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Amino acids injure mesangial cells by advanced glycation end products, oxidative stress, and protein kinase C

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Amino acids injure mesangial cells by advanced glycation end products, oxidative stress, and protein kinase C.

Background. In diabetes, high intake of dietary protein exacerbates responses associated with kidney damage. Increased levels of amino acids could injure cells by providing free amino groups for glycation reactions leading to advanced glycation end products (AGEs).

Methods. Rat mesangial cells were cultured with increased amino acids designed to resemble protein feeding, high glucose (30.5 mmol/L), and, the combination, amino acids/high glucose. AGEs, reactive oxygen species (ROS), protein kinase C (PKC) activity and production, and mitogen-activated protein (MAP) kinase-extracellular signal regulated kinase (ERK) 1,2 activity were measured. Inhibitors were used to determine roles of these processes in fibrosis and/or AGE formation.

Results. AGE immunostaining increased when cells were cultured in amino acids and was comparable to that observed with high glucose. In amino acids/high glucose, AGE immunostaining appeared even greater. Amino acids, high glucose, and amino acids/high glucose induced ROS production. Aminoguanidine and vitamin E prevented AGE accumulation and induction of protein and mRNA for fibrosis markers [transforming growth factor-β1 (TGF-β1), fibronectin, and collagen IV]. PKC and ERK 1,2 activity increased with amino acids, high glucose, and amino acids/high glucose. PKC-β inhibition prevented ERK 1,2 activation and fibrosis induction. ERK 1,2 inhibition also blocked the fibrosis response.

Conclusion. A profibrotic injury response occurred in mesangial cells exposed to amino acids, with or without high glucose, by formation of AGE, oxidative stress, and activation of the PKC-β and MAP kinase-ERK 1,2 signal pathway. These observations provide new insight into cellular mechanisms of kidney damage produced by excess dietary protein, particularly in diabetes.

Key words: diabetic nephropathy, high protein diet, hyperglycemia, mitogen-activated protein kinases, fibrosis.

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Diabetes is the leading cause of chronic kidney disease and end-stage renal disease in the developed world [1, 2]. Nutritional strategies are particularly challenging due to the complexity of controlling glycemia and other considerations, including kidney disease risk. The diabetic kidney has enhanced sensitivity to effects of dietary protein. In population-based studies, higher intake of dietary protein is associated with increased risk of kidney damage in people with diabetes and hypertension [3]. Conversely, in meta-analyses of clinical trials, protective effects of dietary protein restriction are more apparent for diabetic nephropathy than for other kidney diseases [4, 5]. Currently, the effects of dietary protein on the kidney are especially germane due to the popularity of high protein diets for weight loss and/or control of hyperglycemia [6–9]. In experimental models, high protein diets cause glomerular hyperfiltration and hypertension, and kidney damage, even more so in diabetic than in nondiabetic rats [10, 11]. Similarly, in human studies, patients with type 1 or type 2 diabetes have an augmented glomerular hyperfiltration response to amino acid infusion that can be corrected by strict glycemic control [12–14]. The mechanisms are unknown for the renal interactions between hyperglycemia and increased dietary protein or amino acids.

In order to better understand the unique sensitivity of the diabetic kidney to dietary protein, we have recently studied cellular mechanisms of glomerular injury induced by amino acids, with or without high glucose. The mesangial cell is a key cell involved in glomerular hemodynamics, as well as the fibrotic and proliferative mesangial expansion typical of diabetes. In mesangial cell culture, an amino acid mixture, similar to the profile observed in the circulation after a protein meal, induced a number of responses associated with injury: production and activation of transforming growth factor-β (TGF-β), increased matrix proteins, cellular proliferation, increased expression of the angiotensin type 1 receptor, and reduced nitric oxide [15, 16]. Most of these responses were remarkably similar to those observed when

mesangial cells were cultured in high glucose [15]. In addition, when mesangial cells were cultured with both high levels of amino acids and glucose, cellular proliferation was further enhanced [15]. Therefore, mechanisms leading to glucose- and amino acid–mediated cellular responses may be shared.

Advanced glycation end products (AGEs) are well-recognized mediators of diabetic vascular complications. They are formed by nonenzymatic glycation of free amino groups, followed by a complex series of sequential glycation and oxidation reactions. Previous research has focused on hyperglycemia as the main causal factor. AGE formation occurs intracellularly and extracellularly, culminating in oxidative stress and tissue injury [17, 18]. Reciprocally, oxidative stress is known to induce AGE [19]. Protein kinase C (PKC) and mitogen-activated protein (MAP) kinase-extracellular signal regulated kinases (ERK) 1,2 can be activated by oxidative stress and signal profibrotic responses in kidney cells [19–24]. We hypothesize that an excess of amino acids, with or without hyperglycemia, can enhance formation of AGE by increasing availability of free amino groups. We also propose that amino acid-induced AGE formation leads to oxidative stress and activates PKC and MAP kinase-ERK 1,2 to induce fibrosis. The aims of this study were to evaluate effects of increased amino acids, with or without high glucose, on formation of AGE and reactive oxygen species (ROS), activity and production of PKC, and activation of ERK 1,2 using a mesangial cell culture system. Inhibitors of these processes were used to establish their causal roles in fibrosis and/or AGE formation in this system.

METHODS

Mesangial cell isolation and culture

Mesangial cells were recovered from glomeruli, isolated by sieving the cortex of kidneys excised from 6-month-old female Sprague-Dawley rats (gift from Donald Lightfoot, Ph.D.). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco Life Technologies, Gaithersburg, MD, USA), supplemented with penicillin-streptomycin (100 U/mL) and 10% heat-inactivated fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO, USA) in 5% carbon dioxide and a humidified atmosphere at 37°C. They were passaged at confluence using 0.025% trypsin (Gibco Life Technologies) in phosphate-buffered saline (PBS) and 0.2 g/L ethylenediaminetetraacetic acid (EDTA). Mesangial cells (passages 4 to 22) were seeded at 10,000 cells/cm^2 into culture dishes (Nunclon, Cambridge, MA, USA): 100 mm size for mRNA analyses and PKC activity assay, and 60 mm size for proteins and microscopy (with coverslips). At cellular confluence, media was changed to serum-free DMEM for 48 hours prior to experiments. Serum-free media was used to make cells quiescent. Otherwise, cell culture procedures were not altered during exposure to the experimental conditions.

Table 1. Amino acid and glucose concentrations for experimental groups in mesangial cell culture

	Control *mmol/L*	High glucose *mmol/L*	Amino acids *mmol/L*	Amino acids/ high glucose *mmol/L*
Glucose	5.5	30.5	5.5	30.5
L-Alanine	0	0	3.10	3.10
L-Arginine	0.40	0.40	2.48	2.48
L-Cystine	0.32	0.32	0.32	0.32
L-Glutamine	4.00	4.00	4.00	4.00
L-Glycine	0.40	0.40	2.23	2.23
L-Histidine	0.20	0.20	0.61	0.61
L-Isoleucine	0.80	0.80	1.41	1.41
L-Leucine	0.80	0.80	1.54	1.54
L-Lysine	0.80	0.80	1.22	1.22
L-Methionine	0.20	0.20	0.56	0.56
L-Phenylalanine	0.40	0.40	0.86	0.86
L-Proline	0	0	0.79	0.79
L-Serine	0.40	0.40	1.04	1.04
L-Threonine	0.80	0.80	1.27	1.27
L-Tryptophan	0.08	0.08	0.20	0.20
L-Tyrosine	0.42	0.42	0.45	0.45
L-Valine	0.80	0.80	1.45	1.45

Main experimental conditions

Mesangial cells were exposed to the following conditions for 48 hours (Table 1): (*1*) control, serum-free DMEM (glucose 5.5 mmol/L); (*2*) amino acids, increased amino acids, serum-free DMEM (glucose 5.5 mmol/L) supplemented with 10% Travasol mixed amino acid solution (Baxter, Deerfield, IL, USA) and L-arginine; (*3*) high glucose, serum-free DMEM (glucose 5.5 mmol/L) supplemented with glucose (25 mmol/L) to achieve a final concentration of 30.5 mmol/L; and (*4*) combination of amino acids and high glucose. Amino acids were designed to resemble the distribution of amino acids and relative concentration changes produced in the circulation after a protein meal [15, 16, 25].

Studies of AGE formation and inhibition

In response to the main experimental conditions, the appearance of carboxymethyllysine (CML), a prominent AGE, was evaluated in mesangial cells. CML was imaged by immunostaining and scanning laser confocal microscopy. The main experimental conditions were also performed with and without addition of aminoguanidine (0.5 mmol/L), an inhibitor of AGE formation. Aminoguanidine was added at the onset of the experiments and continued throughout the 48-hour experimental period. Protein and mRNA for the fibrosis markers, TGF-β1, fibronectin, and collagen IV, were measured in the conditioned media and/or cell lysates. The effect of aminoguanidine treatment on mesangial cell CML was also evaluated.

