Ginsenosides block HIV protease inhibitor ritonavir-induced vascular dysfunction of porcine coronary arteries

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Chai, Hong, Wei Zhou, Peter Lin, Alan Lumsden, Qizhi Yao, and Changyi Chen. Ginsenosides block HIV protease inhibitor ritonavir-induced vascular dysfunction of porcine coronary arteries. Am J Physiol Heart Circ Physiol 288: H2965–H2971, 2005.—Human immunodeficiency virus (HIV) protease inhibitor ritonavir (RTV) may induce vascular dysfunction through oxidative stress. Ginsenosides have been shown to have potential benefits on the cardiovascular system through diverse mechanisms, including antioxidant property. The objective of this study was to determine whether ginsenosides could prevent coronary arteries from RTV-induced dysfunction. Porcine coronary artery rings were incubated with RTV and ginsenosides Rb1, Rc, and Re for 24 h. Vasomotor function was recorded by a myograph tension system. In response to the thromboxane A2 analog U-46619, the contraction of the vessel rings was significantly reduced. When cocultured with Rb1, Rc, and Re, the contractility significantly increased. In response to bradykinin at 10−8 M, the endothelium-dependent relaxation of vessel rings was significantly reduced by 59% for RTV compared with controls (P < 0.05). When cocultured with Rb1, Rc, and Re, the relaxation significantly increased 100%, 90%, and 134%, respectively, compared with the RTV-alone groups (P > 0.05). In response to sodium nitroprusside, RTV significantly reduced vasorelaxation. In addition, the endothelial nitric oxide synthase (eNOS) mRNA levels were significantly reduced by 78% for RTV group (P < 0.05) by real-time PCR analysis. The eNOS protein levels measured by Western blot analysis and nitrite concentrations measured by Griess assay were also decreased, whereas O2− production by lucigenin-enhanced chemiluminescence was significantly increased in the RTV-treated group. These effects of RTV were effectively blocked by ginsenosides. Thus HIV protease inhibitor RTV significantly impaired the vasomotor function of porcine coronary arteries. This effect may be mediated by the downregulation of eNOS and overproduction of O2−. These results suggest that ginsenosides can effectively block RTV-induced vascular dysfunction.

oxidative stress; ginseng root; vasomotor; endothelial nitric oxide; superoxide anion; human immunodeficiency virus

HUMAN IMMUNODEFICIENCY VIRUS (HIV) infection is characterized by persistent viral replication and progressive immune dysfunction. HIV protease inhibitors (PIs) confer striking immunologic and clinical benefits that have led to their widespread acceptance as key components of highly active antiretroviral therapy (HAART) in patients with HIV infection. Whether HIV itself or HAART leads to cardiovascular diseases is still under investigation. Still, up to 60% of patients receiving HIV PIs develop dyslipidemia, hyperglycemia, and central obesity (4, 10, 11). These metabolic changes adversely affect several risk factors for atherosclerotic vascular disease (1, 21, 30).

Ginseng root is used extensively in traditional Chinese medicine for its alleged tonic effect and possible curative and restorative properties. There are increasing data in the literature on ginseng and its potential role in treating cardiovascular diseases. In the published studies involving cell cultures and animal models, ginseng was shown to have potential benefits on the cardiovascular system through diverse mechanisms such as having antioxidant (13, 19), modifying vasomotor function (15, 26), improving lipid profiles (18), and involving glucose metabolism (22, 31).

Chen’s group previously showed that ritonavir (RTV), one of the clinically used PIs, can cause endothelial injury or dysfunction in human endothelial cells (37) and porcine carotid arteries (7). This effect is frequently induced by a remarkable increase of O2− production. In this study, we examined whether ginsenosides Rb1, Rc, and Re could block RTV-induced vascular dysfunction in porcine coronary arteries. Specifically, the vasomotor functions, expression of endothelial nitric oxide synthase (eNOS), and the status of oxidative stress were investigated. The results suggest a new therapeutic strategy to control HIV PIs-associated cardiovascular complications.

