Effect of Notoginsenoside R1 on the Synthesis of Tissue-Type Plasminogen Activator and Plasminogen Activator Inhibitor–1 in Cultured Human Umbilical Vein Endothelial Cells

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Abstract Among other Chinese herb drugs, Panax notoginseng is used to treat cardiovascular diseases. To elucidate any possible effects of this drug on the hemostatic system in vitro, we analyzed the influence of one of its major active constituents on fibrinolytic parameters of cultured human umbilical vein endothelial cells (HUVECs). When confluent cultures of HUVECs (passages 2 to 3) were conditioned with purified notoginsenoside R1 (NR1), a dose- (0.01 to 100 μg NR1/mL) and time-dependent increase in tissue-type plasminogen activator (TPA) synthesis was observed, which was significant from 0.1 μg NR1/mL and from 6 hours of incubation with 100 μg NR1/mL on. TPA antigen increased from 3.9±0.2 ng per 10^6 cells per 24 hours to 8.0±0.5 ng per 10^6 cells per 24 hours on addition of 100 μg NR1/mL. In contrast, no change in urokinase-type plasminogen activator and plasminogen activator inhibitor–1 (PAI-1) antigen synthesis was seen. There was also no effect of NR1 on PAI-1 deposition in the extracellular matrix. As judged from fibrin autography and reverse fibrinolytic system functions as a basic defense mechanism to control the deposition of fibrin in both vascular and extravascular systems. Proper functioning of the fibrinolytic system is necessary to prevent not only hemorrhagic as well as thrombotic phenomena but also the formation of interstitial fibrin deposits and subsequent scarring.

Tissue-type plasminogen activator (TPA) is considered to play an important role in initiation of the extrinsic fibrinolytic route through conversion of the zymogen plasminogen to the active enzyme plasmin, which degrades fibrin.1 The fibrinolytic capacity of plasma is thus considered to be strongly dependent on the concentration of circulating TPA. Plasma TPA is presumed to be derived mainly from the vascular wall, where it is localized in endothelial cells (ECs).2 The availability of ECs in culture has provided an opportunity to study the regulation of TPA synthesis by these cells in more detail.3 Insight into the regulation of TPA synthesis may be helpful in developing drugs that may counteract insufficient endogenous TPA by increasing its production.

Because TPA and plasminogen activator inhibitor–1 (PAI-1) are produced by the endothelium, regulation of their synthesis and secretion at the EC level represents a rapid and direct way of modulating the fibrinolytic potential of blood. Previous studies have shown that production of plasminogen activators and inhibitors in various cell types is modulated by several compounds; eg, TPA synthesis in ECs is increased by various stimuli, such as thrombin,4 histamine,5 butyrate,6 retinoic acid,7,8 and tumor-promoting agents like phorbol 12-myristate 13-acetate.9 Factors that modulate PAI-1 expression in ECs include lipopolysaccharides,10 thrombin,11 interleukin-1,12 tumor necrosis factor–α,13 transforming growth factor–β, basic fibroblast growth factor,14 and EC growth supplement (EGGS) combined with heparin.15

In China, the herb drug Panax notoginseng has been used to treat cardiovascular diseases and relieve blood stasis and pain by traditional Chinese medical doctors for thousands of years. To elucidate any possible effects of this drug on the hemostatic system in vitro, we investigated the influence of 20(S)-protopanaxatriol notoginsenoside R1 (NR1), which belongs to the dammane type of triterpenoidal saponins and is a major constituent of Panax notoginseng, on TPA, urokinase-type plasminogen activator (UPA), and PAI-1 synthesis...
in cultured human umbilical vein endothelial cells (HUVECs).