Table 2. Reverse transcription-polymerase chain reaction (RT-PCR) primers for cDNA probe synthesis

Gene	Genbank accession number(s)	Primers	cDNA size *bp*
Fibronectin	X15906	Forward 5′-CTGGGCGAAGGCAATGGGCGTATC-3′ Reverse 5′-CAAACTTCTGATCGGCATCGTAGTTC-3′	727
TGF-β1	X52498/RNTGFβ1	Forward 5′-AATACGTCAGACATTCGGGAAG-3′ Reverse 5′-GTCAATGTACAGCTGCCGTAC-3′	498
Collagen α1(IV)	AA924749	Reverse 5′-GGAGTATTTTCAGGGTTTCG-3′ Reverse 5′-TGGCAACTTCTCTTTCAACG-3′	411
PKC-β	X04440	Forward 5′-CGAGACACCTCCAACTTCGAC-3′ Reverse 5′-GACAAGAGTTCACAGTAACTGC-3′	591
GAPDH	M17701/RGAPDH	Forward 5′-AATGCATCCTGCACCACCAA-3′ Reverse 5′-GCTGTAGCCATATTCATTGTC-3′	519

Abbreviations are: TGF-β1, transforming growth factor-β1; PKC-β, protein kinase C β; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Studies of oxidative stress and antioxidant treatment

As an indicator of oxidative stress, intracellular ROS were evaluated by a fluorescent microscopy technique with the dye, dichlorodihydro-fluorescein diacetate (DCHF-DA), under the main experimental conditions. Treatment with vitamin E, an antioxidant, was used to determine effects on markers of fibrosis. Its analogue, vitamin E succinate, was administered to enhance cellular transport, which facilitates the release of α-tocopherol by membrane-bound esterases [26]. Cells were pre-loaded with vitamin E succinate (25 μmol/L) for 48 hours prior to the experiments to allow time for intracellular transport and release of α-tocopherol [26]. Protein and mRNA for the fibrosis markers, TGF-β1, fibronectin, and collagen IV, were measured in the conditioned media and/or cell lysates. The effect of vitamin E succinate on mesangial cell CML was also evaluated.

Studies of PKC activity, production, and inhibition

In response to the main experimental conditions, active and total PKC were measured by kinase phosphorylation activity, which reflects the sum of various isoforms. Levels of protein and mRNA for the β isoforms 1 and 2 were of particular interest because PKC-β is a key signaling mediator in diabetic vascular complications [27–29]. In order to assess the relative importance of the β isoforms, a general inhibitor, calphostin C (100 nmol/L), and a β isoform inhibitor, LY-379196 (100 nmol/L), were used. These inhibitors were added at the onset of the experimental conditions and continued throughout this 48-hour period. For the fibrosis markers (TGF-β1, fibronectin, and collagen IV), protein was measured in the conditioned media and/or cell lysates. The effects of calphostin C and LY-379196 on ERK 1,2 activation under the main experimental conditions were also assessed (see below).

Studies of ERK 1,2 activation and inhibition

The effect of the main experimental conditions on ERK 1,2 activation was evaluated by measuring the proportion of the phosphorylated form relative to total ERK 1,2 by Western blot in cell lysates. PD98059, an inhibitor of ERK 1,2 activation, was added to the experimental conditions in order to establish that ERK 1,2 is a signal for expression of fibrosis markers. PD98059 (25 μmol/L) was added at the onset of the experimental conditions and continued throughout this 48-hour period. Proteins for TGF-β1, fibronectin, and collagen IV were measured in the conditioned media and/or cell lysates.

Analytic techniques

RNA purification and Northern blots. RNA was purified from mesangial cells using Tri Reagent (Molecular Research Center, Cincinnati, OH, USA) and quantified by ultraviolet spectrophotometry (Beckman DU 640) (Beckman Coulter, Fullerton, CA, USA) [15]. For Northern blots, RNA (5 μg) was denatured, separated by formaldehyde agarose gel electrophoresis, transferred to charged nylon membranes (Schleicher and Schuell, Keene, NH, USA), and baked at 80°C. Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to prepare cDNA probes for Northern blot hybridization (Table 2). Total rat kidney RNA (2.5 μg) was reverse transcribed after annealing with random hexamers (First-Strand cDNA Synthesis Kit) (Madison, WI, USA). PCR was performed using 1 μL of each RT reaction mixture in a reaction of 100 μL. Each reaction contained 200 pmol/L of primer pairs (Integrated DNA Technologies, Coraville, IA, USA) and $MgCl_2$ (3 mmol/L) with PCR reagents (Promega, Madison, WI, USA). PCR was initiated by denaturation at 95°C for 4 minutes, followed by 30 cycles at 60°C, 73°C, and 95°C for 1 minute each. Amplified cDNA was gel purified (Qiagen, Valencia, CA, USA). The cDNA probes were ^{32}P-labeled using ^{32}P-deoxycytidimine triphosphate (dCTP) with the DECAprime II DNA labeling kit (Ambion, Austin, TX, USA). Blots were hybridized in 0.25 mol/L Na_2HPO_4, pH 7.2, 0.25 mol/L NaCl, 7% sodium dodecyl sulfate (SDS), and 1 mmol/L EDTA, with ^{32}P-labeled cDNA at 2×10^6 cpm/mL for 24 hours at 68°C. Blots were then washed at 60°C with 0.1 × standard sodium citrate (SSC)

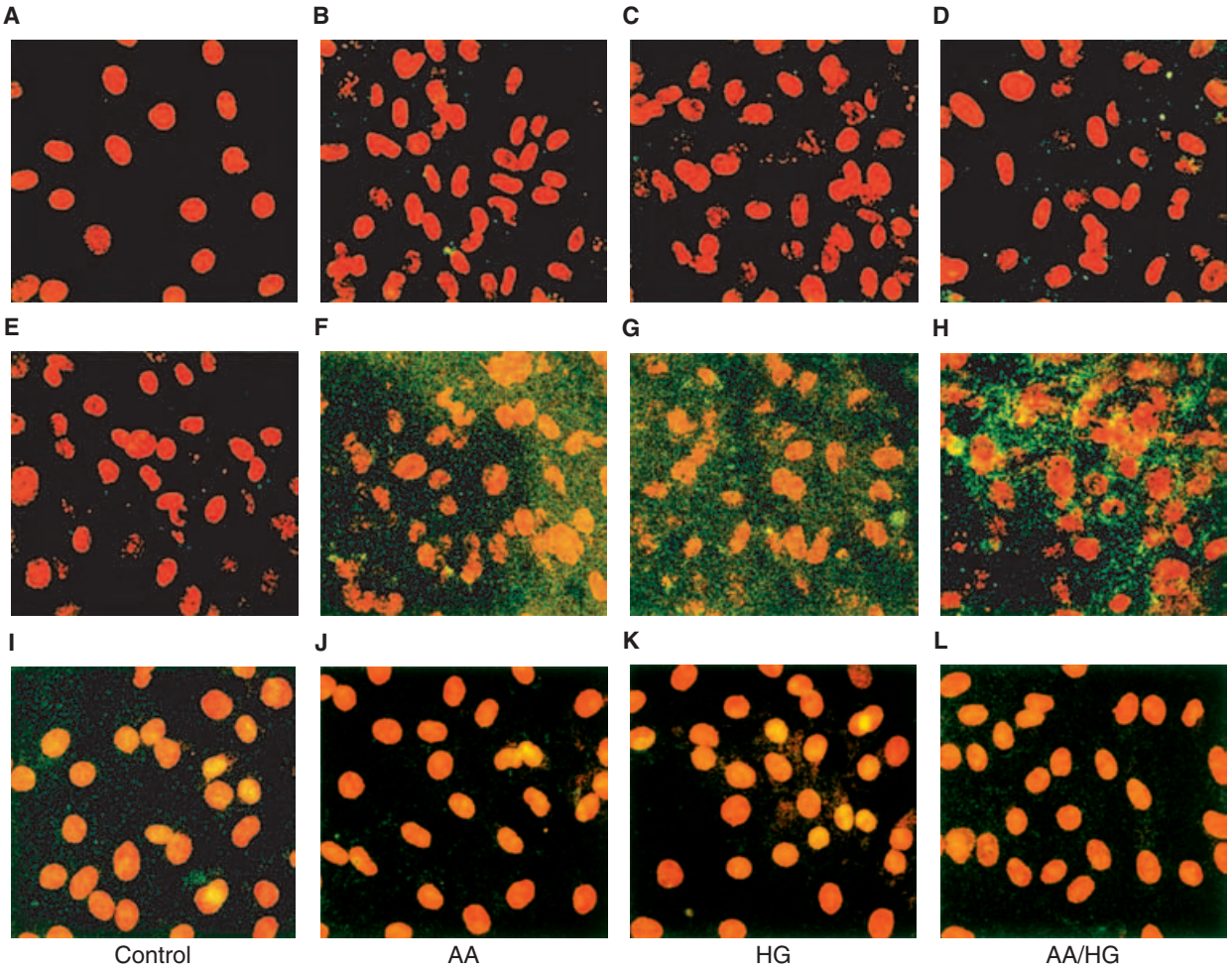

Fig. 1. Carboxymethyllysine (CML) immunostaining. Mesangial cells were exposed to control, amino acids, high glucose, and amino acids/high glucose conditions for 48 hours. The cells were then fixed and processed for scanning laser confocal microscopy. A mouse monoclonal antibody to CML and fluorescein-conjugated goat secondary antibody (green) were used to identify advanced glycation end products (AGEs) in the cells. Nuclei were stained with propidium iodide (red). These photomicrographs show representative fields of confluent mesangial cells. Main experimental conditions are shown with aminoguanidine (*A* to *D*), no addition (*E* to *H*), and vitamin E (*I* to *L*). Abbreviations are: AA, amino acids; HG, high glucose; AA/HG, amino acids/high glucose.

(15 mmol/L NaCl and 1.5 mmol/L sodium citrate) containing 0.1% SDS. Northern blots were quantified from digital images obtained with the Cyclone Storage Phosphor System and Optiquant Image Analysis software (Packard Instruments, Meriden, CT, USA). Quantities of mRNA were expressed as the ratio to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Protein assays. Cell culture conditioned media was collected, and 2 μg/mL each of protease inhibitors, pepstatin, leupeptin, antipain, and aprotinin (Sigma Chemical Co., St. Louis, MO, USA) were added to experimental samples used for enzyme-linked immunosorbent assay (ELISA). Samples were assayed immediately or stored frozen at −70°C until tested. A competition ELISA technique was used to measure fibronectin [15]. TGF-β1 was measured by ELISA with the Duoset Kit (R&D Systems, Minneapolis, MN, USA) [15]. Total protein was determined with the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Absorbances were detected with a BioTek FL-600 plate reader (BioTek Instruments, Winooski, VT, USA). ELISA measurements were calculated as ratios to total protein in media (pg/μg or ng/μg).

