ERK1/2 and ERK1/2. The results were expressed as the mean ± SEM of five independent experiments. $^{*}P < 0.05$ versus BSA. $^{\#}P < 0.05$ versus AGEs.

RAGE in AGEs-induced intracellular ROS generation, RAGE shRNA was used to inhibit RAGE protein expression. RAGE shRNA (but not luciferase shRNA) and NAC attenuated AGEs-induced intracellular ROS level at 5–60 min (Fig. 4B). Thus, AGEs-induced intracellular ROS is dependent on RAGE.

DOSE-DEPENDENT EFFECTS OF HYDROGEN PEROXIDE ON EGFR AND ERK1/2 PROTEIN PHOSPHORYLATION AND RAGE PROTEIN EXPRESSION

Phosphorylation of EGFR and ERK1/2 protein and RAGE protein expression were measured by immunoblotting. We found that

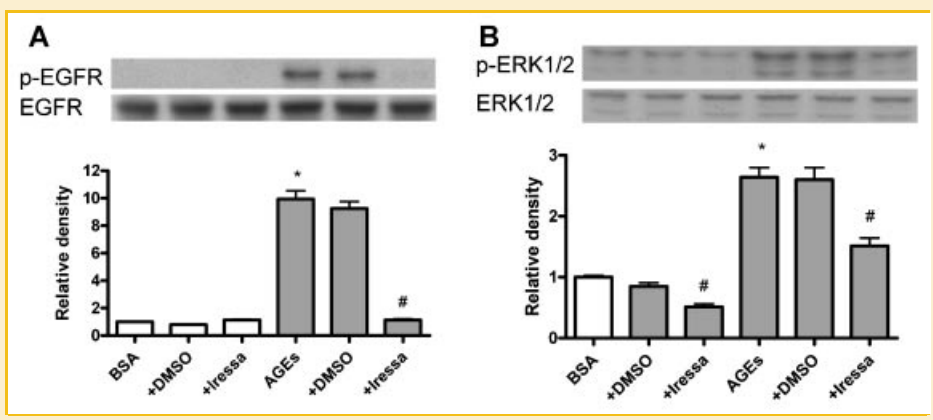

Fig. 3. Role of EGFR in AGEs-induced ERK1/2 protein phosphorylation in NRK-49F cells. Cells (8×10^3/well) were treated with control BSA (100 μg/ml, empty bars) or AGEs (100 μg/ml, gray bars) for 5 min (A) and 15 min (B). Phosphorylation of EGFR (A) and ERK1/2 protein (B) was measured by immunoblotting. Iressa (1 μM in 0.1% DMSO) was pre-treated for 1 h. Relative density was expressed as the ratio between p-EGFR and EGFR or between p-ERK1/2 and ERK1/2. The results were expressed as the mean ± SEM of five independent experiments. $^{*}P < 0.05$ versus BSA. $^{\#}P < 0.05$ versus BSA or AGEs.

