Andrographolide Up-Regulates Cellular-Reduced Glutathione Level and Protects Cardiomyocytes against Hypoxia/Reoxygenation Injury

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ABSTRACT

Recent studies revealed that the herb Andrographis paniculata possesses cardioprotective activities. Using neonatal rat cardiomyocytes, the cardioprotective actions of several diterpene lactones derived from A. paniculata including andrographolide, 14-deoxyandropholide, 14-deoxy-11,12-didehydroandrographolide, and sodium 14-deoxyandrographolide-12-sulfonate were investigated. Pretreatment with andrographolide but not with the other compounds protected the cardiomyocytes against hypoxia/reoxygenation injury and up-regulated the cellular-reduced glutathione (GSH) level and antioxidant enzyme activities. The cardioprotective action of andrographolide was found to coincide in a time-dependent manner with the up-regulation of GSH, indicating the important role of GSH. The cardioprotective action of andrographolide was also completely abolished by buthionine sulfoximine, which acts as a specific γ-glutamyl cysteine ligase (GCL) inhibitor to deplete cellular GSH level. It was subsequently found that the mRNA and protein levels of the GCL catalytic subunit (GCLC) and modifier subunit (GCLM) were up-regulated by andrographolide. Luciferase reporter assay also demonstrated that andrographolide activated both the GCLC and the GCLM promoters in the transfected rat H9C2 cardiomyocyte cell line. The 12-O-tetradecanoylphorbo-13-acetate response element or the antioxidant response element may be involved in the transactivating actions of andrographolide on the GCLC and GCLM promoters. The present study pinpoints andrographolide as a cardioprotective principle in A. paniculata and reveals its cytoprotective mechanism.

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ABBREVIATIONS: AP, Andrographis paniculata; NRC, neonatal rat cardiomyocyte; H/R, hypoxia/reoxygenation; DMEM, Dulbecco’s modified Eagle’s medium; LDH, lactate dehydrogenase; SOD, superoxide dismutase; GPX, glutathione peroxidase; GR, glutathione reductase; GSSG, oxidized glutathione; GSH, reduced glutathione; PCR, polymerase chain reaction; GCL, γ-glutamyl-cysteine ligase; GCLC, γ-glutamyl-cysteine ligase catalytic subunit; GCLM, γ-glutamyl-cysteine ligase modulatory subunit; EMSA, electrophoretic mobility shift assay; BSO, buthionine sulfoximine; AT-A, 3-amino-1,2,4-triazole; NF, nuclear factor; TRE, 12-O-tetradecanoylphorbo-13-acetate response element; AP-1, activator protein-1; ARE, antioxidant response element; NRF, NF-E2-related factor; ROS, reactive oxygen species; H2O2, hydrogen peroxide; NQO, NAD(P)H:quinone oxidoreductase.

Andrographis paniculata (Burm. f.) Nees (Acanthaceae) (AP) is an herb originated from India and widely distributed in southern China. It has been used in traditional systems of medicine to treat a number of ailments including common cold, fever, diarrhea, liver diseases, and inflammation (World Health Organization, 2002). Recent studies have revealed some cardiovascular effects of this herb (Zhang and Tan, 1996; Thisoda et al., 2006). The crude extract of AP (Guo et al., 1995) and its flavonoid fraction API0134 (Guo et al., 1996) have been shown to protect dog hearts against ischemia/reperfusion. However, the active components in this herb and the mechanism of these cardioprotective actions remain obscure.

Using a primary neonatal rat cardiomyocyte (NRC) model, we have screened a number of medicinal materials used in traditional Chinese medicine for their cardioprotective actions. Our screening results indicated that pretreatment with a crude extract of AP could protect NRCs from hypoxia/reoxygenation (H/R) damage. However, the protective action of AP cannot be explained by the presence of antioxidants in...
the herb as might have suggested by previous studies (Guo et al., 1995, 1996). This is because in our established H/R protocol (Woo et al., 2005), the pretreatment followed by a washing step removes any drug carried over from the pretreatment and thus eliminates any direct antioxidant effect during H/R. In addition, the active cardioprotective ingredient in AP is unknown. Thus, we performed our screening assay on a number of selected compounds derived from AP, including its major component, the diterpene lactone andrographolide (I) and two of its closely related derivatives, 14-deoxyandrographolide (II) and 14-deoxy-11,12-diehydroandrographolide (III) (Medforth et al., 1990; World Health Organization, 2002) as well as sodium 14-deoxyandrographolide-12-sulfonate (IV), a metabolite of andrographolide that has been prepared as a pharmaceutical agent (He et al., 2003) (see Fig. 1 for the structures of the compounds). Our results suggested that the cardioprotective action of AP could be attributed to andrographolide. The present report describes the cardioprotective action of this compound and its mechanism of action.

