EFFECTS OF ICARIIN ON PHOSPHODIESTERASE-5 ACTIVITY IN VITRO AND CYCLIC GUANOSINE MONOPHOSPHATE LEVEL IN CAVERNOUS SMOOTH MUSCLE CELLS

HONGXIU NING, ZHONG-CHENG XIN, GUITING LIN, LIA BANIE, TOM F. LUE, AND CHING-SHWUN LIN

ABSTRACT

Objectives. To investigate the effect of icariin on the cyclic guanosine monophosphate (cGMP)-hydrolytic activity of phosphodiesterase-5 (PDE5) isoforms and the cGMP levels in cavernous smooth muscle cells treated with sodium nitroprusside (SNP).

Methods. PDE5 isoforms (PDE5A1, A2, and A3) were isolated from sf9 insect cells infected with baculoviruses carrying PDE5 isoform cDNA. Icariin was isolated from Epimedii herba. Varying amounts (10⁻⁶ to 10⁻¹¹ M) of icariin or zaprinast were added to reaction mixtures containing PDE5 isoforms and cGMP. The inhibitory effects of icariin and zaprinast were analyzed by GraphPad Software and are expressed as concentration that inhibits 50% (IC₅₀) values. Cavernous smooth muscle cells were isolated from 3-month-old rats, treated with icariin (100 and 200 μM) or zaprinast (200 μM) for 15 minutes, and then with 10 μM SNP for 30, 60, 120, 240, and 360 minutes. The cells were then analyzed for the cGMP concentration using an enzyme immunoassay system.

Results. Icariin inhibited PDE5A1, A2, and A3 with an IC₅₀ value of 1.0, 0.75, and 1.1 μM, respectively. The corresponding IC₅₀ values for zaprinast were 0.33, 0.23, and 0.52 μM. Icariin consistently outperformed the control (SNP-only treatment) in maintaining greater cGMP levels, particularly at the greater concentration of 200 μM. In contrast, zaprinast at 200 μM did better than the control only at 60 and 360 minutes.

Conclusions. Icariin was inhibitory to all three PDE5 isoforms with similar IC₅₀ values, which were approximately three times greater than those for zaprinast. Icariin was able to enhance cGMP levels in SNP-treated cavernous smooth muscle cells.


Current treatment for erectile dysfunction (ED) relies largely on the use of the synthetic compounds sildenafil, vardenafil, and tadalafil, which are collectively called phosphodiesterase-5 (PDE5) inhibitors. PDE5 catalyzes the conversion of cyclic guanosine monophosphate (cGMP) to its linear form, leading to penile detumescence. PDE5 inhibitors bind to the cGMP-catalytic site on PDE5, preventing its destruction of cGMP. The resulting accumulation of cGMP in penile smooth muscle cells allows patients with ED to have an otherwise unattainable erection.¹

In humans, three PDE5 isoforms (PDE5A1, A2, and A3) have been identified.¹ These isoforms are the products of alternatively spliced messenger RNAs and are identical in all of the known regulatory and catalytic domains. The only difference in the amino terminal has not been shown to be associated with a particular function. Although the A1 and A2 isoforms are expressed in many cell types, the A3 isoform is smooth muscle specific, the functional significance of which is unknown. Recently, we reassessed the in vitro cGMP-hydrolytic activities of purified PDE5 isoforms and obtained Kₘ
values of 4.76 ± 0.37 μM for A1, 4.52 ± 0.09 μM for A2, and 11.39 ± 0.22 μM for A3. We also found the IC$_{50}$ value of sildenafil was 1.20 ± 0.34 nM, 2.83 ± 0.56 nM, and 2.28 ± 0.38 nM against PDE5A1, PDE5A2, and PDE5A3, respectively.²

Although sildenafil, vardenafil, and tadalafil are currently the first-line choices for treating ED, many alternative therapeutic approaches are still being investigated worldwide. These approaches invariably seek to treat patients who are either untreatable with these compounds, intolerant of their side effects, unable to afford them, or unwilling to use them (eg, because of their being synthetic). In China, Yin Yang Huo (horny goat weed, *Epimedium herba*) has been used for centuries to enhance sexual drive and performance. Recent studies by Xin and colleagues³ and Liu et al.⁴ have shown that icariin (molecular formula C$_{33}$H$_{40}$O$_{15}$, molecular weight 676.67), a flavonol glycoside and major component of Yin Yang Huo, is able to relax cavernous smooth muscle and enhance erectile function. In vitro tests showed that icariin had an IC$_{50}$ value of 0.432 μM and 73.50 μM against PDE5 and PDE4 (ratio of 167.67), respectively,⁵ indicating a high degree of target selectivity. In the present study, we have determined for the first time the IC$_{50}$ values of icariin against purified PDE5 isoforms, and the cGMP levels in icariin-treated cavernous smooth muscle cells (CSMCs).