MATERIALS AND METHODS

Chemicals and reagents. 9,11-Dideoxy-11α,9α-epoxymethano-prostaglandin F2α (U-46619), bradykinin, sodium nitroprusside (SNP), ginsenosides, β-actin monoclonal antibody, Tri-agent kit, and Tween 20 were obtained from Sigma Chemical (St. Louis, MO). Lucigenin was obtained from Molecular Probes (Eugene, OR). Nitric oxide assay kit was obtained from Calbiochem (San Diego, CA). Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Life Technologies (Grand Island, NY). Antibiotic-antimycotic solution was obtained from Mediatech (Herndon, VA). iScript cDNA Synthesis Kit, the iQ SYBR Green SuperMix Kit, protein assay kit, and precast polyacrylamide gels were obtained from Bio-Rad Laboratories (Hercules, CA). Antibody against human eNOS was obtained from BD Transduction Laboratories (Lexington, KY). Horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibodies and the enhanced chemiluminescence kit were obtained from Amer sham Life Sciences (Buckinghamshire, UK). The biotinylated horse anti-mouse IgG and avidin-biotin complex kit were obtained from Vector labs (Burlingame, CA). RTV was obtained through the acquired immunodeficiency syndrome (AIDS) Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. PCR primers were obtained from Sigma Genosys (Woodlands, TX).

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The eNOS gene expression in each sample was calculated as $2^{\Delta \Delta C_{t}}$ amplification so that sample measurement comparisons were possible. The amount of nitrite formed was normalized for 24 h. Supernatant was collected, and total nitrite levels were measured by Griess reaction using a nitric oxide assay kit (Calbiochem, San Diego, CA). The amount of nitrite formed was normalized to the area of each vessel. Final data were represented as means $\pm$ SE (in nM/mg protein).

Detection of total nitrites. Nitric oxide (NO) release from vessel rings was determined by measuring the accumulation of its stable degradation products nitrite and nitrate. Nitrate was converted to nitrite by using nitrate reductase. Artery rings were cultured in DMEM medium (without phenol red, Biosource, Rockville, MD) with RTV alone or with combinations of RTV and Rb1, Rc, or Re (10 μM) for 24 h. Supernatant was collected, and total nitrite levels were measured by Griess reaction using a nitric oxide assay kit (Calbiochem, San Diego, CA). The amount of nitrite formed was normalized to the area of each ring. Final data were represented as means $\pm$ SE (in μM/mg protein).

Statistical analysis. Maximal contraction and endothelium-dependent and independent vasorelaxation of the rings were compared between the control and treatment groups using one-way ANOVA. Real-time PCR, Western blot, O$_2$- and total nitrite data from the different groups were compared using the paired Student’s t-test (two-tails). Significance was considered if $P < 0.05$. Data were reported as means $\pm$ SE.

RESULTS

Effect of RTV and ginsenosides on vasomotor function in porcine coronary arteries. Porcine coronary artery rings were cultured for 24 h with a clinically relevant dose of 15 μM of RTV, with or without ginsenosides Rb1 (1 and 10 μM), Rc (10 μM), and Re (10 μM), and subsequently subjected to physiological contraction (U-46619) and endothelium-dependent (bradykinin) and endothelium-independent (SNP) relaxation ($n = 8$). In response to U-46619, the contraction of the vessel rings was significantly reduced by 76% for RTV compared with controls ($P < 0.05$, ANOVA, Fig. 1A). When cocultured with Rb1 (1 and 10 μM), Re, or Re, the contractility increased by 82%, 117%, 222%, and 198%, respectively, compared with the RTV-alone groups ($P < 0.01$, ANOVA, Fig. 1A). In response to bradykinin at 10$^{-7}$ M, the endothelium-dependent relaxation of vessel rings was significantly reduced by 59% for RTV compared with controls ($P < 0.05$, ANOVA, Fig. 1B).
and C). When cocultured with Rb1 (1 and 10 μM), Rc, or Re, the endothelium-dependent relaxation significantly increased by 55%, 100%, 90%, and 134%, respectively, compared with the RTV-alone groups (P < 0.05, ANOVA, Fig. 1, B and C). In response to SNP, RTV treatment significantly reduced vasorelaxation by 50% compared with controls (P < 0.05, ANOVA, Fig. 1D). When cocultured with Rb1 (1 and 10 μM), Rc, or Re, the relaxation increased by 33%, 72%, 92%, and 93%, respectively, compared with the RTV-alone groups (Fig. 1D).