Materials and Methods

Animals. The protocols related to animal studies have been approved by the Animal Research Ethics Committee, The Chinese University of Hong Kong. The rats were bred and maintained in the Laboratory Animal Service Center of the Chinese University of Hong Kong.

Materials. The compounds used in the present investigation were of analytical quality with a minimum purity of 98%. Andrographolide (I) was obtained from Sigma-Aldrich (St. Louis, MO). 14-Deoxyandrographolide (II) and 14-deoxy-11,12-diehydroandrographolide (III) were supplied by LKT Laboratories (St. Paul, MN). Sodium 14-deoxyandrographolide-12-sulfonate (IV) was purchased from the Kang Da Pharmaceutical Research Institute of West China University of Medical Science (Chengdu, Sichuan, China). Stock solutions of the compounds at 20 mM in dimethyl sulfoxide (for compounds I–III) or water (for compound IV) were prepared, appropriately diluted, and added to the cell culture media. All other reagents were of the highest analytical grade available.

Culture of NRCs and Cell Line. NRCs were isolated from Sprague-Dawley rats 1 to 2 days after birth and cultured in DMEM medium 199:1 medium with 0.1% insulin-transferrin-selenium G supplement (Invitrogen, Carlsbad, CA), 0.5% horse serum, and 0.3% penicillin-streptomycin as determined by the Lowry protein assay (Lowry et al., 1951). The rat cardiomyocyte cell line H9C2 (ATCC CRL-1446, passage 6–10; American Type Culture Collection, Manassas, VA) was maintained in DMEM with 10% fetal bovine serum and induced to express the myotube phenotype by changing the culture medium to DMEM with 2% horse serum and 0.3% penicillin-streptomycin 14 days before the transfection experiment.

NRCs Subjected to H/R Injury. The cultured NRCs were pretreated with the test compounds or the control vehicle. After the preincubation period, the culture medium was removed, and the cells were washed with a washing buffer (5 mM HEPES, 137 mM NaCl, 4 mM KCl, 1 mM MgCl2, 1.5 mM CaCl2, pH 7.0). The cells were then incubated in an acidic glucose-free DMEM base medium and further subjected to 10 h of hypoxia (oxygen level, <0.5%) followed by 1 h of reoxygenation in DMEM as described previously (Woo et al., 2005).

Lactate Dehydrogenase Assay. Lactate dehydrogenase (LDH; EC 1.1.1.27) leakage from cells is widely used as a marker of irreversible cell injury and necrotic cell death. LDH activities in the hypoxia and the reoxygenation media as well as in the cell lysates were analyzed by determining the rate of NADH oxidation in the presence of pyruvate. The sum total of LDH activities in the hypoxia and reoxygenation media as well as in the cell lysate obtained from the same well would give the total LDH activity. The LDH released during hypoxia or reoxygenation was expressed as a percentage of the total LDH activity. The percentages of LDH released during hypoxia and reoxygenation would be combined to give the percentage of LDH released during H/R. The LDH assay has been shown in our hands to give a higher discriminatory power of cell viability than staining with propidium iodide or trypan blue (Woo et al., 2005).

Assays for Catalase, Superoxide Dismutase, Glutathione Peroxidase, Glutathione Reductase, and Glutathione. Cultured NRCs were washed twice with phosphate-buffered saline containing 0.05 mM EDTA. Cell lysates were then obtained by sonication of the harvested cells. Antioxidant enzyme activities were immediately assayed after brief centrifugation (800g for 5 min at 4°C) to obtain the supernatants. Catalase (EC 1.11.1.6) activity was determined according to Aebi (1984). Superoxide dismutase (SOD; EC 1.15.1.1) activity was determined by the nitroblue tetrazolium method according to Beauchamp and Fridovich (1971). Glutathione peroxidase (GPX; EC 1.11.1.9) activity was assayed using cumene hydroperoxide as substrate (Lawrence and Burk, 1976). Glutathione reductase (GR; EC 1.8.1.7) activity was assayed by the method of Aki (2000). Oxidized glutathione (GSSG) and reduced glutathione (GSH) were assayed immediately after sonication of the cells according to the method of Anderson (1996). The enzyme activities and GSH levels were normalized against the amount of protein present in the samples as determined by the Lowry protein assay (Lowry et al., 1951).