**MATERIAL AND METHODS**

**ISOLATION OF ICARIIN**

The aerial part of *Epimedi herba* was extracted three times with ethanol and the extract dried with vacuum. The dried extract was suspended in water and partitioned successively with n-hexane, CHCl$_3$, and n-BuOH. The n-BuOH fraction was subjected to silica gel column chromatography to isolate icariin, which was further purified by repeated recrystallization with MeOH. The final product was 98.8% icariin as determined by high-performance liquid chromatography analysis.³⁵

**EXPRESSION AND PURIFICATION OF PDE5 ISOFORMS**

The PDE5 isoforms were expressed and purified, as previously described.² In brief, PDE5 isoform cDNA samples were cloned into baculoviral vector with a C-terminal histidine tag (Invitrogen, Carlsbad, Calif). The clones were transfected into Sf9 insect cells for expression of full-length PDE5 isoforms. The transfected cells were lysed and the lysate applied to the ProBond column, which binds to the histidine tag (Invitrogen, San Diego, Calif) for the calculation of IC$_{50}$ values.

**CELLULAR cGMP QUANTIFICATION**

Rat CSMCs were isolated from 3-month-old rats and cultured, as previously described.⁷ The cells were verified for smooth muscle identity by indirect immunofluorescence staining for alpha-smooth muscle actin. The fourth-passage cells were seeded into 96-well plates at 12,000 cells/well overnight, treated with icariin (100 and 200 μM) or zaprinast (200 μM) for 15 minutes, and then with 10 μM sodium nitroprusside (SNP, Sigma) for 30, 60, 120, 240, and 360 minutes. The cells were then analyzed for cGMP concentration with an enzyme immunoassay system (RPN 226, Amersham Pharmacia Biotech, Piscataway, NJ), as follows. After the cells were lysed with lysis reagent 1, 20 μL of the acetylation reagent was added to each cell lysate for 5 minutes. Then, 50 μL of the acetylated cell lysate was mixed with 100 μL of anti-cGMP antiserum in an enzyme immunoassay assay microplate for 2 hours at 4°C. After four washes with washing buffer, each well received 100 μL of the cGMP conjugate, and the microplate was further incubated for 1 hour at 4°C. After another round of washes, 200 μL of the enzyme substrate was added to each well, and the microplate was agitated on a shaker for 30 minutes at room temperature. The development of blue color was stopped by adding 100 μL of 1 M sulfuric acid to each well. The resulting yellow color was then read at 450 nm with a spectrophotometer.

**STATISTICAL ANALYSIS**

Prism 4 (GraphPad Software) was used for statistical analysis. Two-way analysis of variance was used to determine whether the differences between zaprinast and icariin on the cellular cGMP response were significant.

**RESULTS**

**INHIBITORY EFFECTS OF ICARIIN AND ZAPRINAST ON PDE5 ISOFORMS**

The isolation and purification of PDE5 isoforms and icariin have been previously reported.²³ The purified PDE5 isoforms were analyzed for their kinetic changes in the presence of varying amounts of icariin or zaprinast. The results are presented in two alternative configurations in Figure 1. Each plot in the first configuration (Fig. 1A–C) compares icariin and zaprinast against each PDE5 isoform. Each plot in the second configuration (Fig. 1D,E) compares the PDE5 isoforms in the presence of icariin or zaprinast. The calculated IC$_{50}$ values of icariin and zaprinast against PDE5 isoforms are shown in Table I.