Effect of RTV and ginsenosides on eNOS expression. To determine whether eNOS expression was correlated to the reduction of endothelium-dependent vasorelaxation in RTV-treated vessels, the eNOS mRNA levels of the endothelial cells isolated from the vessels were determined by real-time PCR analysis (n = 6). The eNOS mRNA levels were significantly reduced by 78% for the RTV-treated group compared with controls (P < 0.05, ANOVA, Fig. 2). When cocultured with 10 μM of Rb1, Rc, or Re, the eNOS mRNA levels were increased to the control level (Fig. 2).

The eNOS protein level was also analyzed by using Western blot analysis (n = 3). RTV treatment significantly decreased the eNOS protein level by 40% compared with controls, whereas Rb1, Rc, or Re coculture totally reversed RTV-induced eNOS reduction (P < 0.05, t-test, Fig. 3, A and B). Immunohistochemistry staining also confirmed that the eNOS protein level in RTV-treated vessel rings was reduced compared with controls (n = 3). When cocultured with Rb1, Rc, or Re, eNOS immunoreactivity was reversed to the control level (Fig. 4).

Effect of RTV and ginsenosides on NO production. The NO production in rings after RTV and Rb1, Rc, or Re treatment was also analyzed (n = 3). Total nitrite accumulation in the supernatant of cultured vessel rings is shown in Fig. 5. In the control group, the basal nitrite level was 1.12 μM/mm². In the RTV-treated group, the NO production was decreased to 71% of the control group (P < 0.05, t-test). When cocultured with 10 μM of Rb1, Rc, and Re, the NO production increased to 93%, 84%, and 80% of the control group, respectively. The effects of Rb1 and Rc were significant compared with the RTV-alone groups (P < 0.05, t-test).

Fig. 1. Effect of ritonavir (RTV) and ginsenosides on vasomotor function of porcine coronary arteries. Pig right coronary arteries (n = 8) were cultured with DMEM alone (as control) or treated with 15 μM of RTV and with or without ginsenosides Rb1 (1 and 10 μM), Rc (10 μM), and Re (10 μM) for 24 h. A: maximal contraction of the vessel rings in response to thromboxane A₂ analog U-46619 (10⁻⁷ M) was analyzed. RTV-alone group showed a significant reduction in contractility (*P < 0.05, t-test) compared with control samples. When cocultured with Rb1, Rc, and Re, the contractility increased compared with the RTV-alone groups (**P < 0.01, ANOVA).

B: precontracted vessels were tested for relaxation by adding a series of concentrations of bradykinin (10⁻⁹–10⁻⁵ M), which showed a significant reduction in the RTV-treated samples (*P < 0.01, ANOVA). C: in response to bradykinin at 10⁻⁵ M, the RTV-alone group showed a significant decrease of vasorelaxation compared with control samples (*P < 0.05). When cocultured with Rb1 (1 and 10 μM), Rc, and Re, the endothelium-dependent relaxation significantly increased compared with the RTV-alone groups (**P < 0.05, ANOVA). D: endothelium-independent relaxation in response to sodium nitroprusside (SNP, 10⁻⁶ M) was also significantly reduced in the RTV-treated vessels (*P < 0.05). When cocultured with Rb1 (1 and 10 μM), Rc, and Re, the endothelium-independent relaxation increased compared with the RTV-alone groups (***P < 0.05, ANOVA).
Effect of RTV and ginsenosides on O\textsubscript{2}\textsuperscript{-} production. Oxidative stress has been shown to cause endothelial dysfunction and vascular injury. To determine whether this is involved in RTV-induced vasomotor dysfunction, O\textsubscript{2}\textsuperscript{-} production was analyzed by a lucigenin-enhanced chemiluminescence assay \((n = 6)\). The O\textsubscript{2}\textsuperscript{-} levels of the endothelial layer of vessel rings were significantly increased by 151\% for RTV compared with control samples \((P < 0.05, t\text{-test}, \FigRef{6})\). Cocultured with Rb1, Rc, or Re reversed the O\textsubscript{2}\textsuperscript{-} level to the control levels \((P > 0.1, t\text{-test}, \FigRef{6})\).