Real-Time PCR. Total RNA was extracted from the cultured NRCs using TRizol Reagent (Invitrogen) and reverse transcribed using the Superscript First-Strand Synthesis System (Invitrogen). To quantify the gene expression levels in the samples, real-time PCR

![Fig. 1. Structures of diterpene lactones derived from A. paniculata. I, andrographolide; II, 14-deoxyandrographolide; III, 14-deoxy-11,12-diehydroandrographolide; IV, sodium 14-deoxyandrographolide-12-sulfonate.](image-url)
was performed on an ABI Prism 7700 Sequence Detection System using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and primers specific for the catalytic or modifier subunits of glutamate-cysteine ligase (GCL; EC 6.3.2.2) of rats, i.e., GCLC (forward primer, 5'-CTCGGCTATGTTGTTATTGG-3'; reverse primer, 5'-TTGGCTAGTACGGATGGA-3'; amplicon size, 454 bp) and GCLM (forward primer, 5'-CTGACGTTAGGAACGGAGA-3'; reverse primer, 5'-ACAATGCAACACCAACAC-3'; amplicon size, 252 bp). The annealing temperature and the primer concentrations were optimized for amplification efficiency (amplification factors > 1.90 for all the transcripts) after validation of the dissociation curves and satisfactory separation of the PCR products on a 2.5% agarose gel. The optimal thermal cycle protocol for all the samples began with 10-min denaturation at 95°C, followed by 40 cycles of 95°C for 15 s, 62°C for 30 s, and 72°C for 45 s. The concentrations of the primers used for GCLC, GCLM, and β-actin were 200, 300, and 200 μM, respectively. The relative amounts of mRNA for GCLC and GCLM in the andrographolide-treated cells versus the vehicle-treated control were calculated as the relative expression ratios in comparison with β-actin (Paffl, 2001).

Western Blot Analysis. Protein (50 μg) from whole-cell lysates of cultured NRCs was separated on a 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane. Immunoblotting was carried out with polyclonal anti-GCLM antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or polyclonal anti-GCLC antibody (Lab Vision, Suffolk, UK) or monoclonal anti-β-actin antibody (Santa Cruz Biotechnology), followed by incubation with appropriate secondary antibodies coupled with horseradish peroxidase. The blot was developed with a chemiluminescence system (Applied Biosystems) according to the manufacturer’s protocol.

Electrophoretic Mobility Shift Assay. Cultured NRCs (1–2 × 10⁶ cells) were lysed by suspending the harvested cells in an ice-cold hypotonic buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, and Complete protease inhibitor cocktail (at the concentration recommended by the manufacturer; Roche Diagnostics, Indianapolis, IN) followed by mixing with Nonidet P-40 (0.6% final concentration). The nuclear fraction was obtained by centrifugation at 11,000 g for 1 min. A hypotonic buffer containing 20 mM HEPES, pH 7.9, 0.5 M NaCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 25% glycerol, 1 mM dithiothreitol, and protease inhibitors was then added to the resuspended nuclei. Nuclear extracts were obtained after further incubation for 30 min on ice and removal of cell debris by centrifugation. To detect the binding of nuclear proteins to the putative DNA sequences, electrophoretic mobility shift assay (EMSA) was performed. Oligonucleotides were synthesized and the complementary strands were annealed before their use as probes or competitors. The probes were prepared by 5’ end labeling of double-stranded oligonucleotides with [γ-³²P]ATP. Binding reactions were carried out in a buffer containing 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.3 mg/ml bovine serum albumin, and 50 μg/ml double-stranded poly(dI-dC) with 6 μg of nuclear extract at 4°C for 20 min. If unlabeled oligonucleotides (8 pmol) were used as competitors, they were preincubated with the nuclear extract before addition of the labeled probes (80 fmol). DNA-protein complexes were separated on a 5% non-denaturing polyacrylamide gel in 0.5× Tris borate-EDTA buffer with 2.5% glycerol. Gels were dried on filter paper and subsequently scanned on a PhosphorImager (Molecular Imager FX; Bio-Rad Laboratories, Hercules, CA). The band intensities were quantified and compared by the built-in Quantity One 4.4.0 program (Bio-Rad Laboratories).