Western blot techniques were used to measure collagen IV, PKC-β1 and 2, and total and phosphorylated ERK 1,2 in cell lysates. Cells were lysed in RIPA buffer [50 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 0.5 mmol/L EDTA, 1 mmol/Ldithiothreitol (DTT), 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS]. Samples were separated through denaturing SDS-polyacrylamide gel electrophoresis (PAGE) (4% to 20% gradient) and transferred to a nitrocellulose membrane, blocked in Tris-buffered saline containing 0.05% Tween-20, and 5% bovine serum albumin (BSA). The samples were

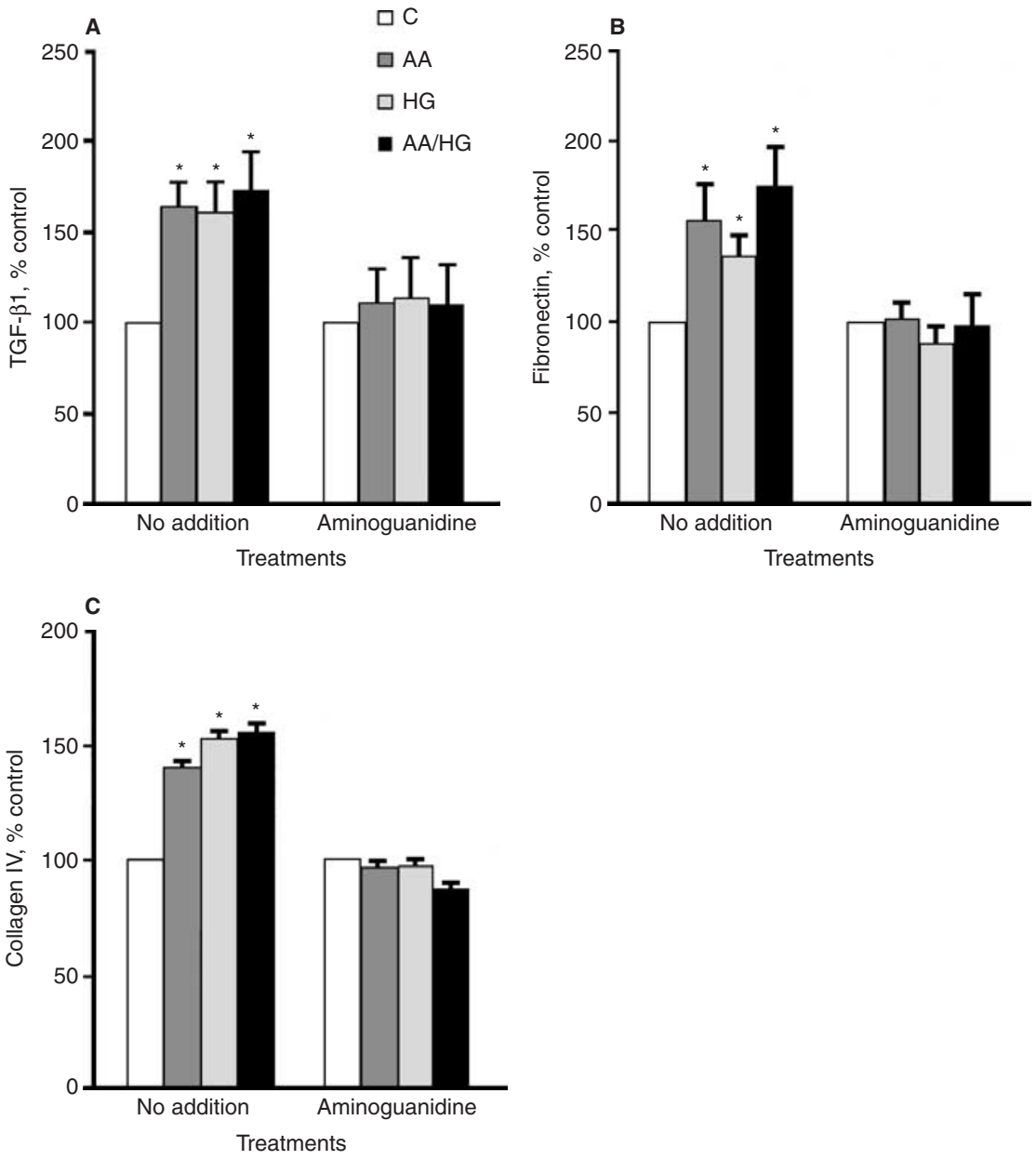

Fig. 2. Protein measurements for mesangial cells cultured under the main experimental conditions with no addition and with aminoguanidine. (*A*) Transforming growth factor-β1 (TGF-β1) (pg/μg). (*B*) Fibronectin (ng/μg). (*C*) Collagen IV (density units/μg).*$P < 0.01$ versus control ($N = 9$–14). Abbreviations are: C, control; AA, amino acids; HG, high glucose; AA/HG, amino acids/high glucose.

then incubated with antibodies that react with rat proteins: collagen IV (goat) (Southern Biotechnology Associates, Birmingham, AL, USA), PKC-β1 and 2 (rabbit) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), ERK 1,2 and dual phosphorylated (Thr^{202}/Thr^{204}) ERK 1,2 (rabbit) (Cell Signaling Technology, Beverly, MA, USA). These antibodies to rat proteins were detected with horseradish peroxidase-conjugated secondary antibodies (mouse antigoat and donkey antirabbit) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), followed by chemiluminescent detection using Super Signal West substrate (Pierce, Rockford, IL, USA). Digitized images were generated and analyzed using a Chemidoc XRS system and Quantity One 1D software (Bio-Rad Laboratories). The DC Protein Assay Kit (BioRad Laboratories) was used to measure total protein in the cell lysates. Western blot measurements were calculated as ratios to total protein (density units/μg).

CML immunostaining and scanning laser confocal microscopy. Mesangial cells were grown on 13 mm round Thermanox coverslips (Nunc, Electron Microscopy Sciences, Ft. Washington, PA, USA), fixed for 5 minutes with 100% methanol, and air-dried before storing at −20°C. Cells were rehydrated and incubated overnight with a

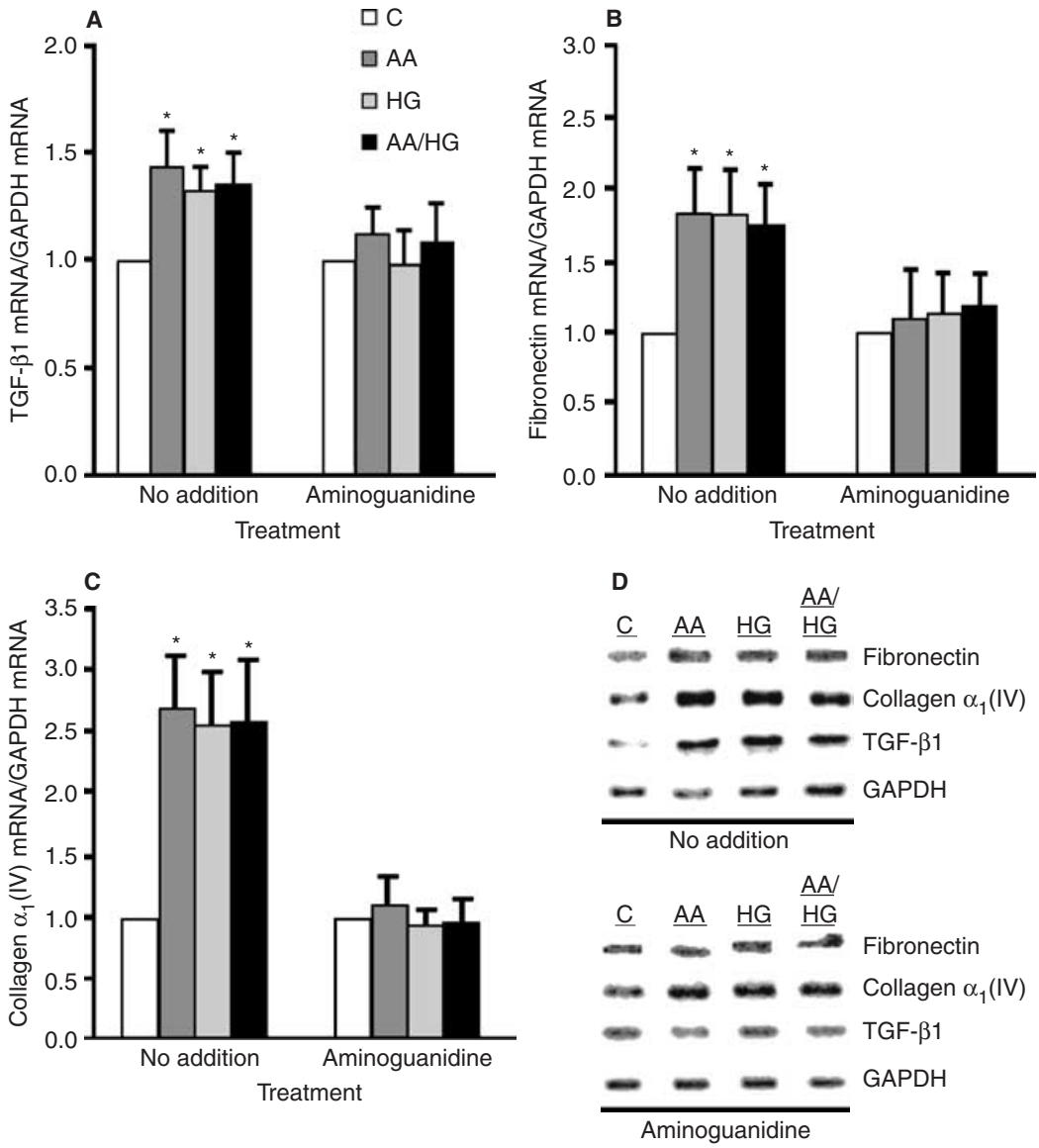

Fig. 3. Quantitative measures of mRNA in mesangial cells cultured under the main experimental conditions with no addition and with aminoguanidine. (*A*) Transforming growth factor-β1 (TGF-β1). (*B*) Fibronectin. (*C*) Collagen α_1(IV). Data are displayed as ratios to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. (*D*) Representative Northern blots. $^*P < 0.05$ versus control ($N = 11$–13). Abbreviations are: C, control; AA, amino acids; HG, high glucose; AA/HG, amino acids/high glucose.

