Andrographolide and 14-deoxy-11,12-didehydroandrographolide from *Andrographis paniculata* attenuate high glucose-induced fibrosis and apoptosis in murine renal mesangial cell lines

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**A R T I C L E   I N F O**

Article history:
Received 25 February 2010
Received in revised form 26 May 2010
Accepted 6 July 2010
Available online 9 September 2010

Keywords:
Andrographolide
14-Deoxy-11,12-didehydroandrographolide
MES-13 cells
Diabetic nephropathy

**A B S T R A C T**

Ethnopharmaceutical relevance: Extracts of *Andrographis paniculata* Nees are used for various ethnomedical conditions including hyperglycemia and hypertension complications.

**Aim of the study:** The purpose of this study is to evaluate the anti-diabetic nephropathy effect of diterpene lactones andrographolide (AP1) and 14-deoxy-11,12-didehydroandrographolide (AP2) from *Andrographis paniculata*.

**Materials and methods:** MES-13, a SV40-transformed murine glomerular mesangial cell line, was cultured in high concentration of glucose to induce diabetic nephropathy phenotypes, which include secretion of extracellular matrix protein fibronectin, cytokine TGF-β, states of oxidative stress, and apoptosis marker caspase-3.

**Results:** Our data suggest that addition of compounds AP1 or AP2 reduces the phenotypes indicating diabetic nephropathy in MES-13 cells. The compound AP2 showed potent activity than AP1 in the reduction of apoptosis marker caspase-3, fibrosis marker TGF-β, and PAI-1. Furthermore, AP1 and AP2 do not have antioxidant ability in acellular environment; however, addition of AP1 and AP2 reduced intracellular oxidative states in high glucose cultured MES-13 cells.

**Conclusion:** This is the first report on anti-diabetic nephropathy effect of AP1 and AP2 in part due to the regulation of intracellular signaling transduction, not mere clearance of reactive oxygen species. Thus, this study may be useful for drug development or food supplement for diabetes and nephropathy from *Andrographis paniculata*.

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1. **Introduction**

*Andrographis paniculata* Nees (Acanthaceae) is a medicinal plant widely cultivated in tropical regions in Asia. Traditionally it is used for the treatment of cold, fever, laryngitis and infection in many Asian countries. Extract of the plant is reported to possess immunological, antibacterial, anti-inflammatory, antithrombotic, hepatoprotective, anti-hypertensive, and anti-diabetic activities (Mishra et al., 2007). The extract of the plant is a rich source for flavonoids and labdane diterpenoids (Rao et al., 2004; Geethangili et al., 2008). Of the diterpenoids, andrographolide (AP1) and 14-deoxy-11,12-didehydroandrographolide (AP2) were isolated from the 95% alcoholic extract obtained from the aerial parts of *Andrographis paniculata* (Rao et al., 2008). AP1 is a diterpene containing a β-lactone ring connected to a decalin ring system via an unsaturated C-2 moiety. AP2 is different from AP1 in dehydrogenation at 11, 12 positions in the diterpene, and deoxygenation in the 14 position of the β-lactone ring (Fig. 1).

AP1 regulates several factors of the innate as well as the adaptive immune response (Chiou et al., 1998; Burgos et al., 2005; Iruretagoyena et al., 2005; Qin et al., 2006), protects the cardiovascular system (Thisoda et al., 2006; Woo et al., 2008), possesses anti-hepatotoxic activities (Kapil et al., 1993; Akowuah et al., 2008), and shows significant anti-viral and anti-cancer activities (Kumar et al., 2004; Kim et al., 2005; Wiart et al., 2005; Ji et al., 2007; Sheeja and Kuttan, 2007; Geethangili et al., 2008; Shi et al., 2008; Zhao et al., 2008). AP2 shows a similar spectrum of activities as found in AP1 including antioxidant (Zhang et al., 1998; Kumar et al., 2004), cardioprotective (Zhang and Tan, 1999; Yoopan et al., 2007; Woo et al., 2008), anti-viral (Wiart et al., 2005), and antitumor abilities (Kumar et al., 2004; Geethangili et al., 2008). Effect of anti-diabetes was reported for AP1. It enhances the uptake of glucose and level of GLUT4 (Yu et al., 2003), and causes reduction in peak blood glucose in diabetic rats (Subramanian et al., 2008). Its action mechanism is suggested via activating α1-angiotensin II receptor to enhance the