Luciferase Reporter Assay. Confluent cultures of differentiated H9C2 cells in 24-well plates were transfected using Lipofectamine reagent (Invitrogen) in a serum-free medium with recombinant plasmids (0.2 μg) containing the human GCLC or GCLM promoters in pGL3-basic vector (Promega, Madison, WI) (i.e., the −3802/GCS₅⁻luc and −1927/GCS₅⁻luc and −1927/GCS₅⁻luc in the original publications of Dr. Timothy Mulcahy’s group (Mulcahy et al., 1997; Moinova and Mulcahy, 1998) generously provided by Dr. Jeffrey Johnson, University of Wisconsin, Madison, WI). Five hours after addition of the DNA-Lipofectamine complexes, the serum-free medium was replaced with normal culture medium containing different concentrations of andrographolide. After a further incubation of 48 h, the cells were washed with phosphate-buffered saline and lysed by the passive lysis buffer (Promega). The lysates were centrifuged at 13,000×g for 2 min at 4°C. The resultant supernatants were saved for luciferase and protein assays. Luciferase activity was determined by luminometry using a luciferase reporter assay system (Promega).

Thiol Depletion Assay. Thiol depletion assay was performed according to an established procedure (Jocelyn, 1987). The test compound was incubated with GSH for 30 min in Tris-HCl with EDTA buffer (0.1 M Tris-HCl, 1 mM EDTA, pH 8.5) at 37°C. The amount of GSH before and after the incubation period was determined spectrophotometrically using the thiol reagent 2,2'-dipyrindyl disulfide by absorbance reading at 343 nm.

Statistical Analyses. All averaged results are presented as mean ± S.D. Statistical analyses were performed either with one-way analysis of variance combined with Bonferroni’s transformation to compare data among three or more groups or with the two-tailed unpaired Student’s t test to compare two groups of data. A P value <0.05 was considered significant.

Results

Andrographolide Protects NRCs against H/R. Selected diterpene lactones I to IV (Fig. 1) from AP were tested for their protective actions on NRCs subjected to H/R damage. LDH activities were used to indicate the extent of cellular damage in the NRCs. Preliminary studies indicated that no observable difference could be noted in the viability (in terms of cell number and LDH released) and the vital quality (in terms of the morphological and functional characteristics such as gap junction integrity and spontaneous contraction rate) of the NRCs treated with the compounds at the highest concentration tested versus the untreated control within the treatment period examined. It was observed that NRCs subjected to H/R insult resulted in an increase in LDH release. The basal LDH release from control cardiomyocytes incubated in unstimulated condition was approximately 20% (Woo et al., 2005). As shown in Fig. 2A, pretreating the cells with andrographolide produced a dose-dependent decrease in the amount of LDH release during H/R demonstrating a protective action of the compound. On the contrary, no protective effects were observed in cells pretreated with the other diterpene lactones (Fig. 2, B–D).

Pretreatment but Not Cotreatment with Andrographolide Affords Protection of NRCs. It was further investigated whether pretreatment (presence of the drug before, but not during, the stress regimen) or cotreatment (presence of the drug during, but not before, the stress regimen) with andrographolide would result in different degrees of protection. Figure 3 shows the results of the different treatment regimens in the NRCs undergoing H/R. It can be seen that pretreatment with andrographolide decreased LDH release by approximately 21%. On the other hand, cotreatment with andrographolide during hypoxia afforded no observable protection on the NRCs. This lack of protection during cotreatment was in fact consistent with the results in which the presence of the drug throughout the pretreatment and stress periods afforded no additional protection compared with the situation in which...
the drug was present only during the pretreatment period. These results imply that andrographolide might exert its cardioprotective effect via induction of some endogenous cellular protective factors rather than acting directly.

**Alteration in Antioxidant Status of NRCs Treated with Andrographolide.** To investigate whether andrographolide exerts its cardioprotective actions through the enhancement of the antioxidant potential of the cardiomyocytes, the activities of a number of antioxidant enzymes and the GSH level in the NRCs treated with andrographolide or its analogs were investigated under baseline condition. Results in Fig. 4 revealed that after treating the cardiomyocytes with andrographolide for 3 days, the enzymatic activities of SOD, catalase, GPX, and GR were increased by 21, 83, 27, 013 1 0

![Fig. 2. Andrographolide but not the other diterpene lactones derived from AP protects cardiomyocytes from H/R injury. NRCs in primary culture were pretreated for 3 days with compounds I to IV (A–D), respectively, at different concentrations shown. After washing, the cells were subjected to H/R as described under Materials and Methods. The percentage of LDH released during H/R was determined. **P < 0.001 compared with the drug-free control (n = 6).**](image)

![Fig. 3. Pretreatment but not cotreatment with andrographolide affords cardioprotection. NRCs in primary culture were divided into groups receiving different treatment regimens with C (control, white bars) or I (10 μM andrographolide, dark bars): +/−, pretreatment for 3 days; −/+, no pretreatment, cotreatment during hypoxia only; +/+, pretreatment for 3 days followed by cotreatment during hypoxia but not reoxygenation. Percentage of LDH released during H/R was determined as described under Materials and Methods. **P < 0.001 compared with the control in the same treatment group (n = 6).**](image)

