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Agmatine Reduces Hydrogen Peroxide in Mesangial Cells under High Glucose Conditions

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Agmatine, an amine and organic cation, reduced H_2O_2 that was generated by hyperglycemia, and transcription factors such as NF-κB and AP-1 activity in the mesangial cells that were exposed to high glucose. However, spermine which shares a strong nucleophilic structure with agmatine decreased the H_2O_2 levels and AP-1, but not the NF-κB activity. Possible roles for agmatine and spermine in decreasing fibronectin are discussed, and the signaling pathway for agmatine-reduced fibronectin accumulation is presented.

Keywords: Agmatine, AP-1, NF-κB, Fibronectin, Hydrogen peroxide

Introduction

Reactive oxygen species (ROS), such as superoxide (O_2^-), alkoxyl ($RO^\bullet$), peroxyl ($ROO^\bullet$), hydroxy radical ($OH^\bullet$), ozone (O_3), and hydrogen peroxide (H_2O_2), injure cells when in excess of normal levels, thereby contributing to the pathogenesis of many renal diseases (Ha and Lee, 2000; Aydin *et al.* 2001; Trolliet *et al.*, 2001; Ha *et al.*, 2002; Onozato *et al.*, 2002; Peng *et al.*, 2002). ROS are effectively eliminated by several antioxidant systems (Ohtake *et al.*, 1997; Kinter *et al.*, 1999; Deng *et al.*, 2001; Sozmen *et al.*, 2001; Koo *et al.*, 2002). However, if excess ROS overwhelms cellular defenses, then these reactive oxygen species display a variety of biochemical phenomena. Oxygen derivatives activate transcription factors, such as NF-κB and AP-1, which can bind the human TGF-β1 promoter and may stimulate the transcription and production of TGF-β1 (Azuma *et al.*, 1999; Sakurai *et al.*, 1999; Chang, 2000; Mezzano *et al.*, 2001). Previous studies (Ha and Kim, 1997; Cruz *et al.*, 2001) showed that H_2O_2 increased the levels of TGF-β1 and extra cellular matrix (ECM) proteins such as fibronectin. Cruz *et al.* verified that H_2O_2 increased ECM mRNA through TGF-β1 in human mesangial cells.

Agmatine, an amine and organic cation, is formed by the decarboxylation of L-arginine by arginine decarboxylase (EC 4.1.1.19). This reaction occurs in plants and bacteria. However, agmatine and arginine decarboxylase were also discovered in the brains of rats and cows (Li *et al.*, 1994). Agmatine is a biologically active substance, but the mode and sites of action have not been fully defined. The administration of agmatine produced increases in the nephron filtration rate and absolute proximal reabsorption (Mark *et al.*, 1996) and reduced collagen accumulation (Marx *et al.*, 1995). We also observed a decrease in fibronectin by placing agmatine in mesangial cells.

We hypothesized that a decrease in the H_2O_2 levels by agmatine does not activate NF-κB and AP-1, but reduces the fibronectin accumulation via a decrease in TGF-β1 activity when compared to cells that are exposed to high glucose. Therefore, our experiments were designed to measure the levels of each of these biologically active factors in mesangial cells that are exposed to high glucose.

Materials and methods

Mesangial cell culture Dulbecco's modified Eagle's medium (DMEM) that contained 5.6 mM glucose was used to culture the mesangial cells (MC), unless otherwise stated. A murine MC line (MES-13, cloned from mice transgenic for the early region of SV-40 virus, passage 25) was obtained from American Type Culture Collection (ATCC, Rockville, USA). These transformed cells exhibited similar characteristics to those of the primary cultures of murine MC (Mackay *et al.*, 1988). The cells were cultured in DMEM that contained 20% fetal bovine serum (FBS, Life