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Diabetic nephropathy (DN) is characterized by renal hypertrophy, accumulation of extracellular matrix components, glomerulosclerosis, tubular atrophy and interstitial fibrosis. DN is due to longstanding diabetes mellitus, and is a prime cause for dialysis in many Western countries. Glomerulosclerosis leads to fluid filtration deficits, hypertension, arteriosclerosis of the renal artery, and proteinuria. For the existing therapy, the first line of treatment is controlling blood-glucose level, however anti-hypertension drugs are suggested drugs. However, renal protection of drug faces the challenge of side effect (dry cough), as well as patient resistance. Renal protection for DN benefits from combination of drugs (Codreanu et al., 2005) and multifactoral treatment (Fioretto and Solini, 2005), therefore development of a new drug or food supplement that acts on multiple factor of DN, is very much needed.

Herbal extracts used in the ancient culture often act on multiple aspects of the disease and were passed on because of their excellent activities. It is possible that extract of Andrographis paniculata Nees was similarly versatile. While both hyperglycemia and hypertension contribute to the diabetic nephropathy, effects of AP1 and AP2 for ameliorating both were reported (Yu et al., 2003; Hsu et al., 2004; Reyes et al., 2006; Yoopan et al., 2007; Yu et al., 2008). Although the constituents of Andrographis paniculata are reported to have anti-diabetic potency, the precise active compounds responsible for diabetic nephropathic activity of this plant have not been clearly identified. In the continuation of our search for the bioactive natural products (Rao et al., 2006, 2009; Lee et al., 2009; Tzeng et al., 2009), we are intended to test how AP1 and its related compound AP2 from Andrographis paniculata act on high glucose afflicted renal cells. High blood glucose-induced advanced glycation end product and transforming growth factor (TGF)-β secretion of tubular cells, and subsequent fibronectin/extracellular matrix (ECM) deposition and cell death are proposed for the etiopathology of DN (Ayo et al., 1990; Ziyadeh et al., 1994; Oh et al., 1998; Ha and Lee, 2000). Plasminogen activator inhibitor type 1 (PAI-1) inhibits fibrinolysis and ECM degradation contributes to TGF-β mediated ECM deposition (Rerolle et al., 2000). Part of the DN pathology could be reproduced in vitro by culturing tubular cell lines or primary mesangial cells in a high glucose concentration. Induction of increased TGF-β, PAI 1, and fibronectin secretion could be measured in the culture medium, and cell apoptosis measured by level of cleaved caspase-3 (MacKay et al., 1988; Wolf et al., 1992; Kang et al., 2003). We took advantage of this system to test the single compounds AP1 and AP2 from Andrographis paniculata for their possible activities on DN. The results are reported herein.

2. Materials and methods

2.1. Extraction and isolation of AP1 and AP2

The compounds andrographolide (AP1) and 14-deoxy-11,12-didehydroandrographolide (AP2) (Fig. 1) were obtained from the 95% alcoholic extract obtained from the air-dried and powdered aerial parts of Andrographis paniculata. The extraction and isolation procedures were described in our previous reports (Rao et al., 2004; Geethangili et al., 2008).

2.2. Murine mesangial cell (MMC) culture

SV40-transformed MMCs (MES-13) were obtained from the Bioresource Collection and Research Center, Food Industry Research and Development Institute, Taiwan, and maintained in a 3:1 mixture of DMEM and Ham’s F-12 medium containing 5% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), HEPES (14 mM), and glucose (100 mg/dl) at 37°C in an atmosphere containing 5% CO2–95% air. After reaching confluence, cells were replated in culture medium for 7 h, followed by subsequent incubation in the above stated medium without FBS (serum free medium) for 2 days. To test the response of high glucose concentration and/or single compound, the cells were then incubated in the serum free medium that was supplemented with 25 mM glucose, with or without the compound for 1 day. The cells or the medium were then collected and the protein was analyzed. The extent of cells undergoing apoptosis was determined by activated caspase-3 levels. The level of fibrosis was determined by the amount of fibronectin, TGF-β, and PAI-1 secreted by the cells.

