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Ascorbic acid and uric acid suppress glucose-induced fibronectin and vascular endothelial growth factor production in human endothelial cells

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Abstract

Human umbilical vein endothelial cells (HUVECs) were used to investigate the individual effect of ascorbic (0.01 and 0.05 mM) and uric acid (3, 5, and 7 mg/dl), as well as the combined effect of these two agents on glucose-induced overexpression of fibronectin (Fn), glutathione peroxidase (GPx) activity, H_2O_2 level and vascular endothelial growth factor (VEGF) protein levels. Under 30 mM glucose stress, GPx activity significantly decreased, and lactate dehydrogenase (LDH) release, H_2O_2 level, and Fn and VEGF production significantly increased in HUVECs ($P<05$). The addition of ascorbic acid at 0.05 mM or uric acid at 5 and 7 mg/dl significantly reduced LDH release, recovered GPx activity, suppressed H_2O_2 production, and decreased Fn and VEGF production ($P<05$). The combination of 0.05 mM ascorbic acid and 3, 5, or 7 mg/dl provided significantly greater effect in enhancing GPx activity and lowering H_2O_2 level than ascorbic acid or uric acid treatment alone did ($P<05$). These data suggest that ascorbic acid alone or combined with uric acid in these concentrations may be beneficial to ameliorate glucose-induced diabetic deterioration.

Keywords: Ascorbic acid; Uric acid; Fibronectin; Vascular endothelial growth factor

1. Introduction

Fibronectin (Fn) is an integral part of the capillary basement membrane and mesangial matrix, and the excessive accumulation of Fn is an important characteristic occurring in diabetic complications (Chen, Mukherjee, Chakraborty, & Chakrabarti, 2003; Roy, Sala, Cagliero, & Lorenzi, 1994). Many cell culture and animal studies have indicated high glucose stress that resulted in a decreased glutathione-dependent H_2O_2 elimination and enhanced cellular exposure to H_2O_2, which further induced Fn biosynthesis and up-regulated vascular endothelial growth factor (VEGF) protein expression (Kashiwagi, Asahina, Ikebuchi, & Tanaka, 1994; Koya, Haneda, Nakagawa, Isshiki, & Sato, 2000; Lin, Sahai, Chugh, Pan, & Elisabeth, 2002; Pinter, Haigh, Nagy, & Madri, 2001). The above adverse reactions certainly enhance the development of diabetic complications and other vascular diseases. Therefore, to ameliorate diabetic deterioration, the application of safe agents with protective effects is necessary.

Ascorbic acid, a natural antioxidant, is able to reduce oxidative stress and affect Fn biosynthesis in cultured cell (Armour, Tyml, Lidington, & Wilson, 2001; Peterszegi, Dagonet, Labat-Robert, & Robert, 2002; Rowe, Ko, Tom, Silverstein, & Richards, 1999). However, the influence of ascorbic acid on high glucose-induced Fn and VEGF production is unknown. In addition, the role of uric acid, another natural antioxidant, in the pathogenic development of diabetes is still controversial (Hassoun et al., 1992; Johnson et al., 2003; Mazzali et al., 2001). Furthermore, the relationship between these antioxidant agents on Fn and VEGF protein levels remains unclear. Therefore, we used human umbilical vein endothelial cells (HUVECs) to investigate the individual effect of ascorbic or uric acid, as

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Table 1
The influence of glucose, ascorbic acid (AA), and uric acid (UA) on total protein and released LDH in HUVECs

	Total protein (mg/ml)	Released LDH (%)
Control	0.49±0.03[a]	9.5±1.8[a]
Glucose, 10 mM	0.52±0.02[a]	18.8±1.5[b]
Glucose, 30 mM (G30)	0.50±0.04[a]	29.8±2.6[d]
G30+0.01 mM AA	0.48±0.05[a]	27.6±2.0[d]
G30+0.05 mM AA	0.51±0.02[a]	24.6±1.5[c]
G30+3 mg/dl UA	0.53±0.04[a]	28.1±2.3[d]
G30+5 mg/dl UA	0.52±0.03[a]	25.7±1.6[c]
G30+7 mg/dl UA	0.48±0.04[a]	24.3±1.4[c]
G30+0.05 mM AA+7 mg/dl UA	0.53±0.02[a]	23.0±2.1[c]

Values are means±S.D.; n=5.
Means in a column without a common letter differ, $P<.05$.

well as the combined effect of these two agents on GPx activity, H_2O_2 level, and Fn and VEGF protein levels.