Methods

Materials

Chemically pure NR1 was purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China, and was dissolved and diluted in incubation medium to yield final concentrations of 0.01 to 100 μg/mL. Acrylamide, bisacylamide, ammonium persulfate, N,N',N'-tetramethylethylenediamine (TEMED), sodium dodecyl sulfate (SDS) (Bio-Rad); morpholinopropanesulfonic acid (Serva); guanidine thiocyanate (Fluka); piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES, Sigma Chemical Co); Seakem LE agarose (FMC Bioproducts); and [α-<sup>32</sup>P]dCTP (ICN Radiochemicals) were obtained from the sources indicated. Other materials used in the methods described below have been specified in detail in the pertinent references.

Cell Culture

ECs were isolated from fresh human umbilical cord veins with a collagenase technique (Sigma) similar to that described by Jaffe et al. Cells from four to six cords were pooled and plated in 75-cm² tissue-culture flasks (Costar) coated with 1% calf skin gelatin (Sigma). Cells were grown to confluence at 37°C in a humidified 95% air–5% CO₂ atmosphere in medium 199 (Sigma) supplemented with 20% heat-inactivated supplemented calf serum (SCS, HyClone), 100 μg/mL streptomycin, 100 IU/mL penicillin, 250 mg/mL amphotericin B, 1 mmol/L glutamine (JHR Biosciences), 2 IU/mL heparin (Liquemin, Hoffmann-La Roche), and 50 μg/mL ECGS (Technolog). Cells were confirmed as endothelial by their “cobblestone” morphology, positive immunofluorescence with anti-von Willebrand factor VIII antibodies, and uptake of acetylated low-density lipoprotein. Primary cultures were harvested at confluence with 0.05% trypsin-0.02% EDTA, and cells were grown to confluence at 37°C in a humidified 95% air–5% CO₂ atmosphere in medium 199 (Sigma) containing 1.25% SCS, 50 μg/mL ECGS, and heparin at 0.1% SDS after a 30-minute incubation at 37°C. The extracts containing described above until confluence was reached. Average cell densities at confluence were 6×10⁴ cells/cm². All experiments used cells between passages 2 and 3.

Preparation of Conditioned Media (CM) and Extracellular Matrix (ECM)

Confluent cultures were rinsed twice with Hank’s balanced salt solution (Sigma) and incubated at 37°C in 1 mL per well with medium 199 containing 1.25% SCS, 50 μg/mL ECGS, and the indicated concentrations of NR1. After incubation, the culture supernatant was collected after removal of cell debris by centrifugation and stored at −70°C until use. Total cell number of the cultures after trypsinization was counted with a hemocytometer. ECM was prepared from these or similarly treated cultures according to the method of Mimuro et al. The monolayers were washed three times with cold phosphate-buffered saline (PBS: 0.01 mol/L sodium phosphate and 0.14 mol/L NaCl, pH 7.4), and cellular components were extracted by incubation for 10 minutes at 37°C with PBS containing 0.5% Triton X-100. The plates were washed once more with distilled water to remove remaining cellular components and then assessed by light-microscopic examination for the presence of cellular debris. These extraction procedures completely removed visible cellular components from the plates, and the ECM was extracted by scraping it into 1 mL PBS containing 0.1% SDS after a 30-minute incubation at 37°C. The extracts were dialyzed overnight at 4°C against PBS.

Assays for TPA, UPA, and PAI-1 Antigens and TPA–PAI-1 Complexes in CM and ECM

TPA, UPA, and PAI-1 antigens and TPA–PAI-1 complex concentrations were determined by specific commercially available enzyme-linked immunosorbent assays (ELISAs) (Technoclone) according to the manufacturer’s instructions. The test ranges for these assays are 0.3 to 2.5 ng/mL for TPA, 0.1 to 10 ng/mL for UPA, 0.3 to 20 ng/mL for TPA–PAI-1 complexes. The TPA ELISA detects free TPA and TPA complexed with PAI-1. The UPA ELISA detects free UPA and UPA complexed with PAI-1. The PAI-1 ELISA measures free, complexed, and latent PAI-1. The ELISA for TPA–PAI-1 complex measures only TPA–PAI-1 complexes.