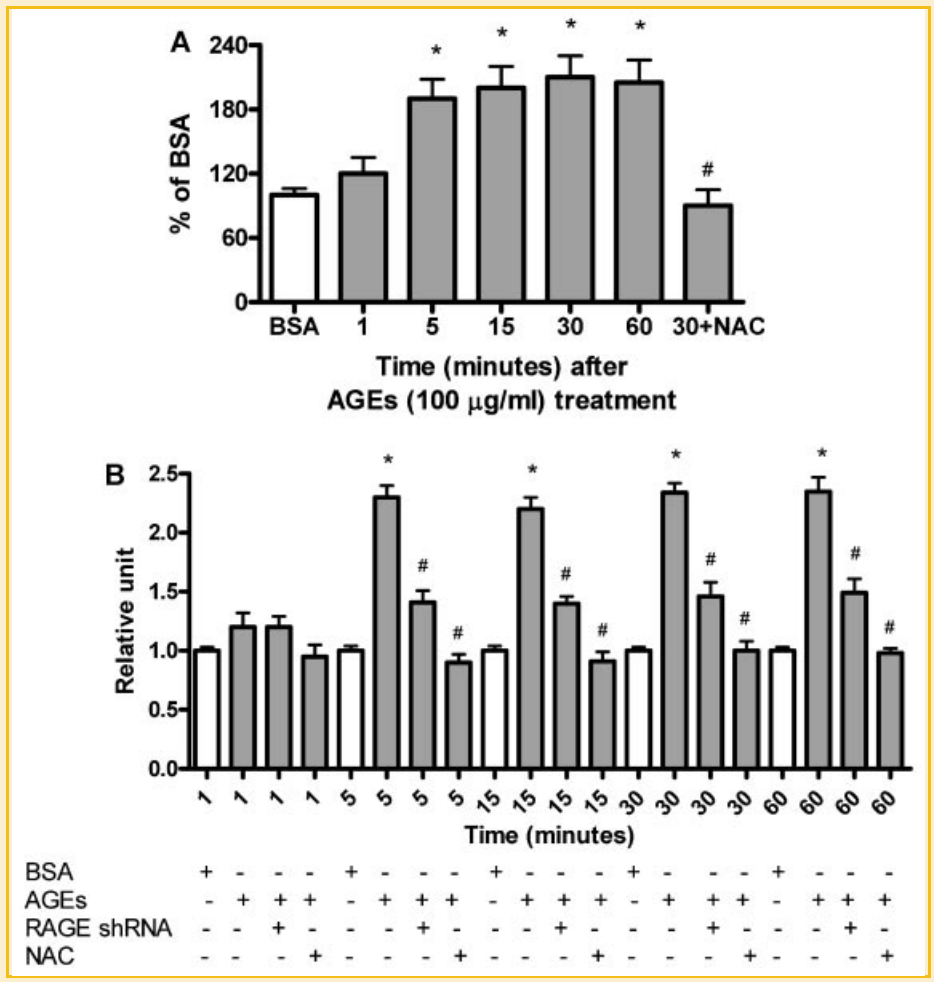

Fig. 4. Time-dependent effects of AGEs on intracellular ROS in NRK-49F cells. Cells (8×10^3/well) were treated with control BSA (100 μg/ml, empty bars) or AGEs (100 μg/ml, gray bars) for 1–60 min. Intracellular ROS was measured by using the fluorescent probe CM-H2DCF-DA (A) or the GSH-Glo Glutathione Assay kit (B). Transient transfection of the RAGE shRNA or the control (luciferase) shRNA plasmids was performed as described in Materials and Methods Section. *N*-acetylcysteine (NAC, 10 mM) was pre-treated for 1 h. The results were expressed as the mean ± SEM of five independent experiments. $^{*}P < 0.05$ versus BSA at each corresponding time. $^{\#}P < 0.05$ versus AGEs alone at each corresponding time.

hydrogen peroxide dose-dependently (5–25 μM) increased the phosphorylation of EGFR at 5 min (Fig. 5A) and increased the phosphorylation of ERK1/2 protein (Fig. 5B) at 15 min. Moreover, hydrogen peroxide dose-dependently (5–25 μM) increased RAGE protein expression at 4 h (Fig. 5C).

ROLE OF ROS IN AGEs-INDUCED MITOGENESIS AND PHOSPHORYLATION OF EGFR AND ERK1/2 PROTEIN

To determine the effects of ROS on mitogenesis [Chiarugi and Fiaschi, 2007; Trachootham et al., 2008], mitogenesis was measured by ^{3}H-thymidine incorporation. We found that low-dose hydrogen peroxide (5 μM) increased whereas high-dose hydrogen peroxide (100 μM) decreased mitogenesis at 3 days (Fig. 7).

The above results show that exogenous ROS activates ERK1/2 protein whereas low-dose and high-dose ROS have contradictory effects on mitogenesis. Thus, it is interesting to elucidate the role of AGEs-induced ROS in AGEs-induced activation of ERK1/2 and mitogenesis. To this end, phosphorylation of EGFR and ERK1/2 protein was measured by immunoblotting. We found that NAC attenuated AGEs-induced mitogenesis (Fig. 7) and phosphorylation of EGFR (Fig. 6A) at 5 min and ERK1/2 protein at 15 min (Fig. 6B). Thus, AGEs-induced mitogenesis and phosphorylation of EGFR and ERK1/2 protein are dependent on AGEs-induced ROS.

ROLE OF ERK1/2 IN AGEs-INDUCED MITOGENESIS

As shown in Figure 7, AGEs increased cell mitogenesis. Becuase the EGFR–ERK1/2 pathway is involved in the regulation of mitogenesis [Melenhorst et al., 2008], we have to determine the role of ERK1/2 in AGEs-induced mitogenesis. To this end, mitogenesis was measured by ^{3}H-thymidine incorporation and the role of ERK1/2 was assessed by PD98059. As shown in Figure 7, PD98059 (20 μM) attenuated AGEs (100 μg/ml)-induced mitogenesis at 3 days. Thus, AGEs-induced mitogenesis is dependent on ERK1/2.

