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Evaluation of *Ginkgo biloba* extract as an activator of human glucocorticoid receptor

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ABSTRACT

Ethnopharmacological relevance: Ginkgo biloba, which is one of the most frequently used herbal medicines, is commonly used in the management of several conditions, including memory impairment. Previously, it was reported to decrease the expression of peripheral benzodiazepine receptor and the biosynthesis of glucocorticoids, thereby regulating glucocorticoid levels. However, it is not known whether *Ginkgo biloba* extract regulates the function of the glucocorticoid receptor.

Aim of the study: We determined whether *Ginkgo biloba* extract and several of its chemical constituents affect the activity of human glucocorticoid receptor (hGR).

Materials and methods: A hGR-dependent reporter gene assay was conducted in HepG2 human hepatocellular carcinoma cells and hGR target gene expression assays were performed in primary cultures of human hepatocytes.

Results: Multiple lots and concentrations of the extract and several of its chemical constituents (ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, and bilobalide) did not increase hGR activity, as assessed by a cell-based luciferase reporter gene assay. The extract did not influence the expression of hGR target genes, including tyrosine aminotransferase (hTAT), constitutive androstane receptor (hCAR), or pregnane X receptor (hPXR), in primary cultures of human hepatocytes. Moreover, hGR antagonism by mifepristone (also known as RU486) did not attenuate the extent of induction of hCAR- and hPXR-regulated target genes CYP2B6 and CYP3A4 by *Ginkgo biloba* extract.

Conclusion: Ginkgo biloba extract, ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, and bilobalide are not activators of hGR. Furthermore, the extract does not influence the hGR-hCAR or the hGR-hPXR signaling pathway in primary cultures of human hepatocytes.

1. Introduction

Glucocorticoid receptor (GR; NR3C1) is a ligand-dependent transcription factor (Hollenberg et al., 1985) belonging to the superfamily of nuclear receptors (Germain et al., 2006). It mediates the action of glucocorticoids and has a broad array of biological roles, including anti-inflammatory function and stress-related homeostasis (Nicolaides et al., 2010). Its mechanism of action involves transcriptional activation or repression of target genes (Veleiro et al., 2010). Activated GR also up-regulates the expression of other transcription factors, such as constitutive androstane receptor (CAR) and pregnane X receptor (PXR), which in turn may increase transcription of their respective target genes, such as CYP2B6 and CYP3A4 (Dvorak and Pavek, 2010). This has been referred to as the GR-CAR-cytochrome P450 enzymes and GR-PXR-cytochrome P450 enzymes cascades (Pascussi et al., 2008).

Ginkgo biloba, which belongs to the family Ginkgoaceae (Nakanishi, 2005), is one of the most frequently used herbal medicines (Barnes et al., 2008). It is commonly used in the management of several conditions, including memory impairment (Fransen et al., 2010). Extracts of *Ginkgo biloba* contain approximately 6% (w/w) terpene trilactones (e.g. ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, and bilobalide) and

Abbreviations: CAR, constitutive androstane receptor; CYP2B6, cytochrome P450 2B6; CYP3A4, cytochrome P450 3A4; DMSO, dimethyl sulfoxide; GR, glucocorticoid receptor; HBSS, Hanks' balanced salt solution; hCAR, human constitutive androstane receptor; hGR, human glucocorticoid receptor; hHPRT, human hypoxanthine phosphoribosyltransferase 1; hPXR, human pregnane X receptor; hTAT, human tyrosine aminotransferase; PCR, polymerase chain reaction; PXR, pregnane X receptor; RU486, mifepristone

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24% (w/w) flavonol glycosides (e.g. those of quercetin, kaempferol, and isorhamnetin) (van Beek and Montoro, 2009). As shown in various experimental models, some of the actions of *Ginkgo biloba* extract include inhibiting beta-amyloid aggregation (Luo et al., 2002), attenuating beta-amyloid-induced neurotoxicity (Shi et al., 2009), scavenging free radicals (Lee et al., 1998), and promoting cerebral (Mashayekh et al., 2011) and coronary (Wu et al., 2008) blood flow. *Ginkgo biloba* extract and ginkgolide B have also been reported to decrease the expression of peripheral benzodiazepine receptor and the biosynthesis of glucocorticoids, thereby regulating glucocorticoid levels and providing mechanistic insights into the anti-stress effects of the extract (Amri et al., 1996, 2003). To date, it is not known whether *Ginkgo biloba* extract regulates the function of the glucocorticoid receptor.

In the current study, we determined whether *Ginkgo biloba* extract and several of its chemical constituents (ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, and bilobalide) activate the human GR (hGR) and investigated their effects on a prototypical hGR target gene (tyrosine aminotransferase; hTAT). Given that hGR has been proposed to regulate the expression of hCAR, hPXR, and their respective target genes (CYP2B6 and CYP3A4) (Pascussi et al., 2008), we also determined the effect of *Ginkgo biloba* extract on the hGR-hCAR-CYP2B6 and the hGR-hPXR-CYP3A4 cascades. The experimental approaches involved a hGR-dependent reporter gene assay conducted in a human hepatoma cell line and hGR target gene expression assays performed in primary cultures of human hepatocytes. The findings are discussed in the context of *Ginkgo biloba* extract and the hGR signaling pathway.