Determination of TPA and PAI-1 Functional Activities

TPA and PAI-1 activities were analyzed by fibrin autography and reverse fibrin autography, respectively, after SDS–polyacrylamide gel electrophoresis (PAGE). SDS polyacrylamide slab gels and buffers were prepared as described by Laemmli. Fibrin autography was performed as described by Granelli-Piperno and Reich. Briefly, 100 μL of each sample was applied to a 10-cm resolving gel containing 10% acrylamide and 2-cm stacking gels of 4% acrylamide and 2-cm stacking gels of 4% acrylamide. The gels were incubated at room temperature for 16 hours, or until the dye had reached the bottom of the gels. After electrophoresis, the gels were soaked in 250 mL 2.5% Triton X-100 (Serva) for 90 minutes (two changes of fluid, 45 minutes each) to neutralize the SDS and then applied to the surface of the fibrin-agar indicator films containing 1.5% agarose type L (Behring), 2 mg/mL plasminogen-rich fibrinogen (Organon Teknika), and 0.2 IU/mL bovine thrombin (Sigma). The gels were incubated at 37°C in a moist chamber and photographed at various times. Reverse fibrin autography was performed by placing the gels on fibrin films, prepared essentially as described above, to which was added 0.4 IU/mL urokinase (Technoclone). Quantitation of TPA and PAI-1 activity in a particular sample was accomplished by photograpthing both lysis zones and lysis-resistant regions in the indicator film. These zones were outlined on tracing paper, and the outlined areas were cut out and weighed on an analytical balance.

To immunologically identify the plasminogen activator in the CM of HUVECs, samples of CM were incubated for 24 hours at 4°C with either a monoclonal anti-TPA antibody (MPW3VPA, Technoclone) or a monoclonal anti-UPA antibody (MPWSUK, Technoclone) bound to cyanogen bromide-activated Sepharose, or as a control, with Sepharose 4B (Pharmacia, Sweden). Thereafter, the Sepharose was removed by centrifugation, and 100 μL of each sample was analyzed by SDS-PAGE followed by fibrin autography as described above.

Quantitation of TPA and PAI-1 mRNA Levels by Northern Blot Analysis

Total cellular RNA was isolated from ECs by acid guanidinium thiocyanate/phenol/chloroform extraction as described by Chomczynski and Sacchi. The final RNA pellet was resuspended in 50 μL 0.5% SDS and the concentration determined at 260 nm. For Northern blot analysis, RNA samples were electroblotted to a 1.2% agarose gel followed by capillary transfer of the fractionated RNA to a Duralon-UV membrane (Stratagene). RNA blots were placed in Seal-a-Meal bags and prehybridized in 50 mmol/L PIPES, 100 mmol/L NaCl, 50 mmol/L sodium phosphate, and 1 mmol/L EDTA, containing 5.0% SDS for at least 3 hours at 37°C. The prehybridization buffer was then discarded and replaced with...
Notoginsenoside R1 concentration (ug/ml)

Fig 1. Semilog line graphs showing effect of notoginsenoside R1 (NR1) on tissue-type plasminogen activator (t-PA) antigen production and 51Cr release by human umbilical vein endothelial cells (HUVECs) (A), TPA-plasminogen activator inhibitor-1 (PAI-1) complexes (B), and PAI-1 antigen (C) production in cultured HUVECs. HUVECs were incubated for 24 hours with different concentrations of NR1. 51Cr release was determined as described in “Methods” section and is given in percent 51Cr released (A, C). Conditioned media were analyzed for TPA antigen (A, •), PAI-1 antigen, and TPA-PAI-1 complexes as described in “Methods.” Results are mean values of three experiments, each performed in triplicate. Values are mean±SD. *P<.05, **P<.01; ***P<.001 compared with control.