**EFFECTS OF ICARIIN AND ZAPRINAST ON cGMP LEVEL IN CSMCs**

To simulate the clinical application of PDE5 inhibitors, icariin or zaprinast was added to CSMCs
for 15 minutes before the addition of the nitric oxide donor SNP. At 30, 60, 120, 240, and 360 minutes after SNP treatment, the cells were harvested for quantification of cGMP. In repeated experiments, SNP treatment alone consistently caused increases of cGMP at 30, 60, 240, and 360 minutes but few increases at 120 minutes (Fig. 2). This appears to be a novel observation and will be discussed in the Comment section. Independent of this biphasic effect of SNP, icariin was able to cause additional increases of cGMP greater than those caused by SNP, and this enhancing effect was more pronounced with the 200-μM than with the 100-μM dosage. In contrast, zaprinast at 200 μM enhanced the cGMP level only at 60 and 360 minutes.

TABLE I. Concentration that inhibits 50% values

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COMMENT

It is unlikely that icariin could compete with sildenafil, vardenafil, or tadalafil as a first-line treatment for ED. However, judging from the tens of thousands of websites posting information and/or selling icariin, it is clear that the demand for this herbal compound is substantial. As such, a better understanding of icariin’s biochemical property is warranted.

On the basis of the study by Xin et al.,3 which determined an IC50 value of 0.432 μM for icariin against PDE5, we chose zaprinast as a reference compound for comparison with icariin in this study. Zaprinast is the first known PDE5-specific inhibitor and both sildenafil and vardenafil were derived from it.1 In our previous study, we showed that zaprinast had an IC50 value of 3.2, 1.3, and 1.6 μM against PDE5A1, PDE5A2, and PDE5A3, respectively.6 These values were greater than the corresponding values (0.33, 0.23, and 0.31 μM) obtained in this study, and the discrepancies probably resulted from the impure cellular extracts used as the PDE5 enzyme preparations in the previous study. In a study by Wang et al.,8 that used purified human PDE5 (from platelets and corpus cavernosum), zaprinast was shown to have an IC50 of 0.33 μM, identical or nearly identical to the IC50 values of zaprinast against purified PDE5 isoforms of the present study. Thus, the purity of the enzyme preparation is important for IC50 value determination. On the basis of the updated IC50 values for zaprinast, it can be seen that icariin was generally three times less potent than zaprinast in suppressing the cGMP-hydrolytic activity of PDE5 isoforms (Table I).

The present study is, to our knowledge, the first to investigate the effect of icariin and zaprinast on cellular cGMP levels. To do so using a method that simulates clinical application of PDE5 inhibitors, CSMCs were treated with icariin or zaprinast for 15 minutes and then with SNP for 30 minutes to 6 hours. In repeated experiments, we observed that SNP treatment resulted in increases of cGMP at 30 and 60 minutes, a dip to nearly basal level at 120 minutes, a peak at 240 minutes, and another dip at 360 minutes. This biphasic modulation of cellular cGMP by SNP, to our knowledge, has not been reported. Previously, SNP has been shown to cause biphasic relaxation and contractions in esophageal longitudinal muscles.9 Although SNP at 1 mM has been shown to induce apoptosis in vascular smooth muscle cells (treated for 24 hours),10 at 10 μM (the same as in our study), it protected endothelial cells from apoptosis (treated for 18 hours).11 In addition, in our study, we observed no differences in cell number or morphology at any point. As such, the biphasic effect of SNP on cellular cGMP level did not result from cellular toxicity and might deserve additional investigation.

Independent of the unexpected observation of the biphasic effect of SNP, icariin consistently outperformed the control (SNP-only treatment) in maintaining greater cGMP levels, particularly at the greater concentration of 200 μM. In contrast, zaprinast at 200 mM did better than the control only at 60 and 360 minutes. This was surprising, because the first part of this study showed that zaprinast was three times as potent as icariin in suppressing PDE5 activities in vitro. The reason for this disparity is unknown but could perhaps be a result of differences between icariin and zaprinast in their influence on additional PDEs in CSMCs.12 Although the exact mechanism awaits additional clarification, it is noteworthy that an ancient herbal medicine could be corroborated for its therapeutic effectiveness at the cellular and molecular levels.

CONCLUSIONS

Icariin was inhibitory to PDE5 isoforms with similar IC50 values, which were approximately three times lower than those of zaprinast. However, icariin was more effective than zaprinast in maintaining greater cGMP levels in SNP-treated CSMCs.

REFERENCES