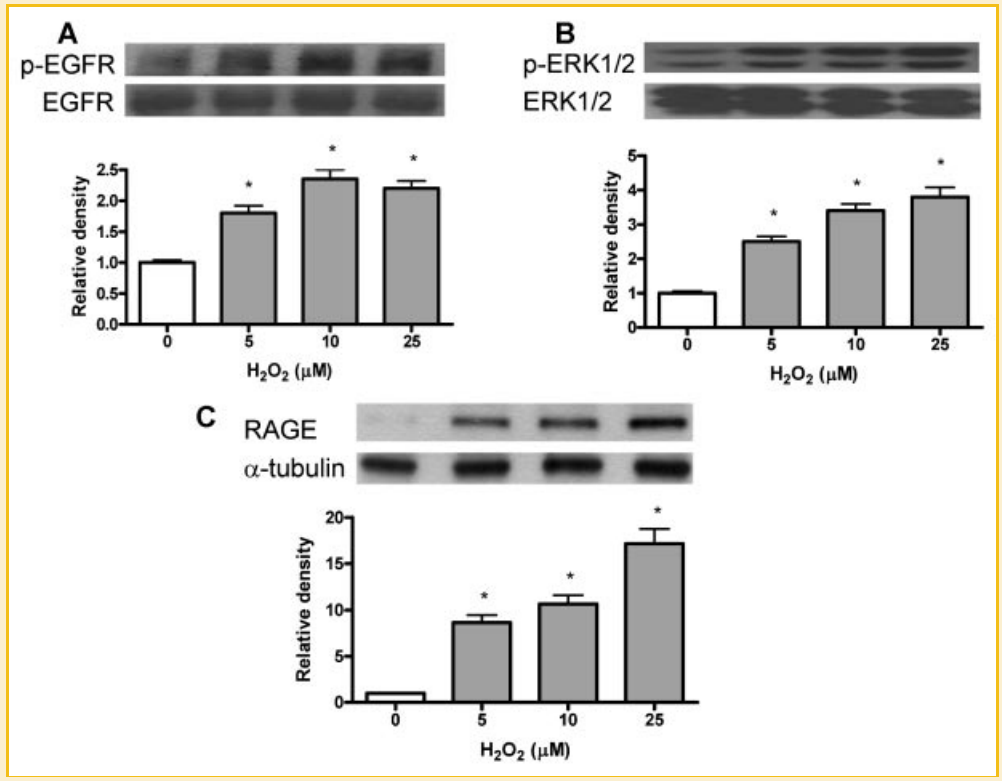

Fig. 5. Dose-dependent effects of H_2O_2 on EGFR and ERK1/2 protein phosphorylation and RAGE protein expression in NRK-49F cells. Cells (8×10^3/well) were treated with hydrogen peroxide (5–25 μM, gray bars) or not (empty bar) for 5 min (A), 15 min (B), and 4 h (C). The EGFR protein, EGFR protein phosphorylation (A), ERK1/2 protein, ERK1/2 protein phosphorylation (B), and RAGE protein (C) were measured by immunoblotting. Relative density was expressed as the ratio between p-EGFR and EGFR, between p-ERK1/2 and ERK1/2 or between RAGE and α-tubulin. The results were expressed as the mean ± SEM of five independent experiments. $^*P < 0.05$ versus lane 1.

To determine the effects of AGEs and ROS on cell death, apoptosis was measured by flow cytometry and necrosis was measured by the release of LDH into the culture media. We found that AGEs did not increase apoptosis until it reached a dose of 300 μg/ml (Fig. 8A), and that hydrogen peroxide increased apoptosis at a dose of 5 μM (Fig. 8A). In contrast, AGEs (50–300 μg/ml) and low-dose hydrogen peroxide (5 μM) did not increase necrosis. Note that the effects of high-dose hydrogen peroxide (25–100 μM) on apoptosis cannot be measured because more than 80% of the cells were necrotic in this case (Fig. 8B).