![Fig. 4. Changes in antioxidant status in NRCs treated with diterpene lactones derived from AP. NRCs in primary culture were pretreated for 3 days with 10 μM compounds I to IV. Vehicle controls were performed in parallel. The amounts of SOD, catalase (CAT), GPX, GR, and GSH in the lysates of the treated cells were measured as described under Materials and Methods. *, P < 0.05 and ***, P < 0.001 compared with the control (n = 5 or 6).](image)
and 99%, respectively. In addition, the cellular GSH level was also increased to 258% of the control level. On the contrary, after treating the cardiomyocytes with 14-deoxyandrographolide, 14-deoxy-11,12-didehydroandrographolide, or sodium 14-deoxyandrographolide-12-sulfonate, no significant increases in antioxidant enzyme activities or GSH level were observed, corroborating with the ineffectiveness of these compounds in protecting the NRCs against H/R damage (Fig. 2, B–D).

Effects of Andrographolide on the Antioxidant Status and Cellular Resistance to H/R in NRCs: A Time-Dependent Study. A time-dependent study of the effects of andrographolide on the antioxidant enzyme activities and the GSH level in cultured NRCs was performed. As shown in Fig. 5A, no significant change could be observed in the activities of any of the antioxidant enzymes 12 h after treatment. Induction of catalase and GR was evident 24 h after treatment with andrographolide, and the degrees of induction followed an increasing trend with time in general. The activity of catalase and GR reached 180 and 200% of the control, respectively, after 88 h of treatment. Induction of SOD activity was observed 48 h after treatment, and the induction level was approximately 130% of the control, which remained unchanged until the end of the investigation period. Increase in GPX activity was observed only after a prolonged period of incubation (88 h) with andrographolide. On the other hand, a significant increase in cellular GSH level (170%) could be observed as early as 12 h after andrographolide treatment. By 24 h, the GSH level had reached over 300% of the control, after which the induction in GSH declines a little bit. A time-dependent study of the protective action of andrographolide on the NRCs against H/R was also performed. The results in Fig. 5B show that a significant cardioprotective effect could be observed as early as 12 h after pretreatment with andrographolide. A gradual increase in the extent of protection appeared as the pretreatment time increased to 33 h. Increase in the pretreatment duration beyond this point seems to produce no additional protection.

Cardioprotective Action of Andrographolide Is Abolished by a Cellular GSH Depletor. The results from the time-dependent study show that cellular GSH level was upregulated as early as 12 h after treatment with andrographolide, whereas no such effect could be observed in any of the antioxidant enzymes. It is interesting to note that the cardioprotective effect of andrographolide was also evident 12 h after treatment. Thus, it is deduced that the increase in cellular GSH level may be responsible for the cardioprotective action of andrographolide. To substantiate this hypothesis, buthionine sulfoximine (BSO), a specific GCL inhibitor, was employed to deplete cellular GSH to see whether this could abolish the cardioprotective action of andrographolide. In addition, a specific catalase inhibitor, 3-amino-1,2,4-triazole (ATA), was also used to see whether catalase may play a role in the cardioprotective action of andrographolide. Preliminary studies were conducted to determine the concentrations and treatment periods of BSO and ATA that could bring the andrographolide-induced GSH level or catalase activity back to the control level. No cytotoxic effects of the inhibitors could be observed at the concentrations used within the treatment period. Figure 6A shows that treatment with 10 mM ATA for 90 min brought the increased catalase activity of the andrographolide-treated NRCs back to the control level and dramatically reduced the catalase activity in the untreated cells. However, the same treatment with ATA could not abolish the cardioprotective effect of andrographolide (Fig. 6A2; $P > 0.05$ compared with I-blank). Cotreatment with 0.15 mM BSO during the last 8 h of the pretreatment period brought the increased cellular GSH level of the andrographolide-treated cardiomyocytes back to the control level and dramatically reduced the GSH level in the untreated cells (Fig. 6B1). The same treatment with BSO completely abolished the cardioprotective effect of andrographolide (Fig. 6B2; $P > 0.05$ compared with C-blank), suggesting the pivotal role of GSH in the cardioprotective effect of andrographolide. The inverse correlation between the cellular GSH level and the cardioprotective action was also substantiated in the elevation of LDH release during H/R in the BSO-treated control (Fig. 6B2; $P < 0.001$ compared with C-blank).