2. Materials and methods

2.1. Materials

Medium, plates, antibiotics, and chemicals used for cell culture were purchased from Difco Laboratory (Detroit, MI). Enzymes and antibodies were purchased from Sigma (St. Louis, MO). Other chemicals were purchased from Bio-Rad Chemical (Hercules, CA).

2.2. Cell culture

HUVECs, obtained from the Food Industry Research and Development Institute (Shin-chu City, Taiwan), were grown on 0.2% gelatin-coated tissue culture plates in an endothelial cell growth medium at 37 °C in a humidified 5% CO_2 atmosphere. The media were supplemented with 200 U/ml penicillin and 0.2 g/l streptomycin. Subconfluent HUVECs were starved in endothelial cell basal medium containing 1% bovine serum albumin for 16 h. HUVECs of fourth to fifth passages were used for the experiments. HUVECs were plated at 4×10^5 cells/well in 2% gelatin-coated 12-well, flat-bottom plates and subsequently treated with glucose and other agents.

2.3. Treatments

Glucose at 0, 10, and 30 mM was added into HUVECs medium. Then, ascorbic acid at 0.01 and 0.05 mM or uric acid at 3, 5, and 7 mg/dl was added into HUVECs medium to study the individual effect of these agents. The combined effect of 0.05 mM ascorbic acid and uric acid at 3, 5, and 7 mg/dl was also examined. After 72 h incubation at 37 °C with a 5% CO_2 humidified atmosphere, HUVECs with various treatments were then incubated in 0.25% BSA/Dulbecco's Modified Eagle Medium to harvest cells, and culture media were collected for the following assays.

2.4. Total protein determination and lactate dehydrogenase (LDH) release

The total protein level (mg/ml) in HUVEC lysates was determined by a colorimetric assay (Bradford, 1976). According to the method of Korzeniewski and Callewaert (1983), LDH activity (slope=ΔOD_{490}/min) was measured by using a nonradioactive cytotoxicity assay kit (Promega, Madison, WI). The absorbance at 492 nm was measured by an ELISA reader. The percent of LDH released from HUVECs was measured by using the formula: % release= LDH activity in supernatant/(LDH activity in supernatant+LDH activity in cell lysate).

2.5. Glutathione peroxidase (GPx) assay, H_2O_2 assay, and Fn measurement

The GPx activity (U/mg protein) in HUVECs was determined by using GPx assay kit (EMD Biosciences, San Diego, CA). The H_2O_2 level in HUVECs was assayed by a phenol red/horseradish peroxidase technique (Kettle, Carr, & Winterbourn, 1994). Fn level in HUVECs was determined by rabbit antihuman Fn antiserum and goat antirabbit IgG horseradish peroxidase conjugated antibody and was finally quantified by ELISA (Sakata, Meng, & Takebayashi, 2000).

2.6. Measurement of VEGF protein level

HUVECs were scraped off into HBSS/BSA solution and pelleted after centrifugation. Pellets were lysed in 0.05 mol/l Tris (pH 7.4), 0.15 mol/l NaCl, and 1% Nonidet P40 and followed by a 10-s sonication. The cell lysate was

Table 2
Fn level (Fn/protein, mg/mg) in HUVECs treated with glucose (0 and 30 mM) plus ascorbic (0.01 and 0.05 mM) and/or uric acid (3, 5, and 7 mg/dl)

	Fibronectin	
Treatment (glucose)	0 mM	30 mM
Control	0.041±0.004[a]	0.074±0.008[d*]
AA		
0.01 mM	0.042±0.002[a]	0.045±0.003[b]
0.05 mM	0.040±0.002[a]	0.039±0.001[a]
UA		
3 mg/dl	0.041±0.003[a]	0.057±0.003[c*]
5 mg/dl	0.044±0.003[a]	0.051±0.001[c]
7 mg/dl	0.042±0.002[a]	0.048±0.002[c]
0.05 mM AA		
+3 mg/dl UA	0.045±0.002[a]	0.050±0.005[c]
+5 mg/dl UA	0.043±0.002[a]	0.046±0.001[b]
+7 mg/dl UA	0.046±0.003[a]	0.043±0.001[b]

Values are means+S.D.; n=5.
Means in a column without a common letter differ, $P<.05$.
* $P<.05$ statistically different from corresponding 0 mM glucose group.