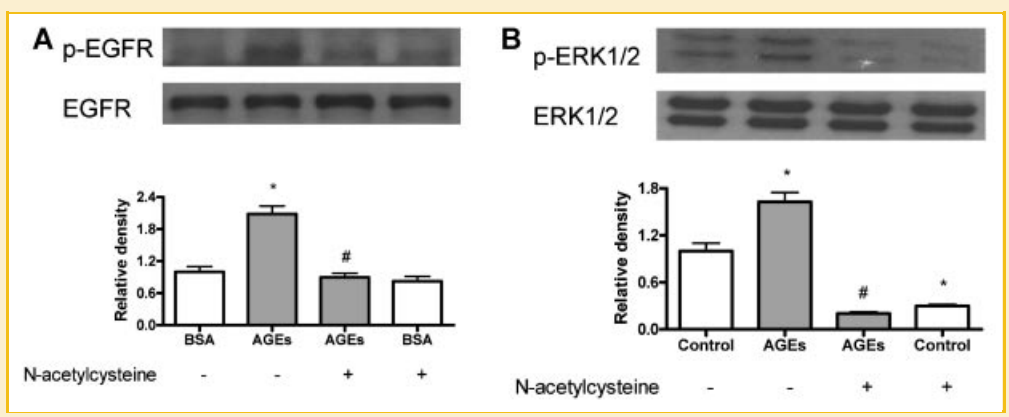

Fig. 6. Role of ROS in AGEs-induced phosphorylation of EGFR and ERK1/2 protein in NRK-49F cells. Cells (8×10^3/well) were treated with control BSA (100 μg/ml, empty bars) or AGEs (100 μg/ml, gray bars) for 5 min (A) and 15 min (B). *N*-acetylcysteine (NAC, 10 mM) was pre-treated for 1 h. Phosphorylation of EGFR (A) and ERK1/2 protein (B) was measured by immunoblotting. Relative density was expressed as the ratio between p-EGFR and EGFR or between p-ERK1/2 and ERK1/2. The results were expressed as the mean ± SEM of five independent experiments. $^*P < 0.05$ versus lane 1. $^\#P < 0.05$ versus lane 2.

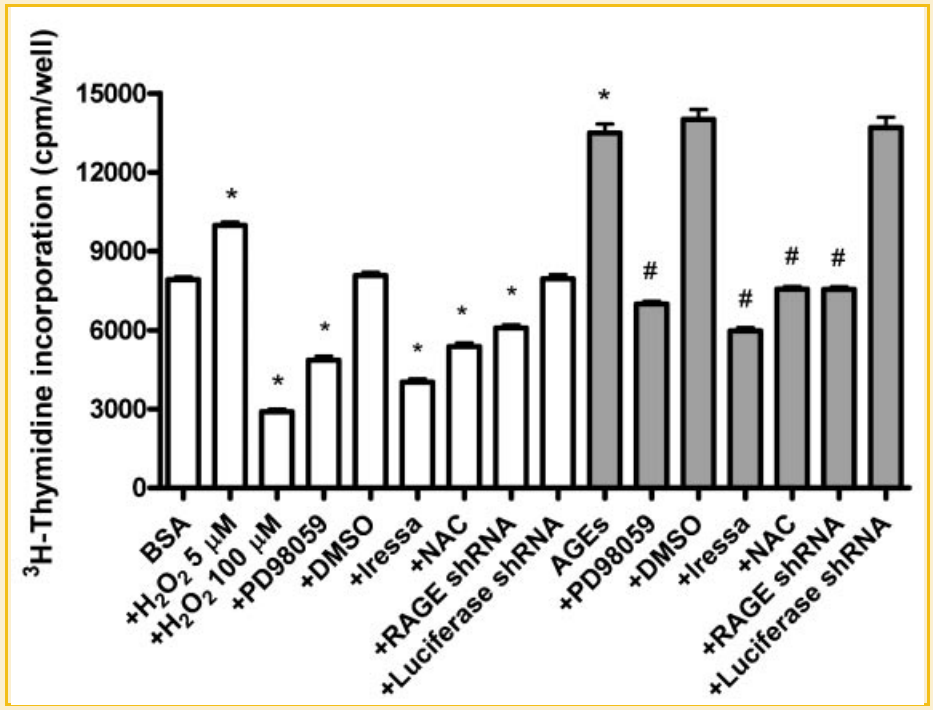

Fig. 7. Role of EGF, ERK1/2, and ROS in AGEs-induced mitogenesis in NRK-49F cells. Cells (8×10^3/well) were treated with control BSA (100 μg/ml, empty bars), AGEs (100 μg/ml, gray bars), or hydrogen peroxide (5 or 100 μM, empty bars) for 3 days. Mitogenesis was measured by ^{3}H-thymidine incorporation into DNA. PD98059 (20 μM in 0.1% DMSO), Iressa (1 μM in 0.1% DMSO), and *N*-acetylcysteine (10 mM) were pre-treated for 1 h. The results were expressed as the mean ± SEM of five independent experiments. $^*P < 0.05$ versus BSA. $^\#P < 0.05$ versus AGEs.