2. Materials and methods

2.1. Ginkgo biloba extract, chemicals, and reagents

Individual lots of *Ginkgo biloba* extract, designated as lot A, lot B, lot C, lot D, and lot E, were supplied in a dry powder form by Indena S.p.A. (Milan, Italy). The contents of terpene trilactones and flavonol glycosides in each lot are shown in Table 1. Ginkgolide A, ginkgolide B, ginkgolide C, and (-)-bilobalide were obtained from LKT Laboratories (St. Paul, MN), and ginkgolide J was from ChromaDex (Irvine, CA). Dexamethasone, mifepristone (also known as RU486), bupropion hydrochloride, testosterone, rifampicin, and triprolidine hydrochloride were purchased from Sigma–Aldrich (St Louis, MO), hydroxybupropion from Toronto Research Chemicals, Inc. (North York, ON, Canada), and 6β-hydroxytestosterone from Steraloids, Inc. (Newport, RI). The suppliers of reagents for human hepatocytes isolation and culture (LeCluyse et al., 2005), for reverse transcription and real-time polymerase chain reaction (PCR) analyses (Chang et al., 2006), and for cell culture and reporter gene assays (Lau et al., 2010) were specified previously.

2.2. Plasmids

pCMV6-XL5-hGR and pCMV6-XL5 were purchased from OriGene Technologies, Inc. (Rockville, MD). *Renilla reniformis* luciferase pGL4.74 [*hRluc*/TK] was obtained from Promega. PathDetect GRE *cis*-Reporting System (pGRE-luc reporter plasmid) was purchased from Agilent Technologies (Santa Clara, CA).

2.3. Cell line, transient transfection, and reporter gene assay

hGR-dependent reporter gene assay was conducted using HepG2 cells (American Type Culture Collection, Manassas, VA) cultured in minimum essential medium supplemented with 10% (v/v) charcoal-stripped heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin. Cells were seeded onto 24-well microplates at a density of 100,000 cells per well and in a volume of 0.5 ml culture medium. At 24 h after plating, cells were transfected with 20 μl of a transfection master mix containing FuGENE 6 transfection reagent (3 μl/μg of DNA), serum-free Opti-MEM (20 μl/well), pGL4.74[*hRluc*/TK] internal control plasmid (1 ng/well), pGRE-luc reporter plasmid (100 ng/well), and either pCMV6-XL5-hGR receptor expression plasmid (100 ng/well) or pCMV6-XL5 (empty vector; 100 ng/well) for 24 h. Transfected cells were treated with 0.5 ml of fresh supplemented culture medium containing *Ginkgo biloba* extract, ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, bilobalide, dexamethasone (hGR agonist) (Pascussi et al., 2001), RU486 (hGR antagonist) (Pascussi et al., 2001), both RU486 and dexamethasone, rifampicin (negative control) (Herr et al., 2000), or vehicle (culture medium for *Ginkgo biloba* extract or 0.1% (v/v) DMSO for chemicals). At the end of the 24 h treatment period, cells were lysed. Firefly luciferase and *Renilla reniformis* luciferase activities were determined using a Dual-Luciferase Reporter Assay System. Luminescence was measured using a GloMax 96 microplate luminometer (Promega Corporation). Luciferase activity was expressed as a normalized ratio of firefly luciferase to *Renilla reniformis* luciferase activity. Fold increase was calculated by dividing the normalized luciferase activity of the treatment group by that of the vehicle-treated control group.

Table 1
Quantity of ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, bilobalide, quercetin, kaempferol, and isorhamnetin in five individual lots of *Ginkgo biloba* extract.

Chemical	Quantity in *Ginkgo biloba* Extract %w/w				
	Lot A	Lot B	Lot C	Lot D	Lot E
Terpene trilactone					
Ginkgolide A	1.1	0.9	1.3	1.5	1.3
Ginkgolide B	0.3	0.3	0.6	0.6	0.6
Ginkgolide C	1.4	1.5	1.4	1.4	1.4
Ginkgolide J	0.6	0.6	0.5	0.6	0.5
Bilobalide	2.8	2.9	3.0	3.0	3.0
Total terpene trilactones	6.2	6.2	6.8	7.1	6.8
Flavonol glycosides (sum of aglycone and glycosides)					
Quercetin glycosides	10.6	10.9	N/A	N/A	N/A
Kaempferol glycosides	6.3	11.2	N/A	N/A	N/A
Isorhamnetin glycosides	4.1	2.3	N/A	N/A	N/A
Total flavonol glycosides	21.0	24.4	24.4	24.3	24.4

The levels of terpene trilactones and flavonol glycosides were quantified by gas chromatography (Indena S.A., Milan, Italy) and liquid chromatography-mass spectrometry (ChromaDex, Inc., Santa Ana, CA), respectively.
N/A, not available.