Inhibition of cGMP-Phosphodiesterase-5 By Biflavones of Ginkgo biloba

Mario Dell’Agli, Germana V. Galli, Enrica Bosiosis

Abstract

Ginkgo biloba dimeric flavonoids (GBDF) were shown to inhibit cAMP phosphodiesterase activity and to promote vasorelaxation. In particular, amentoflavone exhibited endothelium-dependent relaxation of rat aorta rings via enhanced generation and/or increased biological activity of nitric oxide, leading to elevated cGMP levels. The aim of this study was to investigate whether GBDF were able to inhibit cGMP-specific phosphodiesterase-5 (PDE5) as well. Human recombinant PDE5A1 was prepared by expression of the full-length cDNA of PDE5A1 in COS-7 cells. The PDE activity was determined in the presence of biflavones at 0.1 – 100 μM. All biflavones inhibited PDE5A1 in a concentration-dependent fashion, ginkgetin being the most potent (IC₅₀ = 0.59 μM). The ability to inhibit the enzyme followed this order: ginkgetin > bilobetin > sciadopitysin > amentoflavone > sequoiaflavone. These data suggest that GBDF could exert a vasodilating effect through a mechanism independent of NO release.

Ginkgo biloba L. is being used worldwide as a herbal medicine for the treatment of circulation disorders, cerebrovascular insufficiency [1], and peripheral vascular disease such as arterial occlusive diseases [2]. In addition, a relaxing effect on human and rabbit corpus cavernosum tissue has been reported [3].

Dimeric flavonoids isolated from Ginkgo biloba L. leaves (GBDF) (Fig. 1) possess a multitude of biological activities, including anti-inflammatory and vasokinetic properties [4]. GBDF-Phytosome® (GBDF 1:2 complex with phospholipids), when tested on the vasomotor activity and skin microcirculation of the cheeks, hands, limbs and female breast in human subjects, evoked a statistically significant increase in the sphygmic activity of the smallest arteries, associated with increased blood flow at capillary level [4]. Amentoflavone, one component of GBDF, induced a concentration-dependent relaxation of the phenylephrine-precontracted aorta via an endothelium-dependent nitric oxide-cGMP signalling pathway [5]. When nitric oxide is released by the endothelial cells, the direct activation of guanylyl cyclase leads to the accumulation of cGMP and the consequent activation of protein kinase G [6] thereby inducing a decrease of smooth muscle tone via an alteration in calcium signalling [7]. The duration and amplitude of the signal are controlled by the degradation of cGMP by cyclic nucleotide phosphodiesterase (PDE). cGMP-specific PDE5 in vascular smooth muscle cells regulates cGMP levels and consequently the extent and the duration of the vasorelaxation. Accordingly, the PDE5 isofrom represents the pharmacological target of the drugs for the treatment of erectile dysfunction, such as sildenafil (Viagra®) [8], [9].

GBDF inhibited beef heart PDEs [10] and cAMP-PDE in rat adipose tissue [11]. The latter effect correlated with the stimulation of lipolysis [12]. The present investigation was carried out to verify whether GBDF, in combination or as individual components, could also inhibit human recombinant cGMP-specific PDE5A1 leading to an increase of vascular cGMP levels through a process complementary to NO availability.

The basal level of PDE5A1 activity in control samples was 69.71 ± 8.89 pmol × min⁻¹ × mg prot⁻¹ (mean ± s.d., n = 14). All biflavones inhibited PDE5A1 in a concentration-dependent fashion. From the concentration-inhibition curves (Fig. 2), IC₅₀ values were calculated (Table 1). Among the tested biflavones, ginkgetin was found to be the most potent (IC₅₀ = 0.59 ± 0.07 μM, mean ± s.d.) followed by bilobetin > sciadopitysin > amentoflavone > sequoiaflavone. The mixture of biflavones, prepared by extraction from the leaves and containing all the compounds in the proportion as described in the Materials and Methods section, at 100 μM caused only 50% inhibition (Fig. 2). As compared to the individual compounds, the mixture (containing 35.7% biflavones) exerted a lower effect. An explanation for this result could be the co-presence of compounds counteracting the inhibition of biflavones. IC₅₀ values of the reference compounds sildenafil and zaprinast were 0.075 and 9.8 μM, respectively, close to the values reported in the literature [13]. Regarding amentoflavone, the concentration for inhibiting the enzyme (IC₅₀ = 11.7 μM) is close to that required for contrasting aorta contraction by phenylephrine (70% relaxant effect at 10 μM) [5]. Kang et al. [5] attributed the relaxant effects to the NO cascade pathway. Our results suggest that amentoflavone acts at the vascular level also at a later stage by inhibiting cGMP degradation and these two mecha-
nisms are contributing to the relaxant effect observed on precontracted aorta.