mouse monoclonal anti-CML antibody (Research Diagnostics, Flanders, NJ, USA). A secondary goat antibody conjugated with fluorescein was used to identify the anti-CML antibody. Cells were washed and mounted on coverslips using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) containing propidium iodide to stain cell nuclei for visualization. Cellular fluorescence was imaged with a Bio-Rad 1024 scanning laser confocal microscope (Bio-Rad Laboratories).

ROS detection by fluorescence microscopy. ROS generation (predominantly hydrogen peroxide) was assessed using the fluorescent dye, DCHF-DA (Molecular Probes, Eugene, OR, USA). Mesangial cells were grown on 13 mm round Thermanox coverslips (Nunc, Electron Microscopy Sciences). After 4 and 48 hours of growth in the main experimental conditions, cells were washed with DMEM and incubated with DCHF-DA (10 μmol/L) for 30 minutes. Subsequently, cells were washed again with DMEM, and the coverslips were mounted on a glass slide using a mixture of PBS and Vectashield (Vector Laboratories). A positive control for ROS was made by culture of mesangial cells in control conditions followed by exposure to hydrogen peroxide (100 μmol/L) for 15 minutes prior to addition of DCHF-DA. To demonstrate that hydrogen peroxide was the source of the fluorescence, under each condition (positive control and experimental) catalase (80 U/mL) was administered 30 minutes prior to addition of DCHF-DA to degrade hydrogen peroxide.

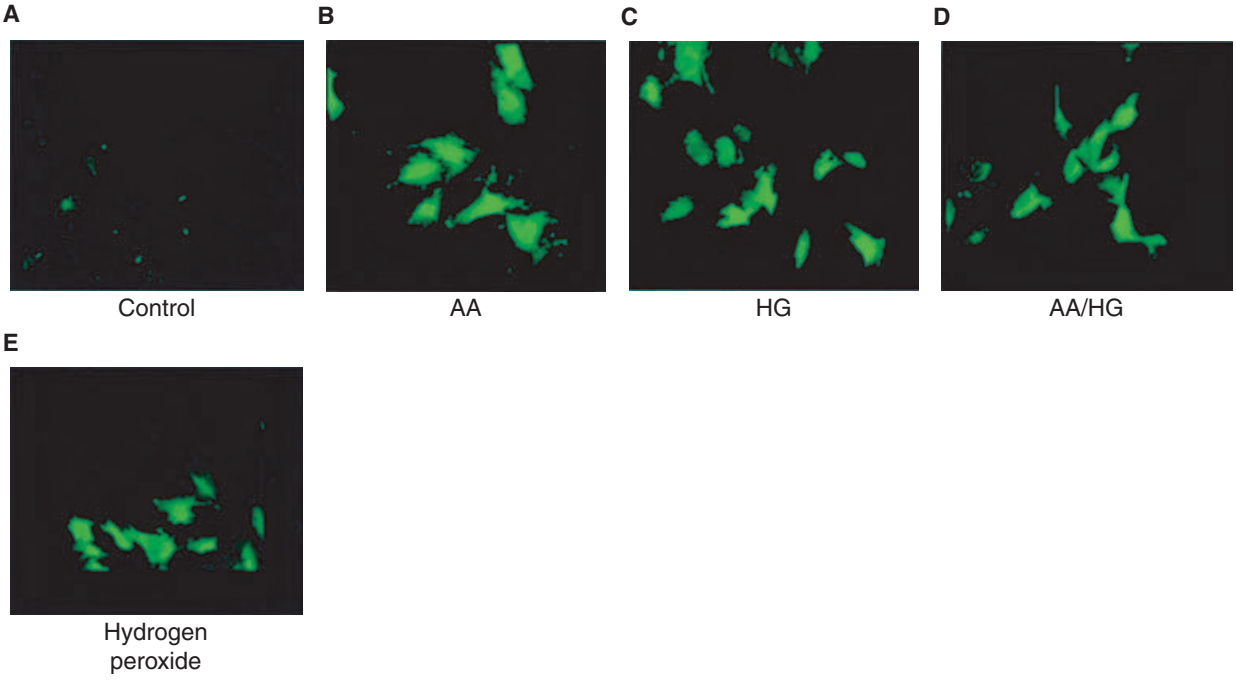

Fig. 4. Reactive oxygen species (ROS) detection by fluorescence microscopy. Mesangial cells were exposed to the main experimental conditions for 48 hours. The fluorescent dye, dichlorodihydro-fluorescein diacetate (DCHF-DA), was used to identify ROS in representative fields of mesangial cells cultured under control (*A*), amino acids (AA) (*B*), high glucose (HG) (*C*), and amino acids/high glucose (AA/HG) (*D*) conditions. For a positive control, mesangial cells were exposed to hydrogen peroxide (100 μmol/L) prior to addition of DCHF-DA (*E*).

Mesangial cells were viewed with an Axioplan II fluorescence microscope (Zeiss, Gottingen, Germany) equipped with a triple-bandpass filter. Digital images were captured and stored using Isis software version 3.4.0 (Metasystems, Altlussheim, Germany).

PKC activity assay. Cells were washed with 7 mL of ice-cold PBS and scraped into 0.5 mL of ice-cold extraction buffer (25 mmol/L Tris-HCl, pH 7.4, 0.5 mmol/L EDTA, 0.5 mmol/L EGTA, 0.05% Triton X-100, 10 mmol/L β-mercaptoethanol, 1 μg/mL leupeptin, 1 μg/mL aprotinin, and 100 mmol/L 4,2-aminoethylbenzenesulfonylfluoride HCl). The mixture was sonicated with two 5-second bursts using a probe sonicator (Sonic Dismembranator 60) (Fisher Scientific, Pittsburgh, PA, USA). Cell lysates were centrifuged for 5 minutes at 14,000 × *g* to remove nuclei and debris. Supernatants were recovered and assayed for kinase phosphorylation activity by measuring incorporation of [γ-^{32}P]-adenosine monophosphate (ATP) into the substrate, neurogranin, using the SignaTECT PKC Assay System (Promega, Madison, WI, USA). To derive a measure of total PKC, activators (phosphatidylserine, 320 μg/mL, and diacylglycerol, 32 μg/mL) were added to cell lysates to convert the inactive form to active PKC. The DC Protein Assay Kit (Bio-Rad Laboratories) was used to measure total protein in the cell lysates. Results were expressed as pmol ATP/minute/μg of total protein.

Statistics

Data were expressed as mean ± SE. One-way repeated measures analysis of variance (ANOVA) was used to evaluate outcome measurements in response to the main experimental conditions. Two-way repeated measures ANOVA was used to assess differences in outcome measurements between groups when the experimental conditions were performed with and without inhibitors of the various processes. Specific tests were conducted using a priori contrasts. Statistical significance was set at two-tailed probabilities less than 5% ($P < 0.05$). SPSS version 10 (SPSS, Chicago, IL, USA) was the software used for the statistical analyses.

RESULTS

AGE formation and inhibition

In mesangial cells cultured in the amino acid condition, immunostaining for CML clearly increased compared to control (Fig. 1E and F). This increase in cellular CML was similar to that observed in the high glucose condition (Fig. 1G). In consecutive experiments, intensity of CML immunostaining appeared greater in amino acid/high glucose than that in either condition alone (Fig. 1H). Treatment with an inhibitor of AGE formation, aminoguanidine, prevented the increase in cellular CML under each of these conditions (Fig. 1A to D). These