Induction of GCL Subunits mRNA and Protein Levels in Andrographolide-Treated NRCs. Our preliminary
reporter assays were performed on the rat cardiomyocyte cell line H9C2 transfected with the luciferase reporter construct containing either the human GCLC promoter or the GCLM promoter. The results show that both the GCLC and GCLM promoter/enhancer activities in the transfected cells were increased after incubation with andrographolide (5 or 10 μM), whereas no activation could be observed in cells transfected with the empty pGL3-basic vector (Fig. 8).

**Involvement of the 12-O-Tetradecanoylphorbo-13-Acetate Response Element and Antioxidant Response Element in the Andrographolide-Induced Activation of GCLC and GCLM Genes.** The real-time PCR results and the luciferase reporter assay results suggest that andrographolide induces the expression of GCL at the transcription level. It was speculated that andrographolide might induce the transcription of its target genes through activation of the redox-sensitive or stress-induced transcription factors. EMSA was therefore performed to investigate the possible involvement of the following enhancer elements: κB element [for the binding of nuclear factor (NF)-κB], 12-O-tetradecanoylphorbo-13-acetate response element [TRE; for activator protein-1 (AP-1) binding], and antioxidant response element [ARE, also known as electrophile response element; activated by both the AP-1 family transcription factors and the NF-E2-related factors (Nrfs)] (Dickinson et al., 2004) in the transactivation effects of andrographolide. Synthetic oligonucleotides with the consensus sequences for the NF-κB and AP-1 binding sites and the DNA sequences identified to be functional ARES in the 5′-flanking regions of the rat GCLC and GCLM were labeled and used as probes (Table 1). The EMSA results show that andrographolide significantly induced AP-1 and ARE, but not NF-κB responses (Fig. 9, A and B), and the percentage of induction on AP-1 and ARE responses seemed to be concentration-dependent, with a maximal response of approximately 40% over the control at 10 μM andrographolide (Fig. 9B). It is interesting to note that the induction effects of andrographolide appeared to be higher in the control level concomitant with abolition of the cardioprotective effect of andrographolide: the different effects of ATA and BSO. NRCs in primary culture were pretreated for 3 days with control vehicle (C) or 10 μM andrographolide (I). The pretreated cardiomyocytes were subdivided into two groups subjected to A, further treatment for 90 min with plain medium (blank) or ATA (10 mM); or B, cotreatment with BSO (0.15 mM) during the last 8 h of the pretreatment period or sham treatments (blank) performed in parallel as controls. Catalase activities were then assayed for cells in treatment group A (A1), and GSH levels were assayed in treatment group B (B1). In another subset of study, the pretreated cells in all these groups were also subjected to H/R for 8 h as described under Materials and Methods, and the percentage of LDH released was determined for the respective groups (A2 and B2). ***P < 0.001 and #, P > 0.05 compared with C-blank (n = 4 for catalase; n = 5 for GSH; n = 6 for LDH).

**Fig. 6.** Reversibility of the cardioprotective effect of andrographolide: the different effects of ATA and BSO. NRCs in primary culture were pretreated for 3 days with control vehicle (C) or 10 μM andrographolide (I). The pretreated cardiomyocytes were subdivided into two groups subjected to A, further treatment for 90 min with plain medium (blank) or ATA (10 mM); or B, cotreatment with BSO (0.15 mM) during the last 8 h of the pretreatment period or sham treatments (blank) performed in parallel as controls. Catalase activities were then assayed for cells in treatment group A (A1), and GSH levels were assayed in treatment group B (B1). In another subset of study, the pretreated cells in all these groups were also subjected to H/R for 8 h as described under Materials and Methods, and the percentage of LDH released was determined for the respective groups (A2 and B2). ***P < 0.001 and #, P > 0.05 compared with C-blank (n = 4 for catalase; n = 5 for GSH; n = 6 for LDH).
for GCLM-ARE1 than for GCLC-ARE4 at the two concentrations
tested, corroborating with the higher induction effect of
andrographolide on GCLM mRNA at 8 h (Fig. 7A).

**Discussion**

The anti-ischemic action of AP extracts has been attributed
to the alleviation of calcium overload and the scavenging of...
Andrographolide Protects Cardiomyocytes

Table 1: Sequence information of DNA probes for detecting NF-κB, AP-1, and ARE responses