2.3. Western blot

MES-13 cells were trypsinised and the cell pellet was collected. The cells were lysed in lysis buffer containing 1 mM Tris–HCl, 0.1 mM EDTA, pH 8.0 and 200 mM PMSF, 1 mg/ml aprotinin, and supernatant was collected. The culture medium was collected and freeze-dried, and resuspended in 1/10 of original volume with lysis buffer. After immunoblotting, the concentrated medium or the cell lysate were boiled in lysis buffer with β-mercaptoethanol for 5 min. The cell lysates or the culture media were separated on a 7.5%–10% SDS-PAGE, and transferred to polyvinylidene fluoride (PVDF) membrane. The PVDF membranes were incubated in 0.1% milk in PBS (phosphate-buffered saline with 0.1% Tween-20) for 1 h to rid them of background staining, followed by overnight incubation in the primary antibodies at 4°C. Primary antibodies used were: anti-TGF-β (MBL), anti-fibronectin (Sigma), anti-rat PAI-1 (American Diagnostica, Greenwich, CT, USA; 1:1000), anti-cleaved caspase-3 (Cell signaling), and anti-actin (Chemicon). The secondary antibody used was anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (Amersham) for all. After the first antibody, the blots were stripped and actin was stained and used as normalization standard. After washing, blotted proteins were visualized using a Western blotting detection system (ECL Plus, Amersham, UK) and quantified with NIH Image software.
2.4. Measurement of cellular redox potential

Cellular redox potential was examined following the distribution and oxidation of RedoxSensor Red CC-1 (Molecular Probes, Eugene, OR) in mitochondria. Cells were grown on coverslips (1 × 10^4 cells/100 μl) at 37 °C. After replating and cultured in serum free medium as described, cells were cultured in normal or high concentration of glucose supplemented with various concentrations of test compounds for 1 day. After different treatment, cells were incubated with 2 μM RedoxSensor Red CC-1 and 200 mM Mito Tracker Green FM (Molecular Probes) for 15 min. The cells were then washed with phosphate-buffered saline (PBS) and were fixed with 4% paraformaldehyde in PBS for 15 min and examined under a Zeiss Axiocam CCD camera mounted on Zeiss Axioscope 2. The images were captured with appropriate filter sets. Mitochondria were stained by Mito Tracker Green FM. To evaluate the intracellular oxidative state of the cells, estimation of the fluorescence intensity of RedoxSensor Red CC-1 was performed using NIH image software. The mean intensity of the six randomly chosen fixed-sized squares from the fluorescence photography was obtained for estimating the fluorescence intensity. The difference between low glucose control and the sample treated was statistically analyzed.

2.5. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay

The DPPH radical scavenging ability of compounds AP1 and AP2 was determined according to the previously described procedure (Rao et al., 2007) with slight modification. The AP1 and AP2 solution (100 μg/ml) was mixed with 400 μM DPPH (Sigma Chemical Co., St Louis, MO) methanol solution at a ratio of 1:3. The mixture was left in the dark at room temperature for 30 min. The absorbance was measured by a spectrophotometer at 517 nm. The capability of scavenging DPPH radicals was then calculated by the following equation: scavenging response% = [1 − (A517 of sample/A517 of control)] × 100.

2.6. Ferric reducing antioxidant power (FRAP) assay

The FRAP reagent contained 2.5 ml of a 10 mM/l, 2,4,6-tripyridyl-s-triazine (TPTZ) (Sigma) solution in 40 mM HCl plus 2.5 ml of 20 mM FeCl3·6H2O and 25 ml of 0.3 ml acetate buffer, pH 3.6. Aliquots of 6 μl of AP1 or AP2 were mixed with 180 μl FRAP reagent and the absorbance of the reaction mixture at 593 nm was measured spectrophotometrically after incubation at room temperature for 3 min. The FRAP activity was calculated by the equation:

\[ \text{FA} = \left( \frac{A_C}{A_A} \right) \times 100\% \]

where FA is the FRAP activity, \( A_C \) is the absorbance of the each component at 1 mM, \( A_A \) is the absorbance of the FeCl2 at 1 mM. Trolox at the same concentration as FeCl2 (1 mM) was used as the positive sample. Results were expressed as percentage of 1 mM FeCl2 equivalent.