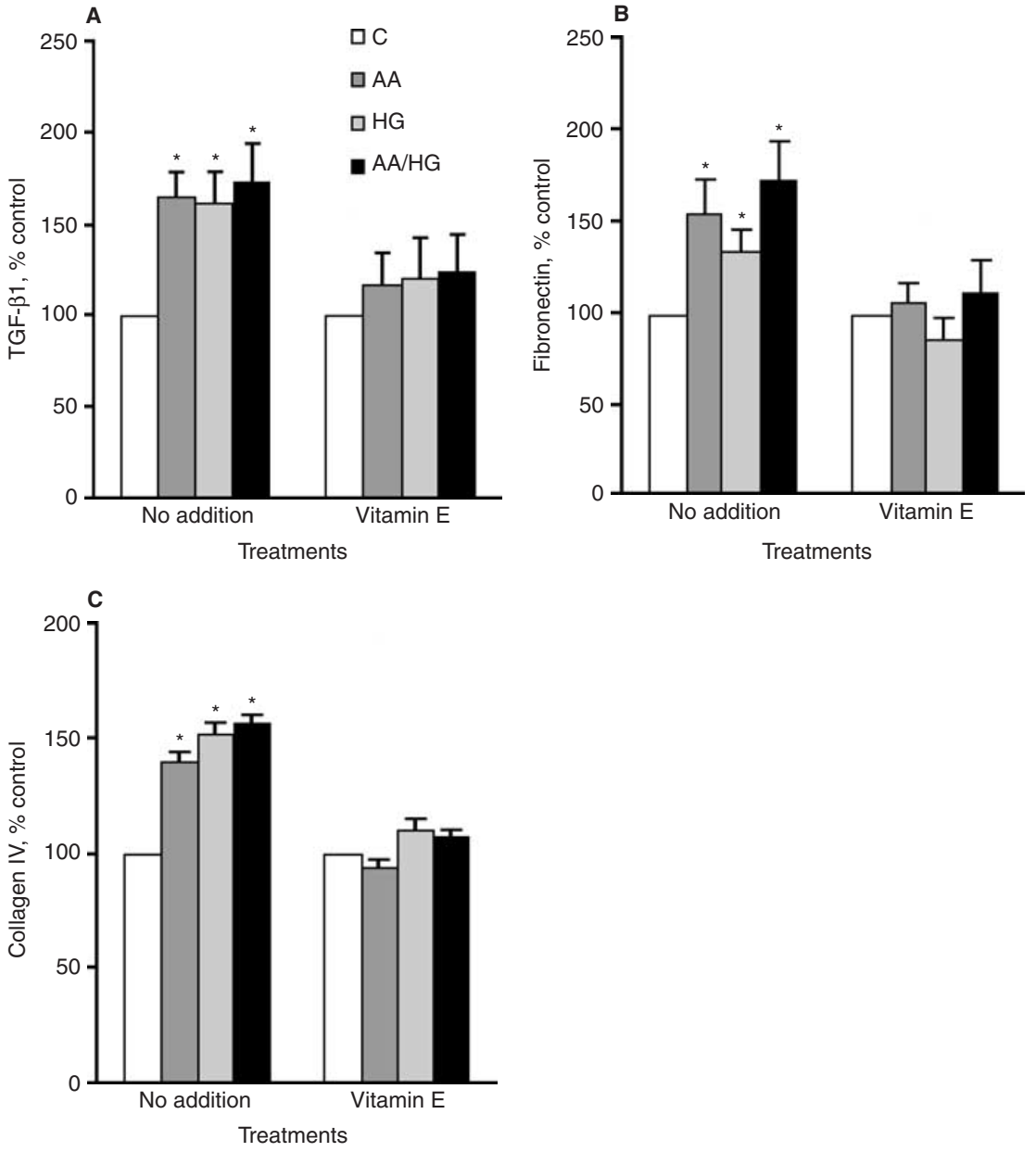

Fig. 5. Protein measurements for mesangial cells cultured under the main experimental conditions with no addition and with vitamin E. (*A*) Transforming growth factor-β1 (TGF-β1) (pg/μg). (*B*) Fibronectin (ng/μg). (*C*) Collagen IV (density units/μg). $^*P < 0.01$ versus control ($N = 9$–14). Abbreviations are: C, control; AA, amino acids; HG, high glucose; AA/HG, amino acids/high glucose.

images (Fig. 1) are representative of those obtained in the series of experiments ($N = 7$). Protein and mRNA for TGF-β1, fibronectin, and collagen IV increased significantly in response to amino acids, high glucose, and amino acids/high glucose (Figs. 2 and 3). Aminoguanidine treatment reduced both protein and mRNA for these fibrosis markers to control levels.

Oxidative stress and antioxidant treatment

The amino acid condition induced mesangial cell production of ROS (Fig. 4A and B). Similarly, high glucose and amino acids/high glucose induced ROS production (Fig. 4C and D). ROS were observed in mesangial cells cultured with amino acids, high glucose, and amino acids/high glucose at both the 4-hour (data not shown) and 48-hour time points. Treatment of mesangial cells cultured under the main experimental conditions with vitamin E succinate prevented increased expression of TGF-β1, fibronectin, and collagen IV at both the protein and mRNA levels (Figs. 5 and 6). The increases in mesangial cell CML induced by amino acids, high glucose, and amino acids/high glucose were also inhibited by treatment with vitamin E succinate (Fig. 1I to L).

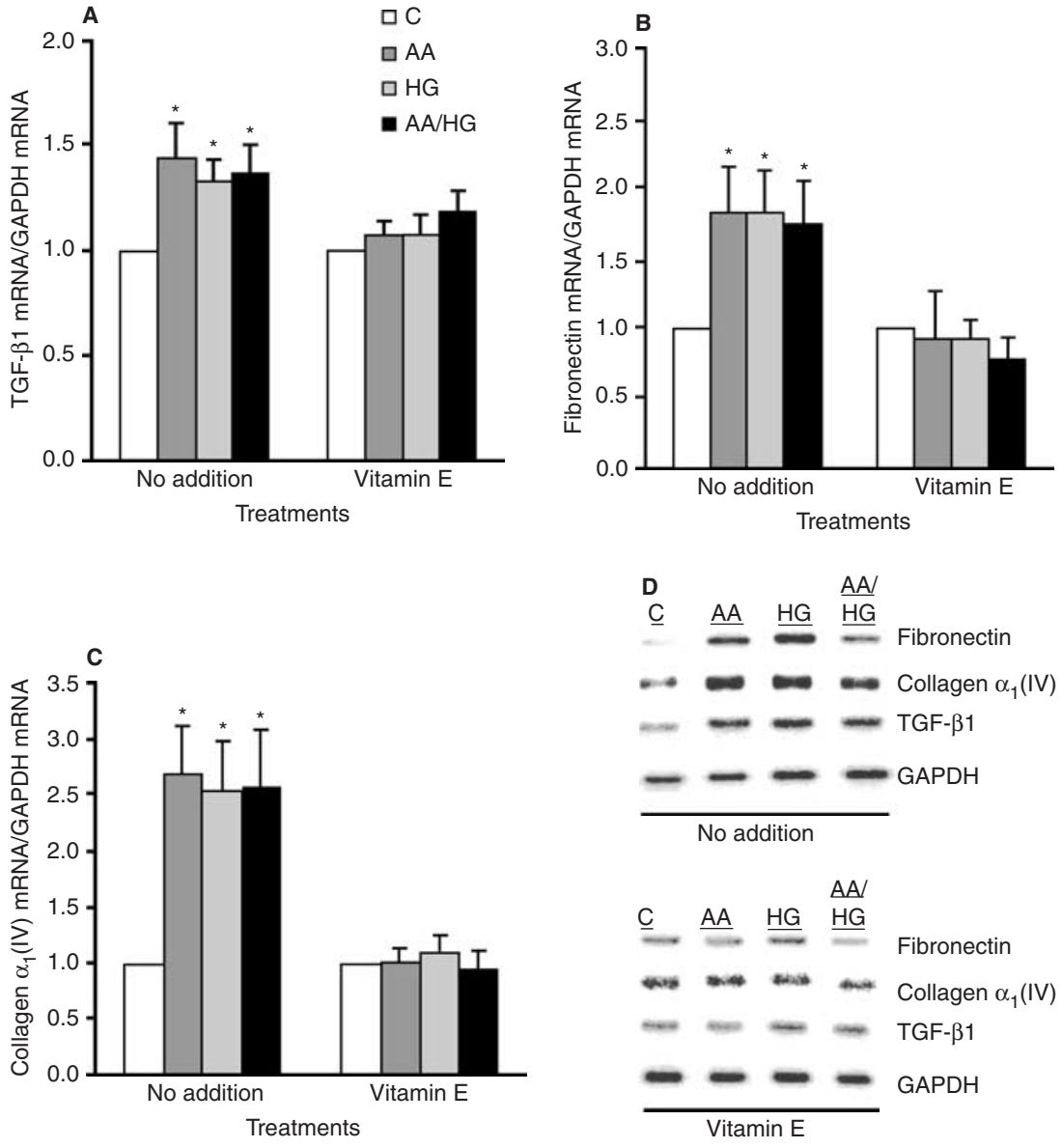

Fig. 6. Quantitative measures of mRNA in mesangial cells cultured under the main experimental conditions with no addition and with vitamin E. (*A*) Transforming growth factor-β1 (TGF-β1). (*B*) Fibronectin. (*C*) Collagen α_1(IV). Data are displayed as ratios to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. (*D*) Representative Northern blots. $^*P < 0.05$ versus control ($N = 11$–13). Abbreviations are: C, control; AA, amino acids; HG, high glucose; AA/HG, amino acids/high glucose.