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Technologies BRL, Gaithersburg, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, 44 mM $NaHCO_3$, and 14 mM N-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES). For MES-13, DMEM that contained 5% FBS was used. The cells were cultured in 100-mm culture dishes for electrophoretic mobility shift assays (EMSA). Near-confluent MC were incubated with serum-free media for 24 h to arrest and synchronize cell growth. Media was then changed to fresh serum-free DMEM that contained 5.6 (control) or 30 mM (high) glucose, and incubated up to 48 h. Incubation of the cells in the control, as well as high glucose media (HG) in serum-free conditions for up to 96 h, did not significantly effect cell viability as determined by the lactate dehydrogenase (LDH) release (data not shown). In some of the experiments, the cells were pretreated with polyamines for 1 h and incubated with the control and HG media for a given period. Preliminary studies demonstrated that up to 2 μM cytochalasin B caused no cytotoxicity as determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Effective concentrations of cytochalasin B were decided on the basis of published data, as well as our own preliminary study. The cells were processed for nuclear protein extraction, then measured for ROS and protein expression as will be described later.

Preparation of nuclear extracts Nuclear extracts were prepared according to the method described by Lee *et al.* (1988) with modifications (Kwon *et al.*, 2001). In brief, the cells were washed twice with ice-cold phosphate-buffered saline after incubations and suspended in one packed cell volume (PCV) of buffer A that contained 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 1 μg/ml leupeptin and aprotinin, pH 7.9, on ice for 10 min, then lysed by passing the cell suspension through a 27-gauge needle 5 times. Crude nuclei were washed twice with buffer A in order to prevent cytosolic contamination. The nuclear proteins were extracted with two-thirds PCV of ice-cold buffer B