DISCUSSION

A principal finding of this study is that a clinically relevant dose of RTV significantly reduced vasocontractility and endothelium-dependent and independent vasorelaxation in porcine coronary artery cultures. RTV also significantly decreased eNOS mRNA and protein levels, as well as NO release, but increased O\textsubscript{2}\textsuperscript{-} production. Meanwhile, ginsenosides Rb1, Rc, and Re can effectively block these detrimental effects of RTV in porcine coronary arteries. The results from this study reveal several potential mechanisms of RTV-induced vascular dysfunction and possible therapeutic values of ginsenosides in preventing the side effects of HIV PIIs in clinical practices.

Since the introduction of the HAART regimen in 1996, the mortality and morbidity of HIV-infected patients have declined sharply. These patients showed a longer lifespan and improved quality of life \((24)\). However, despite these benefits, all anti-retroviral agents, including HIV PIs, nucleoside reverse transcriptase inhibitor (NRTI), and nonnucleoside reverse transcriptase inhibitor (NNRTI), have been associated with cardiovascular complications \((3, 8)\). Although the mechanisms of these complications are not fully understood, metabolic abnormalities as risk factors may play crucial roles in HIV PI-associated cardiovascular lesion formation. In the current investigation, a clinically relevant dose of RTV (15 \(\mu\)M) significantly induced vasomotor dysfunction, including decreasing vessel contractility and endothelium-dependent vasorelaxation as well as independent vasorelaxation. These results are consistent with several clinical studies \((8, 29)\). HIV-infected individuals receiving HAART showed impaired vasodilation by using a flow-mediated brachial artery vasodilation assay, a noninvasive technique that relies on high-resolution ultrasound of the brachial artery. In this study, we used only one concentration of U-46619 for constrictions of these arterial rings. This could be a limitation. A full dose response to U-46619 may demonstrate whether this finding is a shift in sensitivity to the agonist or whether maximal constrictions are altered. It could be possible that the different levels of preconstriction may contribute to the altered relaxation responses observed. These issues warrant further investigations.

NO produced from eNOS is a key regulator of vascular homeostasis, including basal vascular tone (blood flow) and blood pressure \((34)\), as well as acting as an anti-thrombogenic agent \((32)\). An impairment of endothelium-dependent relaxation is present in atherosclerotic vessels even before vascular structural changes occur and represents the reduced eNOS-derived NO activity \((16)\). Bradykinin induces vasodilation via endothelial bradykinin type 2 (B2) receptors. This effect can be blocked partly by inhibitors of NO synthase, suggesting a role for de novo synthesis of NO from L-arginine by NO synthase \((2, 25)\). It has been reported that bradykinin-induced relaxations of porcine coronary artery rings precontracted with U-46619 were attenuated by the NO synthase inhibitor \(\text{N}^\text{G}\)-nitro-L-arginine methyl ester \((\text{-NAME})\) \((33)\). Our real-time PCR, Western blot, and immunohistochemistry data indicated that eNOS expression in endothelial cells was significantly reduced in RTV-treated pig coronary artery rings. RTV can

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**Fig. 2.** Effect of RTV and ginsenosides on endothelial nitric oxide synthase (eNOS) mRNA levels. Porcine coronary rings were cultured with DMEM alone (as control) or treated with 15 \(\mu\)M of RTV, with or without ginsenosides Rb1 (10 \(\mu\)M), Rc (10 \(\mu\)M), and Re (10 \(\mu\)M) for 24 h. Total mRNA was extracted from endothelial layers from the rings, and mRNA levels of eNOS and GAPDH were quantified with real-time PCR analysis. The eNOS mRNA level in each sample was normalized to that of GAPDH. Relative mRNA level and GAPDH were quantified with real-time PCR analysis. The eNOS mRNA was presented as 2\(^{-}\Delta\text{CT(eNOS)}\), where \(\Delta\text{CT}\) is cycle threshold. RTV treatment significantly decreased the eNOS mRNA expression compared with control samples \((P < 0.05, n = 6)\). When cocultured with 10 \(\mu\)M of Rb1, Rc, and Re, the mRNA level significantly increased compared with the RTV-alone groups \((**P < 0.01, \text{ANOVA})\).