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Sequence</th>
<th>Source of Information</th>
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<tr>
<td>NF-κB</td>
<td>agtttacGGGActTTCCcaggg</td>
<td>Promega</td>
</tr>
<tr>
<td>AP-1</td>
<td>cggttggTGAGTCAcggaa</td>
<td>Promega (modified)</td>
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<tr>
<td>Rat GCLC-ARE4</td>
<td>cagaggtgTggTcaggCGTgAgtgGgcggGcgggcggg</td>
<td>National Center for Biotechnology Information accession no. NW_047799</td>
</tr>
<tr>
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<td>Rat genome 8q31</td>
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<td>−3912 to −3879</td>
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<td></td>
<td></td>
<td>National Center for Biotechnology Information accession no. NW_047627</td>
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<tr>
<td></td>
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<td>Rat genome 2q41</td>
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<td>−303 to −276</td>
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Fig. 9. Andrographolide induces AP-1 and ARE but not NF-κB responses in cardiomyocytes. NRCs in primary culture were treated with 5 or 10 µM I (andrographolide) or control vehicle for 8 h, followed by incubation of the resultant nuclear extracts with the respective probe (radiolabeled oligonucleotides shown in Table 1). The DNA-protein complexes formed were analyzed by EMSA as described under Materials and Methods. A, gel image of a typical experiment. The positions of the gel-band(s) corresponding to the DNA-protein complexes of interest (arrows) were identified in preliminary experiments using competitor techniques. B, summary of the results in A based on digital autoradiographic values generated by the Quality One program over three independent experiments. *, P < 0.05 and ***, P < 0.01 compared with the respective vehicle-treated control.

Free radicals during myocardial ischemia-reperfusion (Guo et al., 1995, 1996). However, a major difference of the current study from these previous studies is that pretreatment but not cotreatment with andrographolide produces the protective action on the cardiomyocytes (Fig. 3). Therefore, our data indicate that the observed cardioprotective effect (Fig. 2A) cannot be attributed to a direct free radical scavenging effect of andrographolide. Protection by pretreatment with andrographolide must act via other mechanisms. We have therefore explored the possibility that andrographolide could produce its cardioprotective action by altering the antioxidant status of the cultured cardiomyocytes.

Treatment with AP extracts has been shown to increase the antioxidant enzyme activities as well as the cellular nonprotein thiol levels in vivo (Singh et al., 2001; Trivedi and Rawal, 2001). Our data (Fig. 4) clearly show that this up-regulation in antioxidant enzyme activities and GSH level can also be demonstrated in vitro on NRCs and that andrographolide is the active component in this herb responsible for this action. The ineffectiveness of other diterpene lactones in inducing these effects suggests that the cellular antioxidant-enhancing action of andrographolide is specific to its chemical structure. It also indicates a causal link between the enhancement of the cellular antioxidant potential and the observed cardioprotective effect of andrographolide.

The results of the time-dependent (Fig. 5) and inhibitor (Fig. 6) studies demonstrate that increased cellular GSH level is responsible for the cardioprotective action of andrographolide. The glutathione antioxidant system is crucial for reactive oxygen species (ROS) defense in the heart. GSH directly detoxifies ROS and repairs ROS-induced damage by scavenging free radicals. In addition, GPX detoxifies a wide variety of ROS at the expense of GSH. GSH also plays an important role in maintaining protein thiol groups, tocopherol, and ascorbate of the cells in reduced states. Thus, the up-regulation of GSH level by more than 2-fold would undoubtedly enhance the antioxidant defense against subsequent oxidative damage during H/R in the andrographolide-treated NRCs. The observed induction of GR by andrographolide in concert with the up-regulation of GSH (Fig. 6) studies demonstrate that increased cellular GSH level is responsible for the cardioprotective action of andrographolide.
In a recent study on H9C2 cells (Seo et al., 2004), pretreatment with a low dose of hydrogen peroxide (\(H_2O_2\)) was found to induce an adaptive tolerance toward a subsequent challenge with a lethal concentration of \(H_2O_2\). It was subsequently shown that the increase in GSH content rather than catalase activity was responsible for the adaptive response because the tolerance to \(H_2O_2\) could be completely abolished by BSO but not by ATA. Likewise, the increase in GSH content was attributed to an increased GCL activity in the \(H_2O_2\)-pretreated cells. Using an \(H/R\) model in the present investigation, we have also demonstrated the important role of GSH in the preconditioning effect of andrographolide in NRCs. Taken together, these results support the notion that increasing GSH synthesis is one of the most crucial mechanisms for cardiomyocytes to guard against oxidative stress.