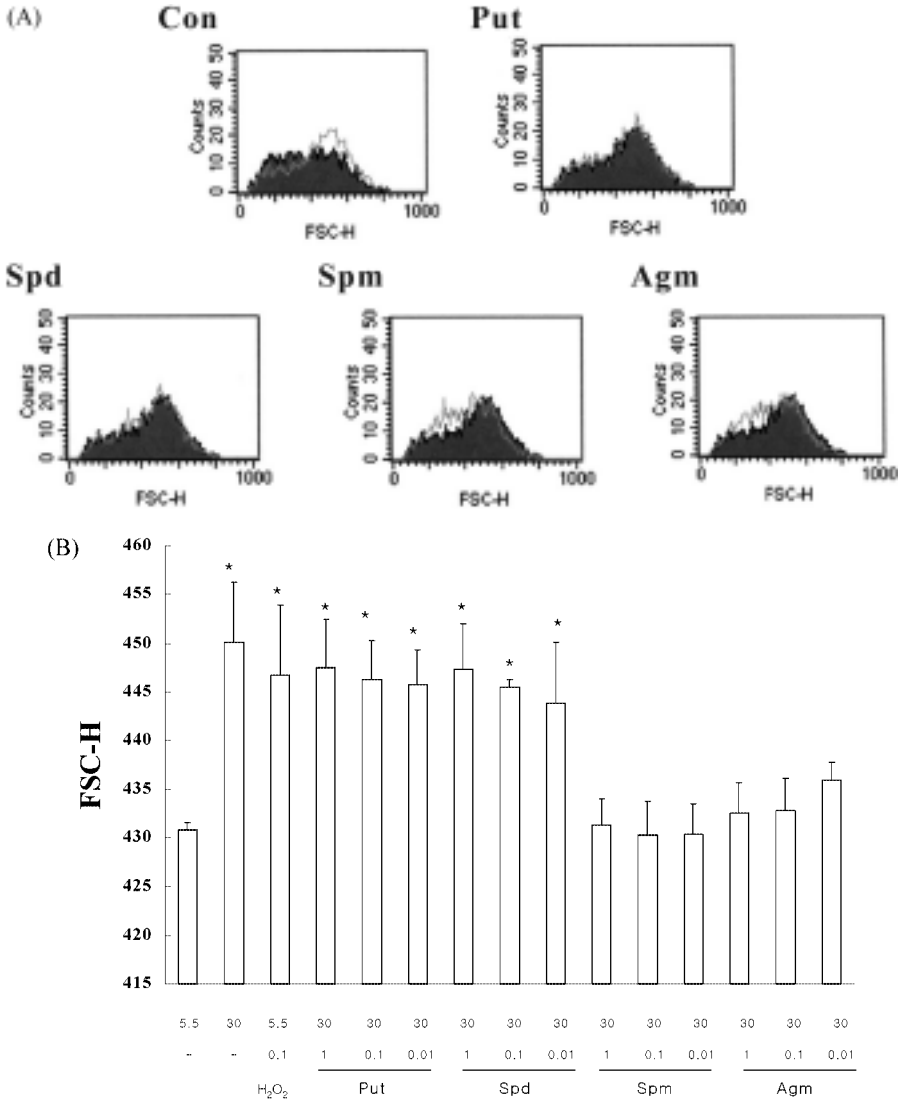

Fig. 1. Specificity of 2',7'-dichlorofluorescein (DCF)-sensitive ROS in mesangial cells that was cultured with various concentrations of polyamines and agmatine under high glucose conditions. Synchronized quiescent MES-13 was incubated under the control glucose and after the addition of 25 mM (for total of 30 mM) of D-glucose for 1 h. Intracellular DCF-sensitive ROS was visualized by FACS.

that contained 20 mM HEPES, 420 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and aprotinin, and 25% glycerol, pH 7.9. A two-thirds PCV of ice-cold buffer C (20 mM HEPES, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and aprotinin, 20% glycerol, pH 7.9) was added. The mixture was then centrifuged at 15,000 × *g* at 4°C for 15 min. The nuclear proteins were transferred to new tubes as aliquots and stored at −70°C until use. The protein concentration was determined by a Bio-Rad assay (Richmond, USA).

Electrophoretic mobility shift assay For the EMSA, the following oligonucleotide with the NF-κB consensus binding sequence was used; 5'-AGTTGAGGGACTTTCCCAGGC-3' (Santa Cruz Biotechnology, Santa Cruz, USA). A mutant motif with G to C substitution (5'-AGTTGAGCGACTTTCCCAGGC-3', Santa Cruz Biotechnology) served as a control. The consensus oligonucleotide was labeled with [γ-^{32}P]ATP (DuPont NEN, Boston, USA) according to the manufacturer's description. The binding reaction was performed in a final volume of 20 μl that contained a binding buffer (10 mM HEPES, 60 mM KCl, 1 mM DTT, 1 mM EDTA, 7% glycerol, pH 7.6), 0.0175 pmol of labeled probe (>10,000 cpm), 20 μg of nuclear protein, and 2 μg of poly dIdC (Amersham Pharmacia Biotech Inc., Piscataway, USA). The reactions began with the addition of nuclear extracts, and were