If we compare these results with those previously obtained on the cAMP-PDE in rat adipose tissue [11], it appears that cyclic nucleotide phosphodiesterases are differently affected by biflavones. While sciadopitysin inhibits cGMP-PDE5, it was inactive on cAMP-PDE.

The results reported herein demonstrate that Ginkgo biloba dimeric flavonoids inhibit the cGMP-specific PDE5A1, suggesting that the increase in blood flow observed in the skin of subjects treated with GBDF-Phytosome® could be associated to the vasodilation induced by biflavones through a mechanism involving cGMP-PDE5 in the smooth muscle cells of the arterial wall.

Materials and Methods

GBDF (35.7% purity), bilobetin (87.8%), sequoiaflavone (7-O-methylamontoflavone, 98.9% purity), sciadopitysin (98% purity), ginkgetin (94.5% purity) were a kind gift of Indena SpA (Milan, Italy). The purity of the biflavones was determined by HPLC.

Table 1 IC50 values (μM ± s. d.) for the inhibition of PDE5A1 activity by the Ginkgo biloba biflavones. IC50 values of sildenafil and zaprinast used as reference compounds were 0.075 ± 0.004 and 9.8 ± 1.32 μM ± s. d., respectively.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequoiaflavone</td>
<td>19.9 ± 0.86</td>
</tr>
<tr>
<td>Amentoflavone</td>
<td>11.7 ± 1.03</td>
</tr>
<tr>
<td>Sciadopitysin</td>
<td>3.24 ± 0.21</td>
</tr>
<tr>
<td>Bilobetin</td>
<td>1.52 ± 0.10</td>
</tr>
<tr>
<td>Ginkgetin</td>
<td>0.59 ± 0.07</td>
</tr>
</tbody>
</table>

The composition of the various biflavonoids in the Ginkgo biloba mixture was as follows: 35% isoginkgetin, 34% ginkgetin, 17% bilobetin, 8% sciadopitysin, 4% sequoiaflavone and 2% amentoflavone [14]. Amentoflavone (99% purity) was purchased from Extrasyntese (Lyon, France). cGMP, zaprinast and all the chemical reagents for cell culture were purchased from Sigma-Aldrich (Milan, Italy). The expression plasmid pcDNA3 containing the full-length cDNA of PDE5A1 was a kind gift of Prof. C. S. Lin (Department of Urology, University of California, San Francisco, USA). Human recombinant PDE5A1 was prepared by expression of the full-length cDNA of PDE5A1 into COS-7 cells as previously described [13, 15]. Cells were homogenised in a Dounce Homogeniser, and the insoluble materials were removed by centrifugation. The supernatant was diluted with an equal volume of glycerol, and the total protein concentration was determined according to the method of Bradford [16]. Cell lysates were stored at -80°C. PDE5A1 activity was determined according to Kincaid and Manganiello [17]. The cell lysate (70 μL at 0.2 mg of protein/ml) was incubated with 0.5 μM cGMP and 60,000 cpm of [3H]-cGMP in 40 mM Tris-HCl, pH 7.4, containing 10 mM MgCl2 and 2 mg/mL bovine albumin; the final reaction volume was 100 μL. [13, 15], PDE5A1 activity was expressed as pmol of product formed × min⁻¹ × mg prot⁻¹.

All biflavones were dissolved in DMSO and assayed in the range 0.1 – 100 μM. The final concentration of the vehicle in control and test samples was 1% of the incubation volume. The molar concentration was calculated taking into account the product purity. For GBDF mixture an average molecular weight of 550 was considered. Sildenafil (0.5 – 500 nM) and zaprinast were used as reference compounds. Concentration-inhibition curves were analysed using Graph Pad Prism 4 and each point represents the mean ± s. d. of 6 replications.

Acknowledgements

The authors gratefully acknowledge Prof. C.S. Lin, Department of Urology, University of California, San Francisco, CA, USA for the kind supply of PDE5A1 cDNA.

References