2.7. Evaluation of anti-lipid peroxidation activity

Lipid peroxidation was promoted by FeCl3–ascorbic acid system. The reaction mixture consists of 10 mg/ml suspension of lecithin (2 ml), 25 mM FeCl3 (0.1 ml), 25 mM ascorbic acid (0.1 ml), 20 mM Na2HPO4–NaH2PO4 buffer (pH 7.4, 1.2 ml), and 0.5 ml sample solution of various concentrations. The reaction was incubated at 37 °C for 2 h before the lipid peroxidation products were measured by the formation of thiobarbituric acid-reactive material, malondialdehyde (Janero, 1990). After incubation, 200 mM BHT 0.1 ml, 0.67% thiobarbituric acid 1 ml and 30% trichloroacetic acid 1 ml were added to the mixture and incubated at 90 °C water bath for 45 min. After cooling the absorbance of supernatant was measured spectrophotometrically at 532 nm. The inhibition of FeCl2/H2O2-stimulated lecithin peroxidation (%) was calculated as follows: inhibition of peroxidation (%) = \( 1 - \left( \frac{A_{\text{sample}}}{A_{\text{control}}} \right) \) × 100. 1,1,3,3-tetraethoxypropane was used as a control.

2.8. Statistical analysis

All determinations were carried out in triplicates. The results are expressed as mean values ± standard deviation (n = 3). Data are compared using one-way ANOVA. p value < 0.05 was considered statistically different. p value < 0.01 was considered significantly different.

3. Results

3.1. AP1 and AP2 protected cells from high glucose-induced apoptosis

Cells were treated with 25 mM glucose (‘high glucose group) in a DMEM/Ham’s F-12 based serum free medium, or high glucose with the addition of 1–20 μM AP1 or AP2, for 1 day, before caspase-3 levels were examined. With high glucose treatment, the cleaved caspase-3 level was up-regulated to 2.5 times of the ‘normal glucose’ treatment (100 mg/dl). AP1 at 10 μM down-regulates the level of activated caspase-3 to be lower than the level with high glucose treatment (p < 0.05), and at 20 μM the inhibition was not significant (Fig. 2A). For AP2, co-incubation of 10 μM AP2 inhibits the elevated activated caspase-3 level to be lower than that with high glucose treatment (p < 0.05). At 20 μM the response is more significant (p < 0.01), and comparable to the level with normal glucose treatment (Fig. 2B). In other words, AP2 is more effective than AP1 in reducing the apoptosis molecular marker caspase-3.

3.2. AP1 and AP2 reduced secreted transforming growth factor (TGF-β) level

Treatment of cells for this test included normal glucose, high glucose, and high glucose with the addition of 1–20 μM AP1 or AP2. Cells were incubated for 1 day before medium was collected, freeze-dried, and TGF-β level examined. With high glucose treatment (25 mM), the secreted TGF-β level was up-regulated to 2.5 times of the one with the low glucose treatment (100 mg/dl). AP1 and AP2 both showed significant effects in reducing TGF-β levels secreted, but AP2 is more potent than AP1 (Fig. 3). AP1 inhibited TGF-β secretion at 10 μM and 20 μM (p < 0.05), and these TGF-β levels were similar to that with normal glucose treatment (Fig. 3A). Noticeably, compound AP2 inhibited TGF-β secretion at 1 μM (p < 0.01), and this TGF-β level was about 50% of the normal glucose control. The inhibition sustained at 10–20 μM (p < 0.01) (Fig. 3B).