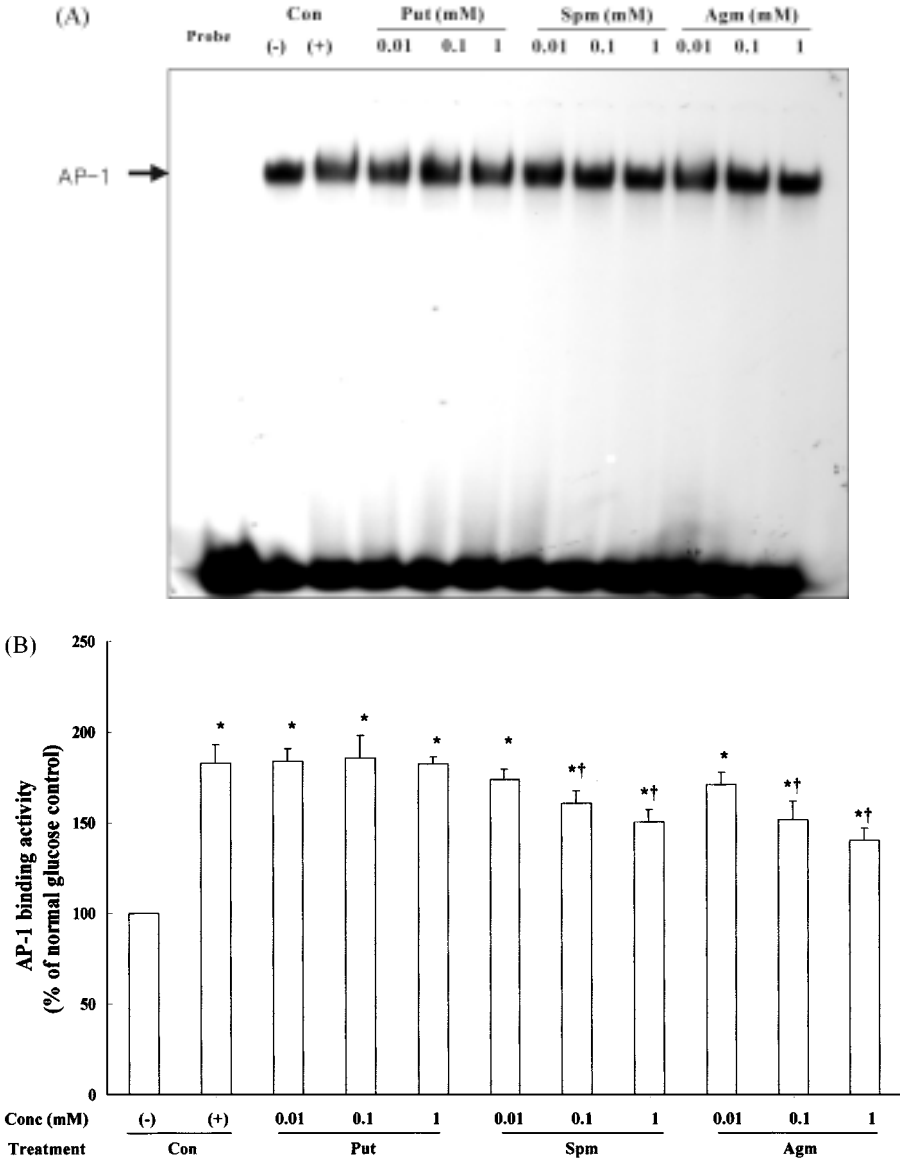

Fig. 2. Effects of polyamines and agmatine on nuclear factor-κB (NF-kappaB) DNA binding activity in mesangial cells. Synchronized quiescent MES-13 were stimulated under experimental conditions for 48 h. Nuclear proteins were prepared and subjected to an electrophoretic mobility shift assay (EMSA), as described in Materials and Methods. (A) A representative EMSA under different concentrations of polyamines and agmatine. (B) Bar graph showing the mean ± SD from three experiments. *, $p<0.05$ compared with 5.6 mM D-glucose + 24.4 mM mannitol control; †, $p<0.05$ compared with 30 mM glucose.

incubated for 30 min at room temperature. The samples were loaded on a 6% polyacrylamide nondenaturing gel and electrophoresed for 2 h at 180 V. The dried gel was exposed to Kodak XR5 film (Eastman Kodak, Rochester, USA) on an intensifying screen for 10 to 20 h at −70°C. For the competition assay, an unlabeled NF-κB oligonucleotide was added in 100-fold excess.

Assay of intracellular hydrogen peroxide Intracellular ROS production was measured by the method of Bass *et al.* (1997) as modified for confocal microscopy (Kim *et al.*, 2001). In brief, after stimulation with HG for 3 h, the confluent cells at various concentrations of polyamines and agmatine were washed with Dulbecco's phosphate-buffered saline. The cells were then incubated in the dark for 5 min in a Krebs-Ringer solution that contained 5 mM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Molecular Probes Inc., Eugene, USA). CM-H_2DCFDA is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed to the nonfluorescent polar derivative 2',7'-dichlorofluorescin (DCFH) and thereby trapped within the cells (Bass *et al.*, 1983). In the presence of a proper oxidant, DCFH is oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF).