The redox-dependent regulation of GSH synthesis is controlled by the induction of the GCL transcripts. The expression of these genes is governed by a number of enhancer elements in their promoters, in particular the \(\alpha\)B element, TRE, and ARE are redox-sensitive and have been shown to be activated by a number of chemicals and stresses (Rahman and MacNee, 2000; Dickinson et al., 2004). Our EMSA results (Fig. 9) suggest that the transactivation effects of andrographolide on the GCLC and GCLM genes (Figs. 7 and 8) may involve AP-1 and ARE but not NF-\(\kappa\)B. The present data provide evidence that andrographolide may induce the GCL subunit genes through TRE and/or ARE activation. Andrographolide has been suggested to interfere with the binding of NF-\(\kappa\)B to DNA, probably through its interaction with cysteine 62 of p50 subunit (Xia et al., 2004). This effect was not observed in NRCs in the present investigation, presumably because the initial NF-\(\kappa\)B response was a basal one and had not been induced to a high level by any stimulatory agent as in the case of other studies.

ARE is present in the promoter regions of various genes encoding for phase II detoxification enzymes and antioxidant enzymes (Nguyen et al., 2003). The transcription factor Nrf2 is believed to be essential for the positive regulation of ARE-mediated gene expression (Nguyen et al., 2003; Jaiswal, 2004). In addition, other transcription factors such as Jun (c-Jun, Jun-D, and Jun B), c-Fos, Fra-1, small Maf (MafG, MafK, and MafF), and Nrf-2 may heterodimerize to regulate ARE-mediated transcription (Venugopal and Jaiswal, 1996; Jardine et al., 2002; Jaiswal, 2004). The transcription factors involved in the andrographolide-induced activation of TRE and ARE are presently unknown. Further studies to identify these transcription factors are highly warranted.

Talalay et al. (1988) have systemically characterized the ability of various chemicals in inducing the ARE-driven NADPH/quinone oxidoreductase (NQO; EC 1.6.99.2) in Hepa1c1c7 cells. These authors concluded that the potencies of these chemicals in inducing NQO activity are dependent on their Michael reaction chemistry and specifically 2-methylene-4-butyrolactone (the lactone ring structure resembles that in andrographolide, Fig. 10A) would be 10 times more potent than \(\gamma\)-crotonolactone (resembles that in 14-deoxyanandrographolide, Fig. 10B) in inducing NQO. It was further proposed that the inducers of ARE being Michael reaction acceptors with the \(\alpha,\beta\)-unsaturated ketone moiety may interact with the sulfhydryl groups of a number of cysteine residues on the cytosolic redox sensor recently identified to be Kelch-like ECH-associated protein 1 with concomitant release and activation of the transcription factor Nrf2 (Dinkova-Kostova et al., 2002; Wakabayashi et al., 2004). It is conceivable from the chemical structures (Fig. 1) that the \(\alpha,\beta\)-unsaturated ketone moiety is present in all the diterpene lactones investigated in the present study. In addition, the presence of an allylic hydroxyl group at C14 of andrographolide significantly increases the electrophilicity of C12 and facilitates addition reaction with thiols. The reaction mechanism of nucleophilic attack on this electrophilic carbon (proposed in Fig. 10C) explains our observation that andrographolide gradually depletes GSH in a weak alkaline solution. This result is in line with our observation that andrographolide treatment induces rather than depletes cellular GSH because the reactivity of andrographolide toward different cellular thiols is heterogeneous. At cellular pH, the reaction between andrographolide and GSH is expected to be very slow. It could be deduced from nucleophilic addition reaction chemistry that basic amino acid residues present in close proximity with the reactive cysteine residues in certain cysteine-bearing redox-sensitive signaling molecules would favor the interaction between andrographolide and the free cysteine residues on these proteins. Thus, it would be interesting to see whether andrographolide may trigger its various cellular effects through interaction with redox sensors such as Kelch-like ECH-associated protein 1. It is noteworthy that 14-deoxy-12-(\(\gamma\)-crotonyl)-andrographolide-3-O-sulfate, a metabolite of andrographolide resembling the nucleophilic addition product of cysteine on C12 of andrographolide, has recently been isolated from human urine (Cui et al., 2004).

Epidemiological and clinical studies on the potential benefits of antioxidant supplementation in the prevention of cardiovascular disease and cancer have come up with ambivalent results (Dutta and Dutta, 2003). On the contrary, antioxidants from natural sources have consistently been shown to produce beneficial effects (Rietveld and Wiseman, 2003; Zern and Fernandez, 2005). Consumption of these natural antioxidants is believed to improve the overall antioxidant status of the body and protect the body against the toxic effects of free radicals. Recent research focus has also shifted toward means that could augment endogenous antioxidant potential because this is expected to offer better protection against oxidative stress than the exogenously administered antioxidants themselves. As a matter of fact, studies on the chemopreventive effects of broccoli and other cruciferous vegetables on cancer have entered preclinical and clinical phases (Clapper et al., 1997; Kensler et al., 2000; Park and Pezzuto,
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