3.3. AP1 and AP2 reduced secreted fibronectin levels

The cells were treated as those for testing TGF-β level as above. With high glucose treatment (25 mM), the secreted fibronectin level was up-regulated to 1.6 times of the one with low glucose treatment. AP1 and AP2 showed similar effects for inhibiting secreted fibronectin (Fig. 4). They were not effective at 1 μM, but significantly reduced the level to be lower than high glucose treatment at 10 μM (p < 0.05) and at 20 μM (p < 0.01, Fig. 4).

3.4. AP1 and AP2 reduced secreted plasminogen activator inhibitor type 1 (PAI-1) levels

The cells were treated as those for testing TGF-β level as above. With high glucose treatment (25 mM), the secreted PAI-1 level was up-regulated to two times of the one of low glucose treatment. AP1
Fig. 2. Effects of AP1 and AP2 on level of cleaved caspase-3 in cells from high glucose-induced apoptosis. The cells were treated with high glucose concentration (25 mM) in a DMEM/Ham’s F-12 based serum free medium (N), or high glucose (H) or high glucose with the addition of 1–20 μM AP1 (A), or 1–20 μM AP2 (B) for 1 day, before caspase-3 levels were examined using Western blotting. Staining of actin was performed after caspase-3 staining and stripping, for validating equal loading of protein. The level of caspase-3 was analysed with NIH image I software, and normalized against the amount for actin. Bar graphs represent the mean values of triplicate determinations ± SD. *p < 0.05; **p < 0.01 compared with the control.

and AP2 showed similar effects for inhibiting secreted PAI-1. They significantly reduced the level of PAI-1 to be lower than that of high glucose treatment at all treatment tested (p < 0.01) (Fig. 5).

3.5. AP1 and AP2 reduced the high glucose-induced oxidative states in MES-13 cells

The cells were treated with high glucose concentration (25 mM) in a serum free medium, with or without 1–20 μM AP1 or AP2, for 1 day, and incubated with the presence of Red CC-1 (Molecular Probes). Without the addition of AP1 or AP2, the incubation of high glucose increased the oxidative state of the MES-13 cells (Fig. 6A). The addition of 0.1% DMSO in the medium marginally elevated the oxidative state (Fig. 6B). The addition of AP1 or AP2 to the high glucose medium reduced the intensity of fluorescence stained by Red CC-1 (Fig. 6A). Incubation of 0.1 μM AP1 reduced the oxidative state (p < 0.05), and 1 μM AP1 was most potent (p < 0.01). The effect was not significant as the concentration rose to 20 μM, possibly caused by higher concentration of DMSO (Fig. 6B). For AP2 0.1 μM gives best results in inhibiting oxidative states (p < 0.01), and also

Fig. 3. Effects of AP1 and AP2 on level of secreted TGF-β in cells cultured in high glucose. The cells were treated as described in Section 2, before medium was collected, freeze-dried, and level of TGF-β was examined using Western blotting. The level of TGF-β was analyzed with NIH image I software, and normalized against the amount for actin. High glucose (H) induced 2.5× of TGF-β than normal glucose (N). The inhibition effects of AP2 were better than those of AP1, as at 1 μM AP2 already inhibited the level to be significantly lower than the high glucose control (B), while AP1 at the same concentration did not show inhibition (A). One-way ANOVA was performed. *p < 0.05; **p < 0.01.
at 1–100 μM (p < 0.05). The oxidative states became higher as concentration of AP2 rose, possibly caused by higher concentration of DMSO (Fig. 6B). The best effective concentration for AP1 (1 μM) is 10 times higher than that for AP2 (0.1 μM), therefore AP2 showed better effect for reducing oxidative states in the MES-13 cells than AP1.

3.6. Capacities of free radical scavenging, ferric ion reducing activities, and anti-lipid peroxidation levels for AP1 and AP2 were low

Capacity of free radical scavenging was tested using DPPH assay, ferric ion reducing activity was tested using FRAP assay, and anti-lipid peroxidation was tested using thiobarbituric acid-reactive substances (TBARS) assay, as described in Section 2. Potent antioxidant trolox was included in the same experiment as a positive control. The values from the three tests were all very low for both AP1 and AP2. In the DPPH assay, 100 μM of AP1 and AP2 yielded 0.21 and 0.17% respectively, while the positive control trolox gave 97.42% (Fig. 7A). For FRAP assay, when the reducing power for 1000 μM trolox equalled 2.15 FRAP unit (1 FRAP unit = 10 μM Fe^{2+}), 1000 μM AP1 and AP2 gave 0.04 and 0.03 FRAP unit (Fig. 7B), respectively. In the TBARS assay, 100 μg/ml of trolox gave 40.62% inhibition, while AP1 and AP2 only gave 1.83, 1.89%, respectively (Fig. 7C).