A preliminary study demonstrated that HG induced intracellular ROS in MC as early as 15 min and gradually increased up to 4 h.

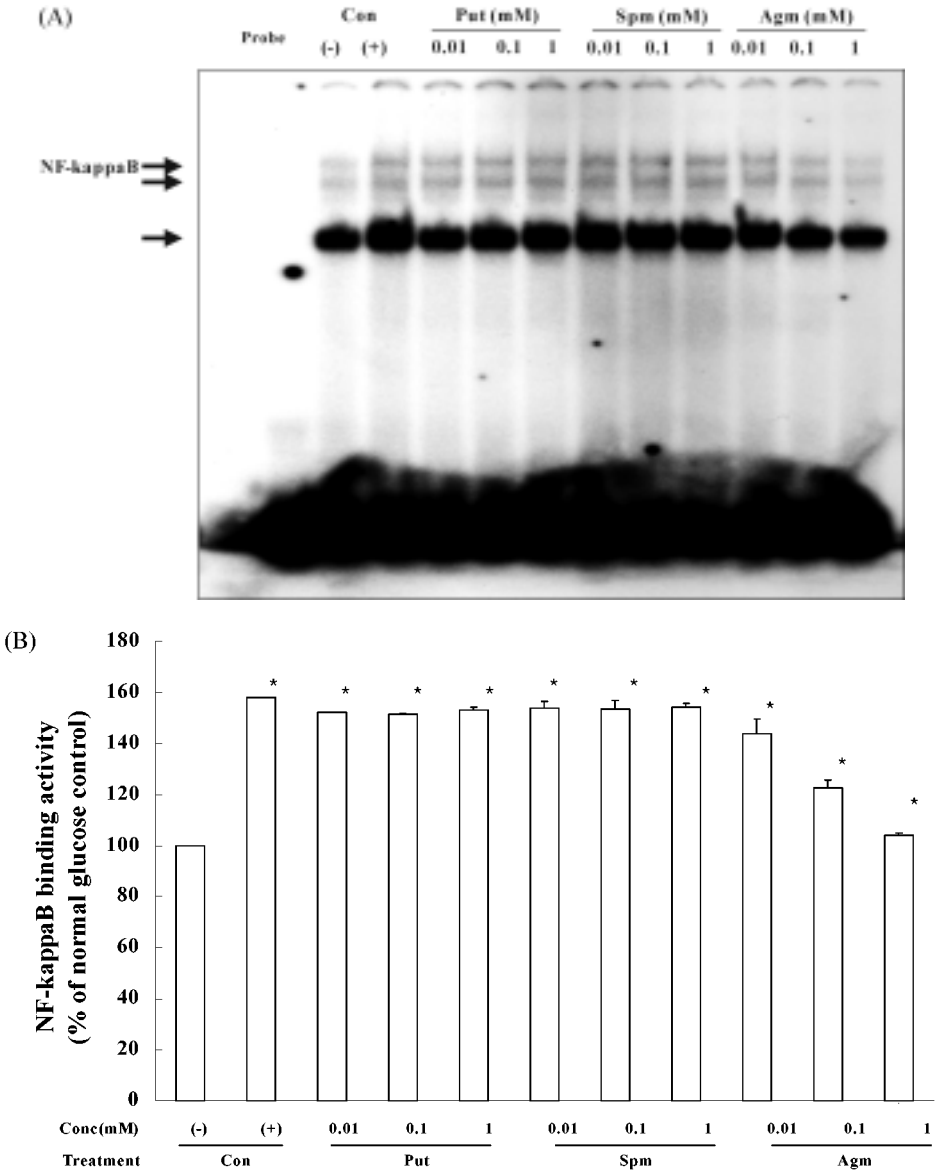

Fig. 3. Effects of polyamines and agmatine on activation factor-1 (AP-1) DNA-binding activity in mesangial cells. Synchronized quiescent MES-13 were stimulated under experimental conditions for 48 h. The nuclear proteins were prepared and subjected to an electrophoretic mobility shift assay (EMSA), as described in Materials and Methods. (A) A representative EMSA under different concentrations of polyamines and agmatine. (B) Bar graph showing the mean ± SD from three experiments. *, $p<0.05$ compared with 5.6 mM D-glucose + 24.4 mM mannitol control; †, $p<0.05$ compared with 30 mM glucose.

Assay for fibronectin protein: Western blot analysis An immunoblot analysis was used to determine fibronectin in the renal cortex homogenate and in the culture media supernatant as previously described (Oh *et al.* 1998). In brief, aliquots of the renal cortex homogenate and conditioned media were mixed with a sample buffer that contained SDS and β-mercaptoethanol and heated at 95°C for 15 min. Respective samples were then applied to a 5% polyacrylamide gel and subjected to electrophoresis. A prestained SDS-PAGE standard (broad range, Bio-Rad) was used as a molecular weight marker. The proteins were transferred onto a nitrocellulose membrane using a transblot chamber with a Tris buffer. The membranes were incubated with rabbit anti-human fibronectin (HRP conjugated, DAKO, Glostrup, Denmark), diluted 1 : 10000 in PBS that contained 1% bovine serum albumin for 2 h at room temperature, and subjected to ECL Western blotting (Amersham Life Science, Little Chalfont, UK). Positive immunoreactive bands were quantified densitometrically and compared to the controls.

Statistical analyses All of the results are expressed as mean ± SD. ANOVA was used to assess the differences between multiple groups. If the *F* statistic was significant, the mean values that were obtained from each group were then compared by Fishers least significant difference method. $P<0.05$ was the criterion for a statistically significant difference.