Cell signaling via PKC and MAP kinase-ERK 1,2 pathway

The active portion of PKC increased in mesangial cells cultured with amino acids, high glucose, and amino acids/high glucose conditions compared to control (Fig. 7A). However, total PKC did not increase (Fig. 7B). No increase in protein for the PKC-β isoforms 1 and 2 was observed in mesangial cells exposed to the main experimental conditions (Fig. 8A and B). Similarly, mRNA expression for PKC-β (probe detects isoforms 1 and 2) was not altered by these conditions (Fig. 8C and D). Two bands (10 kb and 3.6 kb) representing PKC-β mRNA were detected; this pattern is believed to reflect post-transcriptional processing [30, 31]. Inhibition of PKC-β with LY-379196 blocked the profibrotic response of mesangial cells to amino acids, high glucose, and amino acids/high glucose in a manner similar to that observed with the general PKC inhibitor, calphostin C (Fig. 9). Amino acids, high glucose, and amino acids/high glucose increased phosphorylation of ERK 1,2, an index of its activation, in mesangial cells (Fig. 10). Treatment with either LY-379196 or calphostin C inhibited ERK phosphorylation in response to the main experimental conditions (Fig. 10). The inhibitor of ERK 1,2, PD098059, prevented

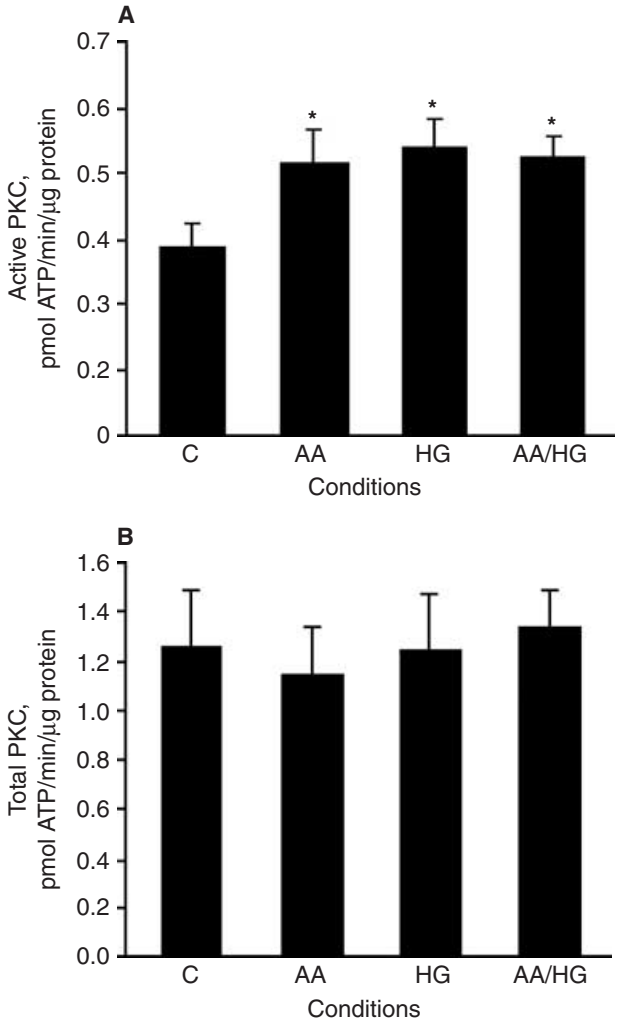

Fig. 7. Active (*A*) and total (*B*) protein kinase C (PKC) as determined by kinase phosphorylation activity in mesangial cells cultured under the main experimental conditions. $^{*}P < 0.01$ versus control ($N = 8$). Abbreviations are: C, control; AA, amino acids; HG, high glucose; AA/HG, amino acids/high glucose.

increases in protein for TGF-β1, fibronectin, and collagen IV in mesangial cells cultured with amino acids, high glucose, and amino acids/high glucose (Fig. 11).

DISCUSSION

When mesangial cells were exposed to elevated levels of amino acids designed to resemble protein feeding, they produced AGEs and ROS in a manner comparable to responses observed when cells were cultured in a glucose concentration typical of hyperglycemia. The immunofluorescent images suggested that even more AGEs could be formed when levels of both amino acids and glucose were increased. An inhibitor of AGE formation, aminoguanidine, prevented induction of CML and indicators of fibrosis (TGF-β1, fibronectin, and collagen IV) when mesangial cells were cultured with amino acids, high glucose, and amino acids/high glucose. Similarly, these responses were prevented by an antioxidant, vitamin E succinate. The cell signaling pathway of PKC and MAP kinase-ERK 1,2 was activated by amino acids, with or without concomitant high glucose. As determined by inhibition studies, PKC-β was largely responsible for ERK 1,2 phosphorylation and the profibrotic response of mesangial cells cultured under these conditions. Since no increase in total PKC-β isoform mRNA or proteins 1 and 2 was observed, the increase in activity appears to be due to activation, rather than production, in this system. The role of ERK 1,2 in the cell signaling sequence was confirmed by inhibition studies demonstrating prevention of fibrosis.

These data support a unifying hypothesis of AGE formation and oxidative stress to potentially explain increased renal sensitivity to dietary protein in diabetes, as well as links between amino acids and mesangial cell injury in nondiabetic disease processes (Fig. 12). To our knowledge, this is the first demonstration that an amino acid mixture, typical of the plasma profile produced by protein feeding, induces formation of AGEs and ROS in mesangial cells. The accumulation of AGEs and the profibrotic response to amino acids were prevented by treatment with an inhibitor of AGE formation. Since inhibition appeared to be complete in both aspects, these data indicate that AGE formation is a primary cause of fibrotic injury induced by amino acids in mesangial cells. Therefore, free amino acids, as well as amino groups on long-lived structural proteins, may participate in glycation reactions leading to cell injury. Although the immunofluorescence pattern suggested greater CML accumulation in response to the combination condition of amino acids/high glucose, confirmation of this impression will require quantitative measurements. These data have implications for understanding effects of high protein diets on diabetic, as well as nondiabetic, kidney diseases. Recently, in the Nurses' Health Study, women with mild renal insufficiency (glomerular filtration rate 55 to 80 mL/minute) were reported to have a 3.5-fold increased risk of losing renal function if dietary protein intake was in the highest (90 g/day) versus lowest quintile (60 g/day) [32]. Similarly, high protein diets have long been known to accelerate kidney disease in nondiabetic animal models such as aging and renal ablation [11, 33]. Glomerular hyperfiltration and hypertension are well-established mechanisms of injury produced by high protein diets [33]. However, accumulating clinical and experimental evidence indicates that nonhemodynamic mechanisms may also contribute to this type of kidney damage [34–37]. Our in vitro system, which was free of influences from hemodynamics or disturbances in other metabolites and hormones, demonstrated that AGE formation is a strong candidate mechanism for nonhemodynamic

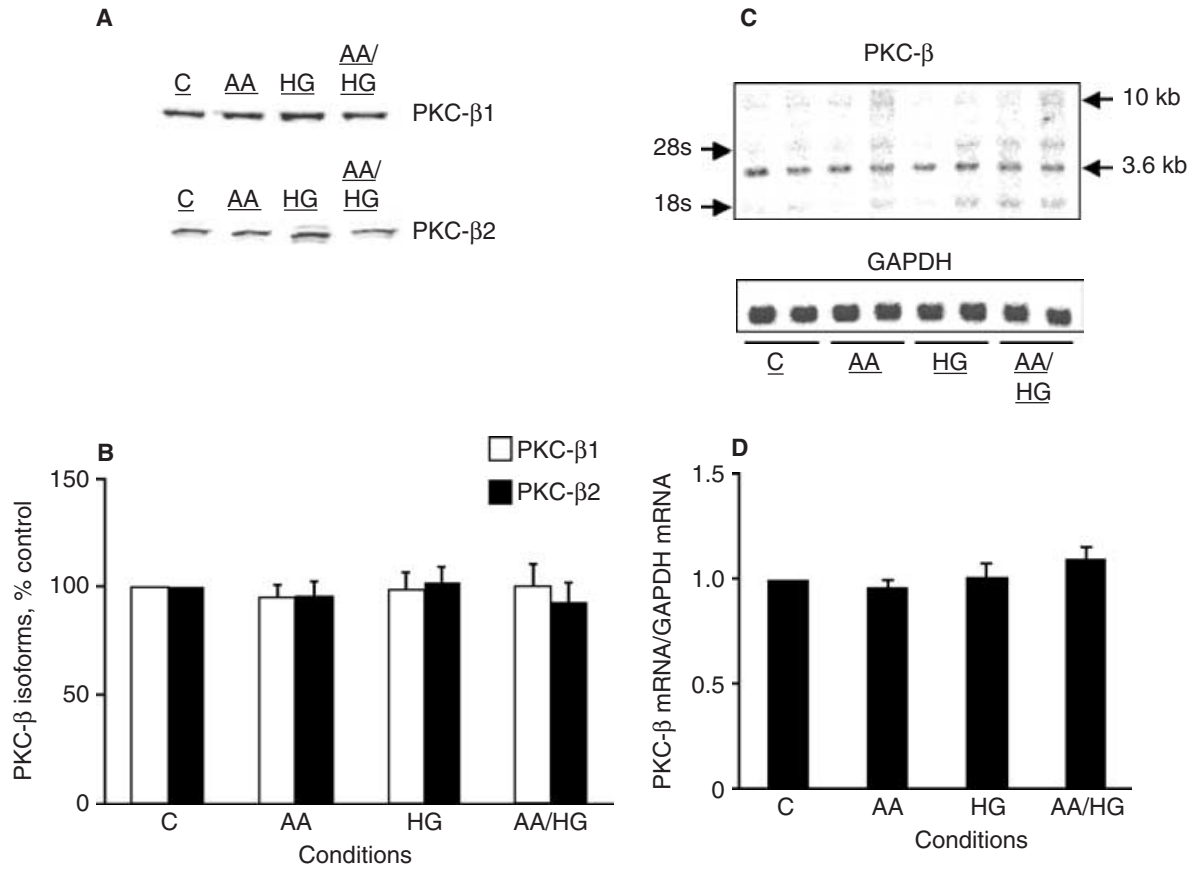

Fig. 8. Expression of protein kinase C β (PKC-β) isoforms 1 and 2 in mesangial cells exposed to the main experimental conditions. (*A*) Representative Western blot. (*B*) Quantitative measures of proteins ($N = 16$). (*C*) Representative Northern blot. (*D*) Quantitative measures of mRNA ($N = 6$). Two bands (10 kb and 3.6 kb) representing PKC-β mRNA were detected; this pattern is believed to reflect posttranscriptional processing [30, 31]. Bands for 18S and 28S RNA are also identified. Abbreviations are: C, control; AA, amino acids; HG, high glucose; AA/HG, amino acids/high glucose; GADPH, glyceraldehyde-3-phosphate dehydrogenase.

injury induced by high amino acid levels typical of protein feeding.