Table 3
GPx activity (nmol NADPH/min/mg protein) and H_2O_2 level (μM) in HUVECs treated with glucose (0 and 30 mM) and ascorbic (AA; 0.01 and 0.05 mM) and/or uric acid (UA, 3, 5, and 7 mg/dl)

	GPx activity		H_2O_2 level	
Treatment (glucose)	0 mM	30 mM	0 mM	30 mM
Control	22.5±1.8[a]	15.2±1.3[a]*	0.5±0.04[b]	0.72±0.09[c]*
AA				
0.01 mM	24.2±2.0[a]	15.5±1.6[a]*	0.55±0.07[b]	0.69±0.06[c]*
0.05 mM	24.2±2.0[a]	17.5±1.6[b]*	0.55+0.07[b]	0.65±0.06[b]
UA				
3 mg/dl	23.5±1.8[a]	15.4±1.0[a]*	0.45±0.07[a]	0.71±0.05[c]*
5 mg/dl	25.4±2.0[a]	19.4±1.5[b]*	0.47±0.04[a]	0.62±0.06[b]*
7 mg/dl	25.2±2.1[a]	20.2±1.8[b]*	0.46±0.08[a]	0.63±0.04[b]*
0.05 mM AA				
+3 mg/dl UA	26.3±2.5[d]	22.6±l.4[c]*	0.46±0.03[a]	0.49±0.06[a]
+5 mg/dl UA	26.6±1.9[d]	24.3±2.2[c]*	0.48±0.05[a]	0.52±0.05[a]
+7 mg/dl UA	28.2±2.0[d]	22.1±1.9[c]*	0.47±0.05[a]	0.47±0.07[a]

Values are means±S.D.; n=5.
Means in a column without a common letter differ, $P<.05$.
* $P<.05$ statistically different from corresponding group with 0 mM glucose treatment.

centrifuged at 10,000×g for 10 min, and the supernatant was collected. VEGF concentration in cell lysate was determined by a quantitative ELISA method (R & D Systems, Minneapolis, MN) according to the manufacturer's instruction (Sartippour et al., 2002). The amount of VEGF immunoreactivity was calculated using recombinant human VEGF standards present on each microtiter plate.

2.7. Statistical analysis

Results were expressed as mean±S.D. The statistical significance of differences between various glucose concentrations, and between various ascorbic and uric acid treatments was determined by using the parametric Student's t test. A P value <.05 was considered significant.

3. Results

The total protein level and released LDH percent in HUVECs under various treatments are presented in Table 1. These treatments did not significantly affect the total protein level ($P>.05$). The released LDH level was significantly increased in 30 mM glucose-treated HUVECs ($P<.05$). The addition of 0.05 mM ascorbic acid, 5 and 7 mg/dl uric acid, and 0.05 mM ascorbic acid, plus 7 mg/dl uric acid, significantly decreased 30 mM glucose-induced LDH release ($P<.05$).

The effect of various treatments on Fn level in HUVECs is presented in Table 2. Under 30 mM glucose stress, HUVECs significantly increased Fn production ($P<.05$); however, the presence of ascorbic and/or uric acid at these test concentrations significantly suppressed 30 mM glucose-induced Fn elevation ($P<.05$).

The effect of various treatments on GPx activity and H_2O_2 levels in HUVECs is presented in Table 3. GPx activity was significantly decreased, and H_2O_2 level was significantly elevated in 30 mM glucose-treated HUVECs ($P<.05$). The addition of 0.05 mM ascorbic acid, or 5 and 7 mg/dl uric acid, and all combined treatments significantly recovered GPx activity ($P<.05$) and reduced H_2O_2 production ($P<.05$). Furthermore, the combination of 0.05 mM ascorbic acid and 3, 5, or 7 mg/dl uric acid showed significantly greater effect in enhancing GPx activity and lowering H_2O_2 level than did ascorbic or uric acid treatment alone ($P<.05$).