Fig. 4. Effects of AP1 and AP2 on secreted fibronectin levels. The cells were treated as described in Section 2, before medium was collected, freeze-dried, and level of fibronectin was examined using Western blotting. The level of fibronectin was analyzed with NIH image I software. High glucose (H) induced 1.6× of fibronectin than normal glucose (N). Addition of AP1 at 10–20 μM inhibits the level of fibronectin. Effect of AP2 on fibronectin secretion is similar to that of AP1. One-way ANOVA was performed. N= 3.*p < 0.05; **p < 0.01.

Fig. 5. Effects of AP1 and AP2 on secreted PAI-1 levels. The cells were treated (as described in Section 2) before medium was collected, freeze-dried, and level of PAI-1 was examined using Western blotting. The level of PAI-1 was analyzed with NIH image I software. High glucose (H) induced 2× of PAI-1 than normal glucose (N). Addition of AP1 at 0.1–20 μM significantly inhibits the level of PAI-1. Effect of AP2 on PAI-1 secretion is similar to that of AP1. One-way ANOVA was performed. N= 3. **p < 0.01.
To test whether the lack of different anti-oxidation activities was due to bad preservation condition for the compounds in our lab, we purchased AP1 standard from Sigma (cat#172060, lot# D00055368), and repeated the three assays. The standard compound from Sigma gave the following results: DPPH assays: 0.08% (trolox = 93.6%), FRAP assay: AP1 = -0.003 unit (trolox = 2.047 unit), TBARs assay: AP1: 2.63% inhibition (trolox = 43.4%). This demonstrated the low antioxidant activity of our isolated compound is not caused by preservation problem, and the AP1 from other source similarly lacks free radical scavenging, ferric iron reducing and anti-lipid peroxidation capacity.

4. Discussion

High glucose increase the level of reactive oxygen species in mesangial cells (Lee et al., 2003; Yamagishi et al., 2005), which in turn stimulate downstream signaling pathways to TGF-β secretion and extracellular matrix (ECM) deposition (Yamagishi et al., 2003), and/or to nuclear factor (NF)-κB mediated apoptosis (Kang et al., 2003). Thus control of blood-glucose level prevents the progression to diabetic nephropathy. In glomerulosclerosis, reduction of intraglomerular capillary pressure reduces proteinuria and preserves renal function. Therefore, the known anti-hyperglycemia...
and anti-hypertension effect of compounds AP1 and AP2 contribute to the delay of development or treatment of symptom for diabetic nephropathy. Longstanding diabetes causes accumulation of ECM components and glomerulosclerosis, which in turn leads to fluid filtration deficits and arteriosclerosis of the renal artery (Schleicher et al., 1988). Deposition of ECM protein such as fibronectin, as well as tubular apoptosis, precedes and results in glomerulosclerosis (Schleicher et al., 1988). TGF-β promotes tubulopithelial cell hypertrophy and the secretion of extracellular matrix protein in tubular and mesangial cells (Sharma and Ziyadeh, 1994). Our data showed that AP1 and AP2 inhibited the level of activated caspase-3 (Fig. 2), TGF-β (Fig. 3), fibronectin (Fig. 4), and PAI-1 (Fig. 5) in mesangial cells. These results indicate that the tested compounds AP1 and AP2 may prevent or delay the progression of DN through not only by the known anti-hyperglycemia and anti-hypertension effects, but also by reducing molecules observed in this study that were direct cause of the glomerulosclerosis.