DISCUSSION

We found that AGEs activate EGFR and ERK1/2 by inducing ROS in NRK-49F cells. Moreover, AGEs-induced ERK1/2 protein phosphorylation and mitogenesis are dependent on the RAGE–ROS–EGFR pathway in NRK-49F cells.

We prepared AGEs from 100 mM methylglyoxal whereas plasma methylglyoxal level is only 0.74 μM in the diabetic patients [Nemet et al., 2005]. However, AGEs are concentrated in the kidney [Miyata et al., 1998] such that a previous study also used 100 mM methylglyoxal-derived AGEs at a dose of 0.25–10 μM to treat mesangial cells [Geoffroy et al., 2004]. Similarly, we used methylglyoxal-derived AGEs at a dose of 100 μg/ml (1.5 μM). Because the content of N^ε-carboxyethyllysine (one of the major AGEs product of methylglyoxal) in 100 mM methylglyoxal-derived AGEs is 0.78 mol/mol of protein [Ahmed and Thornalley, 2002], the content of N^ε-carboxyethyllysine in 1.5 μM of methylglyoxal-derived AGEs is 1.2 μM, which is very similar to plasma N^ε-carboxyethyllysine level (1.2–1.7 μM) [Lieuw et al., 2004] in the diabetic patients.

AGEs mediate effects via cell surface receptors including RAGE, macrophage scavenger receptor types I and II, oligosaccharyl transferase-48 (AGE-R1), 80K-H phosphoprotein (AGE-R2), galectin-3 (AGE-R3), CD-36,4 and ezrin, radixin, and moesin proteins [Tan et al., 2007]. For example, RAGE has been shown to be present in fibroblasts [Owen et al., 1998; Hou et al., 2002]. In this study, AGEs increased RAGE protein level and AGEs-induced mitogenesis and phosphorylation of ERK1/2 protein are dependent on RAGE. These findings are compatible with the notion that the AGEs–RAGE system plays a key role in the pathogenesis of DN [Tan et al., 2007].

Because EGFR can be transactivated by many agents, including G-protein coupled-receptor ligands, growth factors, and cytokines [Higashiyama et al., 2008; Rodland et al., 2008], we next studied the effects of AGEs on the EGFR pathway to find that EGFR was transactivated by AGEs via RAGE. This finding is similar to a previous study which shows that AGEs transactivate EGFR in mesangial cells [Cai et al., 2006]. In this regard, EGFR can be transactivated by many other agents beyond the natural EGFR ligands, including G-protein coupled-receptor ligands, growth factors, and cytokines [Higashiyama et al., 2008; Rodland et al., 2008].

The role of AGEs-activated EGFR was studied by Iresssa, and it was found that AGEs-induced ERK1/2 protein phosphorylation and mitogenesis are dependent on EGFR. Moreover, AGEs-activated ERK1/2 is dependent on EGFR. These findings are corroborated by previous studies which show that AGEs-activated ERK1/2 is dependent on EGFR in some cells [Singh and Harris, 2005; Cai et al., 2006] and that some of the AGEs-induced effects are dependent on EGFR in fibroblasts [Owen et al., 1998]. Given that EGF-induced mitogenesis is dependent on ERK1/2 [Chen et al., 2006], our finding that AGEs-induced mitogenesis is dependent on ERK1/2 supports the notion that AGEs-induced mitogenesis is dependent on the EGFR–ERK1/2 pathway.