Relative VEGF protein levels in HUVECs with various treatments are presented in Table 4. The VEGF protein level in HUVECs treated with 30 mM glucose was significantly higher ($P<.05$), the addition of 0.05 mM ascorbic acid or 5 and 7 mg/dl uric acid and 0.05 mM ascorbic acid plus

Table 4
Relative VEGF protein level (%) in HUVECs treated with 0, 10, and 30 mM glucose (G30), G30 plus 0.01 and 0.05 mM ascorbic acid (AA), G30 plus 3, 5, and 7 mg/dl uric acid (UA), and G30 plus 0.05 mM ascorbic acid and 7 mg/dl uric acid

	Relative VEGF protein level
Control	100[a]
Glucose, 10 mM	118±5.8[b]
Glucose, 30 mM (G30)	142±7.2[d]
G30+0.01 mM AA	137±5.5[d]
G30+0.05 mM AA	129±4.2[c]
G30+3 mg/dl UA	138±6.6[d]
G30+5 mg/dl UA	132±4.8[c]
G30+7 mg/dl UA	125±5.4[b]
G30+0.05 mM AA+7 mg/dl UA	121±6.2[b]

Values are means±S.D.; n=5.
Means in a column without a common letter differ, $P<.05$.

7 mg/dl uric acid significantly decreased VEGF protein level ($P < .05$).

4. Discussion

Reactive oxygen species (ROS) are important contributors toward the pathogenic development of diabetic complications (Kashiwagi et al., 1994; Leehey, Song, Alavi, & Singh, 1995). It has been reported that oxidative stress is directly involved in the up-regulation of Fn and VEGF proteins; thus, the use of ascorbic acid as oxidants scavenger and reducer could prevent oxidative injury to cultured endothelial cells and decrease Fn and VEGF biosynthesis in cultured cells (Armour et al., 2001; Peterszegi et al., 2002). The results of our present study agreed with those previous studies and supported that ascorbic acid could decrease oxidative injury via its enzymatic antioxidant protection and ameliorate glucose-induced Fn and VEGF overproduction. In addition, our present study further found that uric acid alone could also ameliorate high glucose oxidative stress and reduce LDH release, recover GPx activity, decrease H_2O_2 level, and suppress high glucose-induced Fn and VEGF overproduction. Furthermore, the combination of ascorbic and uric acids exhibited markedly antioxidant protection and VEGF suppressive effect. These data from ascorbic and uric acids supported that ROS was directly involved in the glucose-induced expression of Fn and VEGF proteins. Thus, the presence of these two agents could effectively scavenge ROS and consequently decrease ROS stimulated both Fn and VEGF production in cultured HUVECs. These data suggest that ascorbic and/or uric acid could be considered as effective agents to attenuate hyperglycemic stress. Further in vivo study is necessary to verify the protective effect of these two agents.

Uric acid may have detrimental action toward the pathogenic development of cardiovascular diseases because Mazzali et al. (2001) reported that uric acid stimulated vascular smooth muscle cell proliferation and induced endothelial dysfunction. However, it is known that uric acid can act as a peroxynitrite scavenger (Wangsiripaisan et al., 1999), and the results of our present study also found that uric acid enhanced enzymatic antioxidant protection in glucose-treated HUVECs and consequently contributed to the reduction in LDH release and Fn and VEGF protein expression. We believed that this is the first report regarding the contribution of uric acid in suppressing Fn and VEGF production. Because uric acid at these physiological concentrations may be beneficial to retard or delay glucose-induced diabetic deterioration, the role of uric acid in the development of diabetic pathogenesis should be carefully reconsidered.

In conclusion, uric acid alone or combined with ascorbic acid reduced LDH release and exhibited marked antioxidant protection and suppressed oxidation-related Fn and VEGF production. Therefore, these two agents, at these concentrations, may be beneficial to ameliorate glucose-induced diabetic deterioration.

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