A number of specific transporters move glucose and amino acids into cells [38]. Based on the available data, we cannot determine if amino acids were glycated intracellularly or extracellularly and then incorporated into cellular proteins. However, even if incorporation did not occur, reactions with amino acids produce oxidative stress, which have a number of detrimental consequences, including perpetuation of AGE formation on preexisting proteins [39]. In mesangial cells exposed to amino acids, high glucose, and amino acids/high glucose, CML was reduced by an antioxidant, vitamin E, suggesting that oxidative stress facilitates AGE formation in this model. Thus, interactions between AGE and ROS can be viewed as a reciprocal, positive feedback system. Oxidative stress also appears to be a critical component of the mesangial cell injury response to amino acids because vitamin E treatment completely blocked induction of fibrosis.

In order to explore signaling mechanisms responsible for the profibrotic response induced by amino acids, the PKC and MAP kinase-ERK 1,2 pathway was evaluated. This pathway is known to signal cellular injury related to AGE and glucose-induced oxidative stress [20, 21, 23, 24]. PKC-β can be activated by AGE and oxidative stress in mesangial cells, even without exposure to high glucose levels [20]. In response to amino acids with or without high glucose, PKC activity increased. Studies with inhibitors of AGE formation and oxidative stress should be performed to confirm that these mechanisms are also responsible for amino acid-induced PKC activation. Furthermore, even though PKC activity increased, total PKC and the mRNA and protein for the β isoforms did not increase. Since inhibition of ERK 1,2 activation and of the profibrotic response was complete with both the general and β-specific inhibitors, PKC-β appears to account for the increased activity in this system. In addition, inhibition of ERK 1,2 fully prevented the profibrotic response to amino acids, high glucose, and amino acids/high glucose. Therefore, ERK 1,2 also is a critical signal for this cell injury response. PKC-β is one of at least 12 isoforms, which is further subdivided into isoforms 1 and 2 [40].

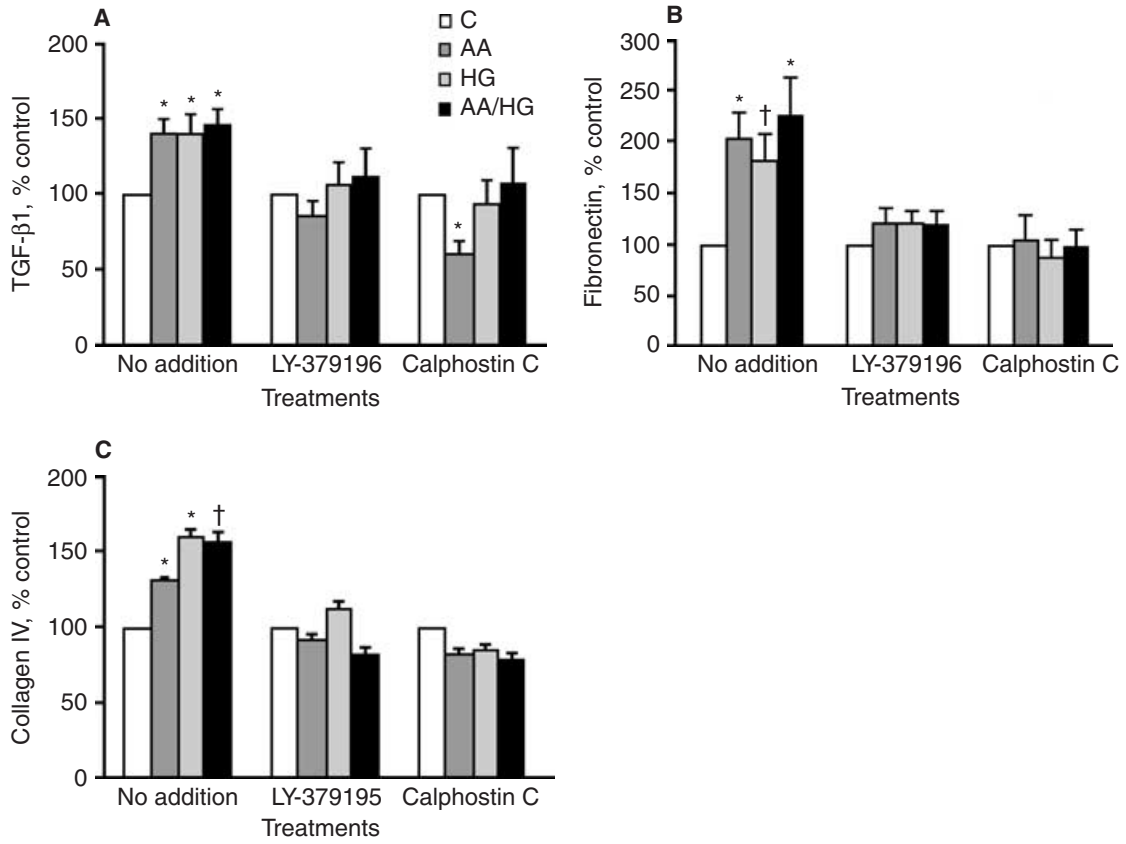

Fig. 9. Protein measurements for mesangial cells cultured under the main experimental conditions with no addition and with LY379196 and calphostin C. (*A*) Transforming growth factor-β1 (TGF-β1) (pg/μg). (*B*) Fibronectin (ng/μg). (*C*) Collagen IV (density units/μg). $^{*}P < 0.01$ versus control; $^{\dagger}P < 0.05$ versus control ($N = 10$–14). Abbreviations are: C, control; AA, amino acids; HG, high glucose; AA/HG, amino acids/high glucose.

These β isoforms are derived from alternative splicing of a single mRNA transcript [41]. In glomeruli and mesangial cells, PKC-β1 is a predominant active form [27, 28]. Although PKC-β2 is largely confined to renal interstitial macrophages in diabetic rats, we and others have found small amounts of this isoform in cultured mesangial cells [27]. The relative contributions of PKC-β1 and 2 to ERK 1,2 activation and fibrosis remain unknown because their expression levels did not change and LY379196 inhibits both β isoforms.

Several other lines of evidence support the hypothesis that amino acids may cause cell and tissue damage via AGE formation and oxidative stress. Anderson et al [42] reported that neutrophils cultured with a physiologic amino acid mixture and a normal glucose concentration produced CML. In another study, neutrophils cultured with an elevated level of the single amino acid glutamine increased production of ROS and nicotinamide adenine dinucleotide phosphate oxidase components, both indicators of oxidative stress [43]. A high methionine diet activated nuclear factor kappaB (NF-κB) and up-regulated inducible nitric oxide synthase (iNOS) expression in kidneys of normal rats, suggesting a role in renal injury [44]. Finally, patients with end-stage renal disease who used a mixed amino acid solution for just one peritoneal

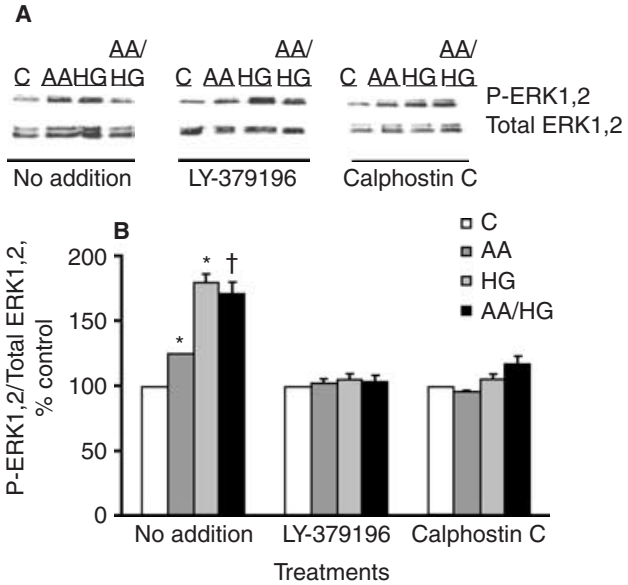

Fig. 10. Extracellular signal-regulated kinase (ERK) 1,2 activation as determined by the ratio of phosphorylated ERK 1,2 (P-ERK1,2) to total ERK1,2 in mesangial cells cultured under the main experimental conditions with no addition and with LY379196 and calphostin C. (*A*) Representative Western blot. (*B*) Quantitative measures of proteins. $^{*}P < 0.01$ versus control; $^{\dagger}P < 0.05$ versus control ($N = 12$). Abbreviations are: C, control; AA, amino acids; HG, high glucose; AA/HG, amino acids/high glucose.