Results and Discussion

Recently, Cruz *et al.* showed that H_2O_2 increased the fibronectin accumulation (Cruz *et al*., 2001). They also proved that the accumulation was mediated by increased TGF-β1 activity. We tried to discover what compound decreases the H_2O_2 level in mesangial cells; H_2O_2 generated by hyperglycemia was decreased by agmatine via FACS (Fig. 1), although the mechanism that is responsible for the decreased-steady state levels of H_2O_2 after agmatine treatment was not clarified. If the decrease in fibronectin by H_2O_2 is due to the increased TGF-β1 activity (Cruz *et al.*, 2001), then the cells that were cultured in the presence of agmatine would be expected to show less TGF-β1 activity when compared to the control, since agmatine reduced the H_2O_2 levels. As expected after agmatine treatment, the TGF-β1 activity in the cell was reduced (Fig. 1).

Oxygen derivatives, such as H_2O_2, have been shown to activate the transcription factors NF-κB and AP-1 (Ishikawa *et al.*, 1997). Also, the TGF-β1 promoter contains the binding sequence for these factors (Eickelberg *et al.*, 1999). In brief, the signaling pathway can be depicted as follows; H_2O_2 -> transcription factors -> TGF-β1 -> ECM production. To determine whether the activities of the transcription factors increased or decreased under decreased H_2O_2 by agmatine, we measured the NF-κB and AP-1 activities via EMSA (Figs. 2, 3). As expected, these two activities decreased.

We observed that the fibronectin level was reduced in the presence of agmatine ranging from 0.01 to 5 mM (Fig. 4). We also discovered that agmatine (0.1-1 mM) did not affect the cell growth, but levels more than 5 mM retarded the growth (data not shown). Therefore, the decrease in fibronectin is not due to decreased cell growth or cell death at the agmatine concentrations that were used in our experiments.