**Fig. 3.** Effect of RTV and ginsenosides on the eNOS protein levels. Total protein was extracted from the rings after treatment. The eNOS protein levels were determined by Western blot using monoclonal antibody against human eNOS \((1:1,000)\) \((A)\). RTV-alone significantly decreased eNOS level, whereas coculture with 10 \(\mu\)M of Rb1, Rc, and Re reversed this effect \((*P < 0.05)\) \((B)\).
also decrease bioavailability of NO, which may be caused by reaction with $O_2^-$ or thiol groups. It is well known that an increase of reactive oxygen species can result in uncoupling of mitochondrial oxidative phosphorylation and eNOS activity, reducing NO availability and generating more reactive oxygen species. Notably, when coronary artery rings were cocultured with RTV and ginsenosides (Rb1, Rc, and Re), these damaging effects of RTV on vasomotor functions were significantly reversed, indicating protective effects of these ginsenosides on vessel walls. Furthermore, ginsenosides can also reverse the effects of RTV on eNOS mRNA and protein expression, as well as NO and $O_2^-/H_2O_2$ production. These data are consistent with a study that showed another ginsenoside Rg1 can enhance NO production, and the expression of eNOS mRNA in tumor necrosis factor-$\alpha$ stimulated human umbilical vein endothelial cells (20). Ginsenoside-induced NO release in cultured endothelial cells has also been well documented. Chen and his colleagues (6) reviewed relaxation effects of ginsenoside Rb1 and Rg1 on pulmonary vessels and discovered that it was eliminated by nitro-L-arginine, an inhibitor of NO synthase. Scott et al. (26) examined the effects of ginsenosides Rb1 and Re on rat cardiac myocytes and discovered that both ginsenosides decreased cardiac contraction in adult rat ventricular myocytes. Additionally, they showed that pretreatment with NO synthase inhibitor attenuated the effects of Rb1 and Re, which further indicated that NO production may be a mediator (26). These data are particularly important because eNOS has been reported to be a target molecule of RTV at the clinically relevant plasma concentration (15 $\mu$M) (7). Future studies...
investigating the phosphorylation of eNOS are warranted to further understand this issue.

Numerous reports have shown that oxidative stress plays a pivotal role in endothelial dysfunction, cardiovascular diseases, and other pathogenic conditions (9, 14). Free radicals have been well documented to play a key role in atherosclerotic plaque formation and endothelial dysfunction (17). Among them, superoxide anions (O$_2^-$) could react with endothelium-derived NO rapidly and inactivate its effect (25), resulting in reduction or loss of endothelium-dependent vasorelaxation and increase of other atherogenic processes. In this study, we showed a significant increase of O$_2^-$ production in the endothelial layer of the RTV-treated porcine coronary artery rings. Thus RTV-induced oxidative stress may elucidate its effects on vascular dysfunction, especially on endothelium-dependent vasorelaxation where NO is an important mediator. We also showed that ginsenoside Rg1 can promote angiogenesis, whereas Rb1 exerts an opposing effect (27). Future studies are warranted to fully understand this issue.

In summary, a clinically relevant dose of RTV (15 μM) can cause vasomotor dysfunction, decrease eNOS expression, and increase O$_2^-$ production. Ginsenosides Rb1, Re, and Rc can effectively block all these detrimental effects of RTV. With the increasing use of HAART regimen in HIV-infected patients, more vascular complications resulting from HIV infection or side effects of antiviral drugs are expected. The data from this study raise the possibility of a new therapeutic strategy for this significant clinical problem.

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