The compounds AP1 and AP2 differed in their individual activities on cellular phenotypes. Although AP1 and AP2 inhibit the fibronectin deposition to a similar extent (Fig. 4), however, AP2 showed slightly better inhibition of the TGF-β secretion to the medium than AP1 (Fig. 3). For the anti-apoptosis parameter, AP2 reduced the amount of activated caspase-3 to the level of normal glucose control at 20 µM, but AP1 did not do so at the highest concentration we tested (Fig. 2). This indicates that AP1 and AP2 work similarly for inhibiting deposition of the ECM, while AP2 was more potent than AP1 in preventing the tubular cell apoptosis. This would be an interesting fact to take into consideration if one desires to modify AP2 for lead optimization. As AP2 is different from AP1 in two position, one being dehydrogenation at 11.12 position in the diterpene, and the other deoxygenation in the 14 position of the β-lactone ring (Fig. 1), of which the variation in the structure caused this differences in the biological response needs further investigation.

Advanced glycation end products (AGE) induced by high glucose concentration in mesangial cells increase the level of reactive oxidative species (ROS) (Haneda et al., 2003), which in turn stimulate downstream signaling pathways to TGF-β and ECM deposition (Yamagishi et al., 2003), and/or to nuclear factor (NF)-κB mediated apoptosis (Kang et al., 2003). Besides the AGE pathway, ROS is produced via PKC–MAPK signaling pathways downstream to high glucose entry to the cell (Lee et al., 2003). ROS induces the activation of NF-κB and P53, which in turn signals through Bcl-2 and stimulates the release of cytochrome c from the mitochondria and increased level of cleaved caspase-3 (Kang et al., 2003). Therefore the production of ROS is proposed to be a major linkage between the signaling pathways leading to ECM deposition and apoptosis (Haneda et al., 2003; Kang et al., 2003; Lee et al., 2003). We also tested whether the protection against mesangial cell apoptosis and fibrosis in high glucose culture was due to the antioxidant activity of AP1 and AP2. We used a staining method in which the intensity of the fluorescence indicates the extent of oxidative stress. While high glucose increase the oxidative state of the MES-13 cells, however co-incubated with AP1 or AP2 reduces the oxidative state (Fig. 6). These data corroborate with the previous observation that reactive oxidative species play an important role in regulating the fibrosis and apoptosis of the mesangial cells (Ha and Lee, 2000; Kang et al., 2003).

Following this, we took a step further to test the mechanism of how AP1 and AP2 regulate the intracellular oxidative state of the MES-13 cells. Our results demonstrated that AP1 and AP2 do not directly clear free radical, or reduced the Fe3+, or reduced lipid peroxidation, in vitro, at the concentration that were effective for protection from apoptosis and fibrosis for MES-13 cells (1–20 µM magnitude, Fig. 7). Previously, it was reported that 50 mg/ml of water extract from Andrographis paniculata shows significant free radical scavenging, xanthine oxidase inhibition and anti-lipid peroxidation activities (Lin et al., 2009). Another report also demonstrated that ingested Andrographis paniculata extract results in reduced lipid peroxidation in urine (Akowuah et al., 2008). In contrast to previous data, the results in this report indicated that compounds AP1 and AP2 do not have the significant response in the tested antioxidant assays. It is known that Andrographis paniculata has different chemical constituents such as flavonoids, phenolic acids, and diterpenoids, which differ among different geographic sources. In the present study, we used pure compounds AP1 and AP2 in MES-13 cells, therefore their antioxidant property may be different from those of crude extracts and tested cell lines. This is the first report to demonstrate that AP1 and AP2 do not have the ability to directly influence the oxidative state in an acellular environment. These data also indicate that the ability of Andrographis paniculata compounds to regulate the oxidative state of the MES-13 cells is probably via regulating the cellular signaling pathways by their other enzymatic activities.