Notoginsenoside R1 concentration (ug/ml)

fresh prehybridization buffer containing 10⁶ cpm/mL of ³²P-labeled cDNA probes for either human TPA, human PAI-1, or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Hybridization was carried out in a water bath overnight at 57°C. After hybridization, blots were removed from the bag and rinsed for 10 minutes in 100 mL of 5% SDS and 0.2x SSC (SSC is saline-sodium citrate) at room temperature. Thereafter the blots were washed for 20 minutes in 400 mL of 5% SDS and 1x SSC at the hybridization temperature. After hybridization, the RNA blots were air dried and exposed to XAR-5 x-ray film (Eastman Kodak) at -70°C. To quantitate differences in specific mRNA expression, the developed films were scanned with a densitometer (Hirschmann Elscript 400, Hirschmann). Scanning data for each specific mRNA message were compared with the intensity of the GAPDH message.

cDNA Probes

The following cDNA fragments were used as probes in the hybridization experiments: a 1.5-kb Sma I/HindIII fragment of human TPA cDNA, a 1.4-kb EcoRI/Bgl II fragment of human PAI-1 cDNA of the 3.2-kb transcript, and a 1.2-kb Pst I fragment of rat GAPDH cDNA, which was used as an internal-standard probe. cDNA fragments were radiolabeled by random priming (random prime DNA labeling kit, Boehringer Mannheim).

Determination of Cell Viability

Cell viability was determined by a chromium release assay as described. Confluent monolayers of HUVECs grown in 24-well plates (Costar) were incubated in medium 199 containing 20% SCS and 50 µg/mL ECGS with ³¹Cr (1 µCi per well) for 2 hours at 37°C. Thereafter the monolayers were rinsed three times with Hank’s balanced salt solution, fresh media with or without the indicated concentrations of NR1 were added, and the cells were incubated for 24 hours at 37°C. At the end of incubation the amounts of ³¹Cr released into the media and bound to the cells were determined by gamma counting.

Statistical Analysis

Results are reported as mean±SD. Student’s unpaired t test was used to determine significance levels.

Results

NR1 Induces TPA but Not PAI-1 Antigen Secretion in Cultured HUVECs

As shown in Fig 1A, treatment of HUVECs with increasing doses of NR1 for 24 hours resulted in a dose-dependent increase of TPA antigen in the CM of such treated cells. Maximal effects were achieved with 100 µg/mL NR1 (100 µg/mL NR1, 8.0±0.5 ng per 10⁵ cells per 24 hours versus control, 3.9±0.2 ng per 10⁵ cells per 24 hours; n=9, P<.001). This effect, however, was not due to a toxic effect of NR1 on HUVECs, as demonstrated by the results of the chromium release assay, which showed that chromium release was constant over the concentration range of NR1 tested (chromium release, 100 µg/mL NR1, 41.3±0.8%; for control, 42.0±6.6%). As shown in Fig 1B, TPA-PAI-1 complexes in CM increased in a similar fashion in the presence of increasing concentrations of NR1 (100 µg/mL NR1, 6.4±0.3 ng per 10⁵ cells per 24 hours versus control, 3.9±0.2 ng per 10⁵ cells per 24 hours; n=9, P<.01). PAI-1 antigen in the CM as well as the ECM of HUVECs treated with NR1 did not change significantly when compared with controls (CM: 100 µg/mL NR1, 2.92±0.32 µg per 10⁵ cells per 24 hours versus control, 2.78±0.45 µg per 10⁵ cells per 24 hours, n=9; ECM: 100 µg/mL NR1, 42.5±3.15 ng/mL per 24 hours versus control, 41.3±0.8%).
Zhang et al. Notoginsenoside Stimulates EC Fibrinolysis

Influence of NR1, RA, and PMA on Tissue-Type Plasminogen Activator Production In HUVECs

Table 1: Comparison of Effect of NR1 on TPA Production by HUVECs With Other Stimulators of TPA Production in ECs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TPA Antigen (ng/10^6 cells/24 h)</th>
<th>Fold Stimulation of TPA Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.2±0.3</td>
<td>...</td>
</tr>
<tr>
<td>NR1, 100 μg/mL</td>
<td>8.0±0.5</td>
<td>-1.9</td>
</tr>
<tr>
<td>RA, 1 μmol/L</td>
<td>8.7±1.8</td>
<td>-2.1</td>
</tr>
<tr>
<td>PMA, 1 ng/mL</td>
<td>12.8±0.8</td>
<td>-3.1</td>
</tr>
</tbody>
</table>

Human umbilical vein endothelial cells (HUVECs) were incubated for 24 hours with the indicated concentrations of notoginsenoside R1 (NR1), retinoic acid (RA), or phorbol 12-myristate 13-acetate (PMA). Conditioned media were analyzed for TPA antigen as described in "Methods." TPA antigen is expressed in nanograms per 10^6 cells per 24 hours. Results are mean±SD values of three experiments, each performed in triplicate.