Our cumulative results lead us to suggest the following: (1) Agmatine reduces H_2O_2 levels, which does not activate these

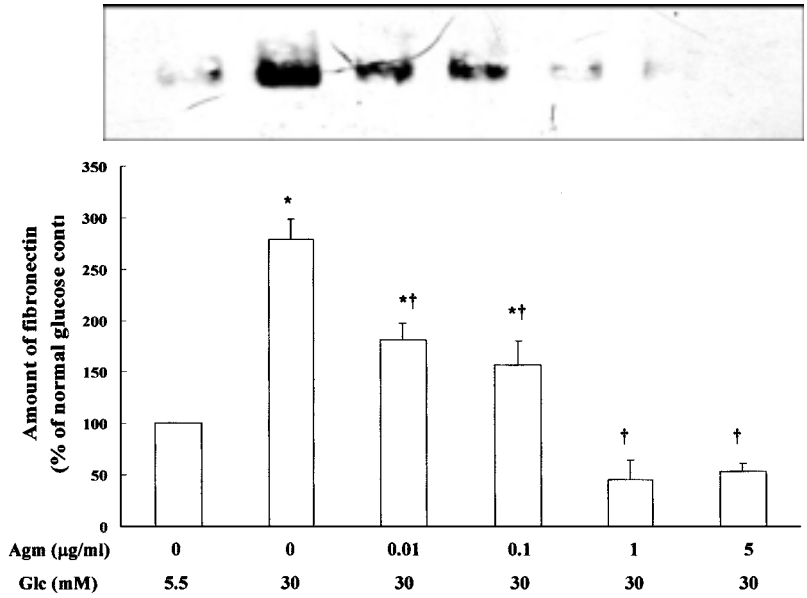

Fig. 4. Western blots of fibronectin protein secreted by mesangial cells. The growth-arrested and synchronized mesangial cells wer grown in different concentrations of agmatine that contained either 5.6 mM D-glucose + 24.4 mM mannitol or 30 mM of D-glucos Values represent means ± SD that were obtained from four individual experiments. *, $p<0.05$ compared with 5.6 mM D-glucose + 24.4 mM mannitol control; †, $p<0.05$ compared with 30 mM glucose.

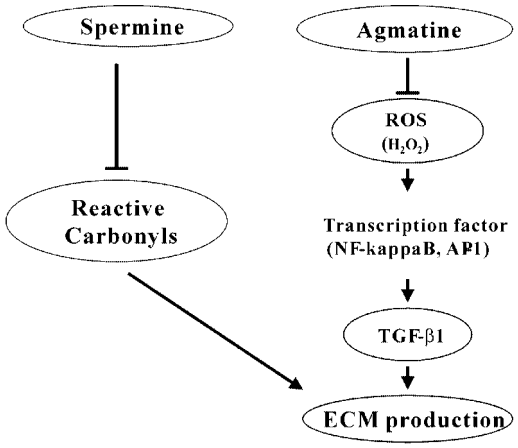

Fig. 5. Pivotal roles of agmatine on generation of reactive oxygen species and nuclear κB (NF-kappaB) and activating factor-1 (AP-1).

transcription factors. (2) It also results in a lowered binding of these factors to the TGF-β1 promoter. (3) Reduced TGF-β1 activity also reduces the fibronectin accumulation when compared to that of the control that was exposed to high glucose alone.

Both polyamine and agmatine have strong nucleophilic structures. Therefore, we expected the same biochemical or physiological roles that were suggested by Marx *et al.* However, we observed different roles among them; only the spermine that ranged from 0.01 to 1 mM reduced the H_2O_2 levels, AP-1 activity, and fibronectin accumulation (Fig. 4). But, the decreased AP-1 activity by spermine seemed to be insufficient to change the TGF-β1 activity. Under the steady-state activity of TGF-β1, spermine could reduce the fibronectin accumulation via another pathway (e.g. reaction with reactive carbonyls), suggested by Marx *et al.* 1995. On the basis of our findings, as well as those of Cruz *et al.* (2001), we propose the possible signaling pathway scheme in Fig. 5.

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