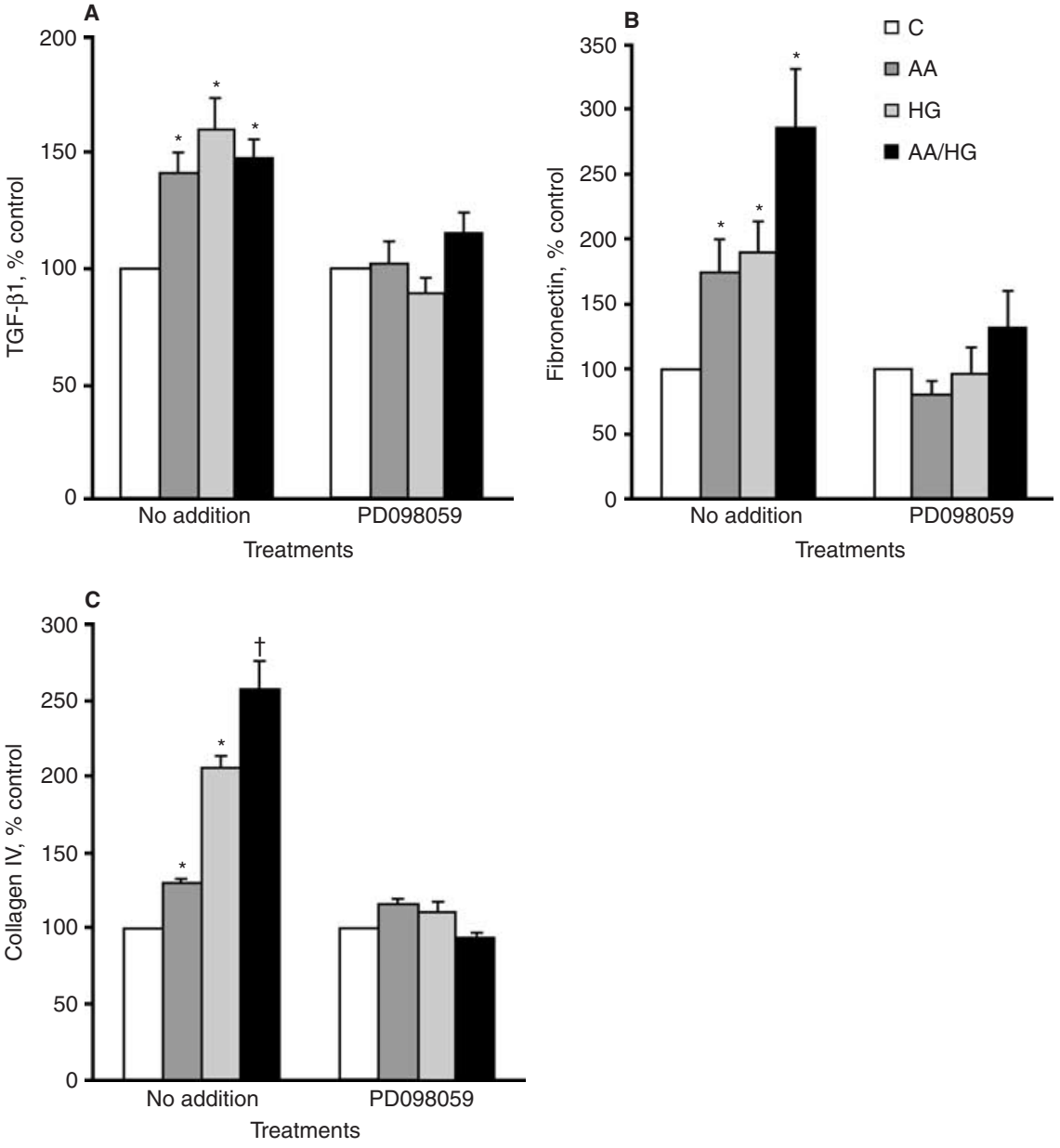

Fig. 11. Protein measurements are shown for mesangial cells cultured under the main experimental conditions with no addition and with PD098059. (*A*) Transforming growth factor-β1 (TGF-β1) (pg/μg). (*B*) Fibronectin (ng/μg). (*C*) Collagen IV (density units/μg). $^{*}P < 0.01$ versus control; $^{\dagger}P < 0.05$ versus control ($N = 7$–10). Abbreviations are: C, control; AA, amino acids; HG, high glucose; AA/HG, amino acids/high glucose.

dialysis exchange developed endothelial dysfunction as determined by impaired brachial artery reactivity [45]. Although these studies did not address the specific mechanisms of NF-κB activation or endothelial dysfunction, such consequences are typical of AGE formation and oxidative stress [17, 18, 46]. Therefore, both in vitro and in vivo, increased exposure to various individual amino acids and combinations produces manifestations of cell and tissue damage, which are consistent with effects of AGE and oxidative stress. These studies also suggest that amino acid-induced injury could be produced in sites other than the kidney, and that the arterial circulation may be particularly vulnerable.

This in vitro study has important limitations. First, we do not know if specific amino acids are more reactive than others because mesangial cells were exposed to a mixed solution. Amino acids with more than one amino group could be particularly reactive due to increased sites for glycation reactions. Previously, we have shown that exposure of mesangial cells to such an individual amino acid, L-arginine, produced a profibrotic response and reduction of nitric oxide in the conditioned media, presumably due to oxidative quenching [15]. Second, cells were exposed to increased amino acid levels continuously. Now that the plausibility of amino acid–induced AGE formation has been established, responses to pulsatile exposure,

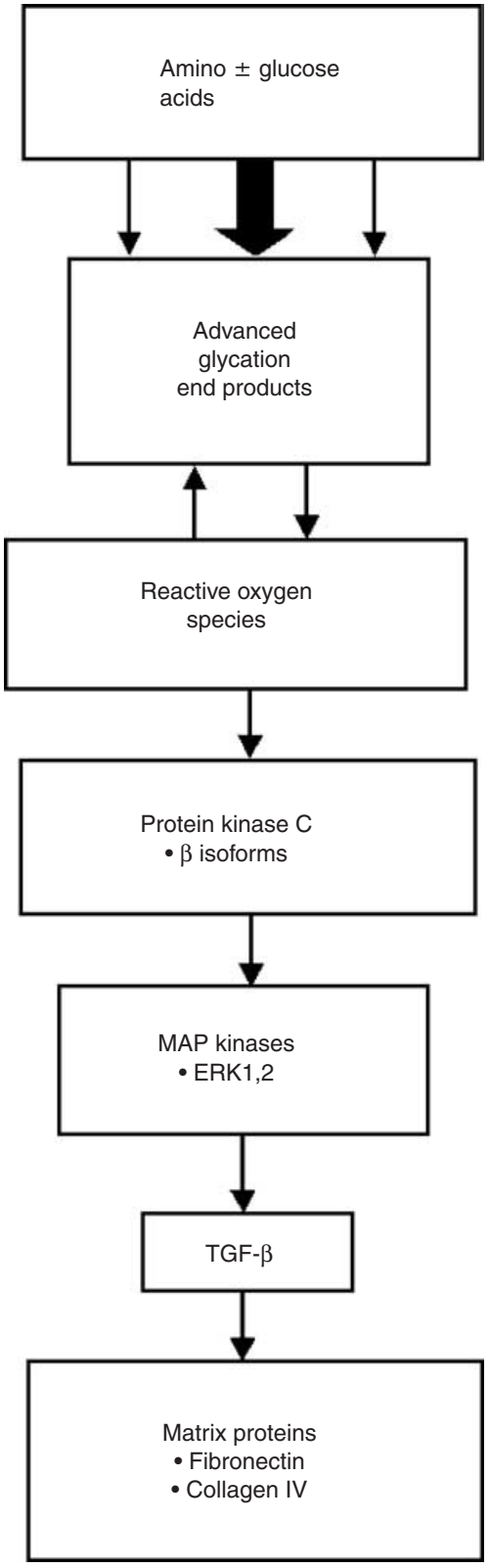

Fig. 12. Unifying hypothesis for fibrotic injury induced by increased amino acids, with or without high glucose, in mesangial cells. Arrows indicate proposed sequence of events. The large arrow represents the potential for an enhanced effect on advanced glycation end products formation by the combination of increased amino acids and high glucose conditions. Bidirectional arrows between advanced glycation end products and reactive oxygen species reflect their relationship as a reciprocal, positive feedback system. Abbreviations are: MAP, mitogen-activated protein; ERK 1,2, extracellular signal-regulated kinase; TGF-β, transforming growth factor-β.

which more closely mimics feeding patterns, would be informative. Third, serum-free media may have been a stress to the cells. However, under the control condition with serum-free media, no evidence of increased oxidative stress or AGE formation was observed. Nevertheless, experiments with the addition of serum would produce a more physiologic condition to examine effects of amino acids on AGE formation, oxidative stress, PKC activation, and cell injury. Fourth, the inhibitors could have actions other than those for which they were intended. Aminoguanidine has antioxidant properties and can inhibit iNOS [47]. However, in our in vitro experimental system, iNOS mRNA has not been detected (unpublished observations). Thus, aminoguanidine is unlikely to function as an iNOS inhibitor in this system. Vitamin E has been reported to inhibit PKC by nonantioxidant molecular interactions [48]. Other inhibitors of AGE formation and oxidative stress should be evaluated to confirm the present results. Although the various kinase inhibitors are believed to be relatively specific at the dosages used, cross-reactivity between them cannot be completely excluded [49]. Despite these limitations, the unifying hypothesis is supported by the consistency of the overall evidence (Fig. 12). Finally, it is possible that the amino acid solution contained preformed AGE. Since the immunofluorescence images of mesangial cells treated with aminoguanidine or vitamin E showed no visible increase in AGEs with addition of amino acids, preformed AGEs were unlikely to have been derived from the solution because these agents work by inhibiting AGE formation. However, even if some preformed AGEs were present in the solution and/or media, this may be relevant to the in vivo state. Consumption of foods with increased amounts of AGE-modified proteins has been reported to increase circulating AGE levels and inflammatory markers in diabetic subjects [50]. In mouse models, diets low in AGEs prevented glomerulosclerosis despite persistent hyperglycemia [51]. Since AGE-modified proteins would be absorbed as glycated amino acids and peptides, these data indicate that such protein fragments, not only AGE-modified intact proteins, have the potential to produce cell and tissue damage.

CONCLUSION

Mesangial cells exposed to a mixture of amino acids, designed to resemble protein feeding, produced a profibrotic response as a consequence of AGE and ROS formation, presumably induced by increased availability of

amino groups for glycation reactions. To our knowledge, this is the first demonstration that amino acids induce AGE formation in mesangial cells and that they may enhance such an effect of glucose. PKC-β and the MAP kinase-ERK 1,2 pathway signal the profibrotic response to amino acids. These observations provide insight into cellular mechanisms of renal injury induced by amino acids and a potential explanation for the enhanced sensitivity of the diabetic kidney to dietary protein.

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