NR1 Increases TPA Activity and Decreases PAI-1 Activity in Cultured HUVECs

When CM harvested from HUVECs incubated for 24 hours under control conditions were analyzed by SDS-PAGE followed by fibrin autography, two predominant lysis zones with apparent molecular masses of 70 and 120 kDa were identified. These lysis zones could be depleted by preincubation with monoclonal anti-TPA antibodies but were not affected by preincubation with monoclonal anti-UPA antibodies (Fig 4). Therefore, we concluded that the lysis zone at 70 kDa was caused by free TPA and that the high-molecular-weight lysis zone (ie, 120 kDa) resulted from TPA complexed with PAI-1.

When CM from HUVECs treated with or without increasing concentrations of NR1 for 24 hours were analyzed by fibrin autography and reverse fibrin autography, a dose-dependent increase in the size of the lysis zones was seen, whereas lysis-resistant zones decreased with increasing amounts of NR1 (Fig 5A and 5B). When the sizes of the lysis zones and lysis-resistant zones were quantitated as outlined in "Methods," as much as a threefold increase in TPA-dependent lysis and a twofold increase in TPA-PAI-1 complex-dependent lysis were seen, whereas PAI-1-dependent lysis resistance

Fig 3. Line plot showing time course of tissue-type plasminogen activator (t-PA) antigen (A) and plasminogen activator inhibitor-1 (PAI-1) antigen (B) production after exposure of cultured human umbilical vein endothelial cells (HUVECs) to notoginsenoside R1 (NR1). HUVECs were incubated for the indicated times in the absence (c) or presence (e) of 100 μg/mL NR1. At specified times, conditioned media were harvested and assayed for TPA and PAI-1 antigens, as outlined in "Methods." Results are mean±SD values of three experiments, each performed in triplicate. *P<.05, **P<.01 compared with control.
Mr
1  2  3  4  5  6
120 kD
70 kD
52 kD

Fig 4. Fibrin autography (FA) of conditioned media (CM) from human umbilical vein endothelial cells (HUVECs). CM from HUVECs that were not treated with notoginsenoside R1 were collected after 24 hours and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by FA as described in “Methods.” Lane 1, untreated CM from HUVECs; lane 2, CM of HUVECs preincubated with monoclonal anti-tissue-type plasminogen activator (TPA) antibodies bound to Sepharose; lane 3, CM of HUVECs preincubated with monoclonal anti-urokinase-type plasminogen activator (UPA) antibodies bound to Sepharose; lane 4, CM of HUVECs preincubated with Sepharose 4B; lane 5, purified human TPA; lane 6, purified human UPA.

decreased to 20% in the presence of 100 μg/mL NR1 compared with control (Fig 5A and 5B).

NR1 Increases Specific TPA mRNA Level

As shown in Fig 6, the stimulating effect of NR1 on TPA secretion in HUVECs was also reflected at the level of specific mRNA expression. TPA-specific message increased as much as twofold in HUVECs treated with 100 μg/mL NR1 for 12 hours, whereas PAI-1-specific mRNA expression was not regulated by NR1 (3.2 kb, 82% of control; 2.2 kb, 86% of control). When Northern blotting experiments were performed in the presence of 10 μg/mL cycloheximide, the stimulating
effect of NR1 on TPA-specific mRNA was abolished (data not shown).