Why the compounds had antitumor efficiency by up-regulating apoptotic proteins and p53 (Yang et al., 2009), but in the MES-13 diabetic nephropathy model the compounds down-regulated the apoptosis proteins? In the former situation the cells were already tumor cells, and apart from p53 mediated apoptosis, the effect of Andrographis paniculata compounds included inhibiting growth of tumor cells by activating c-jun N-terminal kinase (Ji et al., 2007), down-regulating MMP-7 and E-selectin to inhibit metastasis (Jiang et al., 2007; Shi et al., 2008), down-regulating PI3K/akt signaling to inhibit migration and invasion of cancer cells (Lee et al., 2010), and JAK-STAT pathway to enhance chemosensitivity of cancer cells (Zhou et al., 2010). In other words, down-regulation of the activities of cells already showed characteristics of uncontrolled proliferation and metastasis. MES-13 is a murine kidney mesangial cell line obtained from transgenic mice harboring the early region of simian virus 40. The cells retain features characteristic of their nor-
mal counterparts when cultured in normal glucose medium despite their transformed phenotype, and have been used extensively in the study of mesangial cell functions. When MES-13 cells were cultured in high glucose medium, the elevated ROS state caused the cells to deposit ECM and express PAI-1 (Lee et al., 2003), or even going into p53 regulated apoptosis (Kang et al., 2003). Before the treatment the cells were not tumorous or cancerous. In our experiment the compounds AP1 and AP2 were added simultaneously with the high glucose treatment. Therefore the actions of these compounds in mesangial cells were preventing the cells from entering a senescent state or apoptosis fate, while its other action on cells already tumorous or cancerous was by killing or preventing the spreading of these cells.

Our data showed that when cultured at high glucose medium, the mesangial cells expressed up-regulated levels of TGF-β and PAI-1 (Figs. 4 and 5). PAI-1 gene is a target of p53 and essential mediator of senescence (Rerolle et al., 2000). It can be secreted by senescent cells and induce senescence in neighboring cells (Kortlever and Berards, 2006). Senescence is associated with gene expressed in inflammatory responses. The high glucose in mesangial cells also induces p53 activation, and subsequent apoptosis (Kang et al., 2003). Therefore the diabetic nephropathy could be regarded as a senescent state. The fact that the compounds AP1 and AP2 down-regulate TGF-β and PAI-1 (Figs. 4 and 5), suggested that these compounds possess anti-senescence activity. Furthermore, it is proposed that response to DNA damage in senescent cells includes systemic suppression of insulin hormone axis and relevant metabolic changes that lead to type II diabetes (Erol, 2010).

As diabetic nephropathy usually develops at the end stage of type II diabetes, it is less likely that the direct effect of tested compounds on the tubular cells would act systematically to reverse insulin resistance and other metabolic syndrome. However, it is highly possible that the tested compounds AP1 and AP2 act on other tissue to prevent the cells from entering a senescent state, and stop the progression to type II diabetes by blocking the senescence-induced systemic metabolic changes.

5. Conclusion

In conclusion, for the first time our data demonstrated that pure compounds andrographolide and 14-deoxy-11,12-didehydroandrographolide from *Andrographis paniculata* are capable of reducing the production of extracellular molecule fibronectin, cytokine TGF-β, and plasminogen activator inhibitor-1, as well as apoptosis marker caspase-3 in a diabetic nephropathy cell line MES-13 cells. This indicates that apart from anti-hyperglycemia and anti-hypertension, compounds of *Andrographis paniculata* are capable of regulating molecule and this is the direct cause of diabetic nephropathy. Secondly, we demonstrated that 14-deoxy-11,12-didehydroandrographolide is more potent than andrographolide for preventing DN phenotypes. Thirdly, both these compounds reduce ROS states in the MES-13 cells, but do not have antioxidative activity in acellular environment, indicating their anti-diabetic nephropathy effect is regulated via intracellular signaling pathways. Our data are of value to understand the mechanism for single compounds derived from *Andrographis paniculata*, and would benefit the study of drug development or food supplement for diabetes and nephropathy.

Acknowledgment

This study was supported by National Science Council of Taiwan, ROC (NSC 99-2811-M-324-003).

References


Erol, A., 2010. Systemic DNA damage response and metabolic syndrome as a prema-


drenoreceptor by andrographolide to increase glucose uptake in cultured myoblast C2C12 cells. Planta Medica 70, 1230–1233.


Qin, L.H., Kong, L., Shi, G.J., Wang, Z.T., Ce, B.X., 2006. Andrographolide inhibits the production of TNF-α and interleukin-12 in lipopolysaccharide-stimulated...