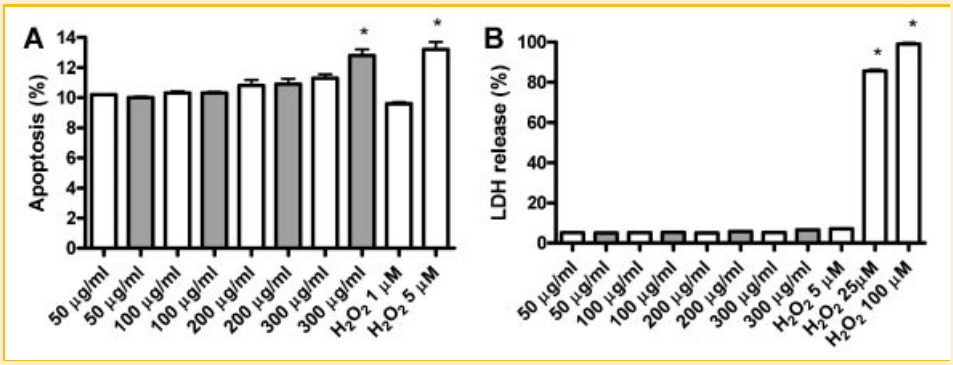

Fig. 8. Effects of AGEs and ROS on apoptosis and necrosis in NRK-49F cells. Cells (8×10^3/well) were treated with control BSA (50–300 μg/ml, empty bars), AGEs (50–300 μg/ml, gray bars), or hydrogen peroxide (empty bars) for 3 days. A: Apoptosis was measured by using the FITC Annexin V Apoptosis Detection Kit by flow cytometry. B: Necrosis was measured by the percentage of lactate dehydrogenenase (LDH) release into the media as compared to the total cell lysates. The results were expressed as the mean ± SEM of five independent experiments. $^{*}P < 0.05$ versus lane 1. $^{\#}P < 0.05$ versus lane 5.

The mechanism by which the AGEs–RAGE system activates EGFR and ERK1/2 is not known because RAGE does not have autophosphorylation sites or specific kinase activity [Cai et al., 2006]. Protein–protein interaction of RAGE and EGFR is an interesting possibility, although a previous study failed to find the interaction between RAGE and EGFR [Oda et al., 2005]. In this study, we found that the AGEs–RAGE system increased intracellular ROS level, which is required for AGEs-induced activation of EGFR and ERK1/2. Moreover, exogenous hydrogen peroxide activated EGFR. These findings are corroborated by previous studies which show that AGEs-induced ROS activates EGFR in mesangial cells [Cai et al., 2006] and that endiothelin-1-induced EGFR activation is dependent on ROS in fibroblasts [Chen et al., 2006].

The mechanism whereby AGEs-induced ROS activates EGFR may be mediated by the oxidation of protein tyrosine phosphatases (PTPs), which inhibit tyrosine kinase activities [Chiarugi and Fiaschi, 2007; Juarez et al., 2008; Monteiro et al., 2008]. Thus, EGFR can be activated by ROS-induced oxidation and inhibition of PTPs [Chiarugi and Fiaschi, 2007; Juarez et al., 2008]. For example, EGFR is activated by ROS-induced oxidation and inhibition of PTPs in endiothelin-1-treated fibroblasts [Chen et al., 2006]. However, there are many other mechanisms transactivating EGFR, such as the shedding of the cell surface EGFR ligands [Higashiyama et al., 2008; Rodland et al., 2008].

We found that low-dose (5 μM) hydrogen peroxide increased mitogenesis and apoptosis. However, high-dose (25–100 μM) hydrogen peroxide decreased cell mitognesis while increasing necrosis. These findings are compatible with current understandings [Trachootham et al., 2008]. In this regard, a previous study found that high-dose hydrogen peroxide (1,000 μM) decreases mitogenesis in cardiac fibroblasts [Colston et al., 2004]. Low-dose hydrogen peroxide also activates EGFR and ERK1/2. This finding is corroborated by a previous study which shows that hydrogen peroxide activates ERK1/2 in fibroblasts [Purdom and Chen, 2005]. Moreover, a previous study found that high-dose hydrogen peroxide (5–100 mM) decreases EGFR internalization in fibroblasts [de Wit et al., 2000]. Interestingly, we found that hydrogen peroxide increased RAGE protein expression. This finding is corroborated by two previous studies which show that anti-oxidants attenuate AGEs-induced RAGE protein expression [Thallas-Bonke et al., 2008; Huang et al., 2009].

We concluded that AGEs-induced mitogenesis is dependent on the RAGE–ROS–EGFR–ERK1/2 pathway whereas AGEs-activated ERK1/2 is dependent on the RAGE–ROS–EGFR pathway in NRK-49F cells.

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