Discussion

In vivo, vascular ECs are thought to be the main source of circulating plasma TPA, which is considered to be mainly responsible for intravascular lysis of fibrin clots. On the other hand, human ECs also express TPA in vitro and therefore provide a useful tool to investigate mechanisms involved in the regulation of TPA expression.

In this study we investigated the effect of NR1 on the fibrinolytic system of cultured HUVECs. When HUVECs were incubated with increasing doses of NR1, a dose-dependent concomitant increase in TPA antigen production and TPA–PAI-1 complex formation by such treated cells was seen, whereas PAI-1 antigen production was not affected. When HUVECs were treated with NR1 for different times, a significant increase in TPA antigen production by such cells was seen at all times tested when compared with control cells. UPA and PAI-1 antigens were not affected by NR1 treatment for the times tested. The stimulating effect of NR1 on TPA production was not due to a cytotoxic effect of NR1, as demonstrated by the fact that 51Cr release was not affected by NR1 at all doses tested. When the effect of NR1 on TPA production by HUVECs was compared with the effects of retinoic acid or PMA, two potent stimulators of TPA production by ECs,26–27 it could be shown that the maximal twofold stimulation of TPA production caused by NR1 was within the same order of magnitude as the effects of retinoic acid and PMA, which caused a maximal twofold and threefold stimulation, respectively.

The results that showed an increase in TPA antigen production and TPA–PAI-1 complex formation caused by NR1 were confirmed when the actual fibrinolytic and antifibrinolytic activity produced by HUVECs in the presence and absence of NR1 was analyzed by fibrin autography and reverse fibrin autography, respectively. As shown by fibrin autography, fibrinolytic activities associated with free TPA and TPA complexed with PAI-1 increased dose dependently as much as threefold and twofold, respectively, when HUVECs were incubated with increasing concentrations of NR1; antifibrinolytic activity associated with PAI-1 decreased concomitantly to approximately 20%. This decrease in PAI-1 activity could be partially explained by an increase in TPA production, which would increase complex formation between TPA and PAI-1 and thereby decrease the amount of free PAI-1. This increase in complex formation between TPA and PAI-1 was indeed confirmed by a specific ELISA and fibrin autography.

The increase in TPA antigen was also reflected at the level of specific mRNA expression as determined by Northern blotting. TPA mRNA increased as much as twofold when HUVECs were treated with NR1, whereas expression of PAI-1-specific mRNA was not significantly affected by NR1. This latter result further confirmed that the increase in TPA–PAI-1 complexes seen on ELISA and fibrin autography was due to enhanced formation of complexes brought about by an increased production of TPA. The NR1-induced increase in TPA mRNA, however, was abolished by cycloheximide, thereby indicating that such increase is dependent on de novo protein synthesis.

However, it is difficult to assess how our results relate to the situation in vivo when Panax notoginseng is used therapeutically in humans. In most studies Panax notoginseng has been used as only one component of a mixture of medical herbs, and no data are available on the exact amount of the active constituent NR1 in this mixture. In studies with rats or rabbits, Panax notoginseng has been used at doses of 100 to 200 mg/kg body weight.29,30 Purified ginsenosides have been used in animal experiments at dilutions of 25 µg/mL in perfusion experiments to prevent ischemic injury and at a dose of 20 mg/kg body weight to restore impaired injury.30,31 Therefore, the dose of NR1 used in this study, despite the lack of actual data on concentrations and pharmacokinetic properties of NR1, should be within the range of concentrations of ginsenosides used in vivo.

In conclusion our data provide evidence that NR1 can increase the fibrinolytic potential of HUVECs in vitro by increasing expression of TPA in such cells. This increase in fibrinolytic potential is due to an increase in the amount of free, active TPA and a concomitant reduction of antifibrinolytic activity by enhanced complex formation between PAI-1 and TPA, thereby leading to a subsequent decrease in PAI-1 available to inhibit TPA. This effect of NR1 on the fibrinolytic system of ECs may also be operative in vivo and contribute to the effect of the Chinese herb drug Panax notoginseng in the treatment of cardiovascular diseases.

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