



Inhibition of plasminogen activator inhibitor-1 release from human endothelial cells by *Angelica keiskei* Koidzumi (Ashitaba) chalcones is structure-dependent

Dear Editor,

The endothelium is a monolayer of cells that lines the inner surface of vessels and plays a crucial role in maintaining hemostatic balance [1]. All blood vessels, from the largest arteries and veins to the smallest venules are lined with endothelial cells, which prevent thrombosis via anticoagulant and antiplatelet mechanisms. Endothelial cells are also involved in hemostatic pathways triggered by vascular injury and limited clot formation in areas where hemostasis is needed. Various conditions such as diabetes, metabolic syndrome, hypertension, and smoking can cause endothelial cell dysfunction [2] that can lead not only to atherosclerosis but also a predisposition toward thrombosis and stroke [1].

Endothelial cells are considered to contribute under various conditions to elevated levels of plasma plasminogen activator inhibitor-1 (PAI-1), which is the major physiological inhibitor of tissue type plasminogen activator (tPA) and urokinase PA (uPA) *in vivo* and it plays a crucial role in the regulation of fibrinolysis [3]. High plasma PAI-1 levels disrupt the fibrinolytic system, resulting in a prothrombotic state that is associated with the development of thrombotic disorders such as stroke, cardiovascular diseases, and deep vein thrombosis [4,5]. Maintaining physiological plasma levels of PAI-1 might represent a promising intervention for these diseases. However, drugs that can suppress PAI-1 release remain unknown and insight into the regulation of PAI-1 release is also inadequate. *Angelica keiskei* Koidzumi (Ashitaba) is a perennial plant that grows mainly along the pacific coast of Japan. Chalcones isolated from Ashitaba have various biological properties [6,7]. The major constituents of the chalcone fraction are xanthoangelol A (XA) (~50%) and 4-hydroxyderricin (4-HD) (~50%). Trace amounts of other chalcones such as xanthoangelols B (XB), D (XD), E (XE), and F (XF) have the basic structure of XA or 4-HD with a slightly modified side chain [Figure 1]. A previous study of mice has shown that Ashitaba exudate inhibits lipopolysaccharide (LPS) induced increases in plasma PAI-1 and that XA inhibits this increase in plasma whereas 4-HD does not [8]. The inhibition activity seemed to be due to the inhibition of nuclear factor kappa B activation. However, the molecular structure of chalcones that are essential for the

suppression of PAI-1 production remained undetermined. Here, the effects of 4-HD XA, XB, XD, XE, and XF on tumor necrosis factors α (TNF α) stimulated PAI-1 production in endothelial cell-like EA.hy926 cells were analyzed and the structure that is critical for such activity is discussed.

EA.hy926 cells (ATCC, Manassas, VA, USA) were seeded in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis MO, USA) at a density of 2.0×10^4 cells per gelatin-coated well in 96-well plates at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Chalcones in dimethyl sulphoxide (DMSO) were added to DMEM containing 1% fetal bovine serum and the cells were incubated for 3 h followed by a 24-h incubation with TNF α (10 ng/mL). Thereafter, PAI-1 concentrations were measured using total PAI-1 ELISA kits (Molecular Innovations, Southfield, MI, USA). Cytotoxic effects of test compounds on EA.hy926 cells were detected using cell counting kit-8 (Dojindo, Kumamoto, Japan). All values are expressed as means \pm standard error of the mean. Differences between the two groups were analyzed using Mann-Whitney U tests and $P < 0.05$ was considered to represent significance.

Xanthoangelol (5 and 10 μ m) dose-dependently suppressed the TNF α -induced PAI-1 increase in the culture medium. The inhibition rates of PAI-1 were $38.7\% \pm 9.7\%$ ($n = 3$) and $75.4\% \pm 1.5\%$ ($n = 3$), respectively. Xanthoangelol was significantly cytotoxic at 25 μ m, but not at either 5 or 10 μ m. Cell viability in the presence of 5 μ m and 10 μ m XA was respectively $120.0\% \pm 11.8\%$ and $120.7\% \pm 13.5\%$ compared with the control value ($n = 3$), whereas that in the presence of 25 μ m XA was only $0.9\% \pm 0.1\%$ of the control value ($n = 3$). We then assessed the effects of 10 μ m XA, XB, XD, XE, XF, and 4-HD on the PAI-1 concentration in the medium [Figure 2]. The inhibition rate of XA was about 77.1%, whereas XB that has a long side chain with a hydroxyl group bound to the A ring was not inhibitory. None of XD, XE, and 4-HD that has a short side chain in the A ring exerted an inhibitory effect. None of XB, XD, XE, and 4-HD were noticeably cytotoxic at a concentration of 10 μ m, whereas XF was extremely cytotoxic, which prevented evaluation of its inhibition rate. Isoliquiritigenin, a chalcone obtained from liquorice root, does not have a long side chain bound to the A ring. This chalcone did not inhibit PAI-1 activity

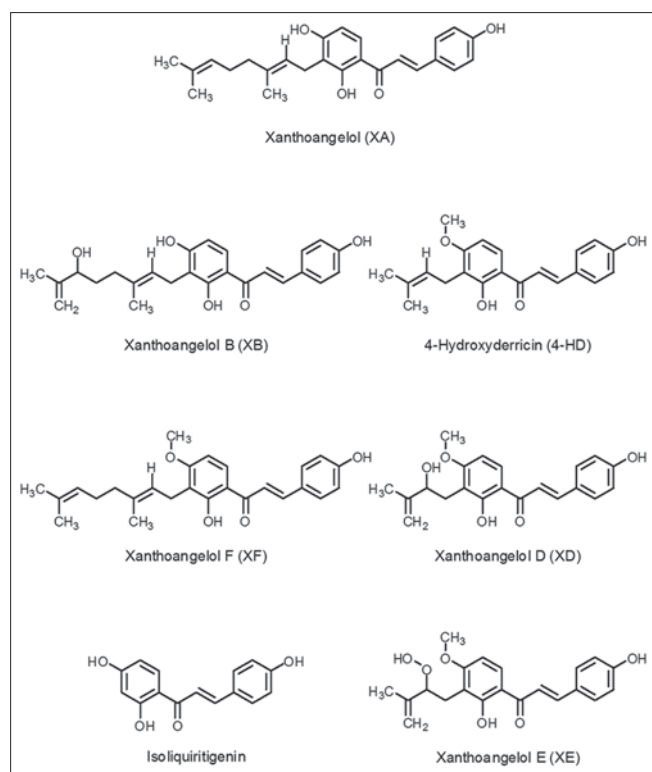


Figure 1: Chemical structure of Ashitaba chalcones and isoliquiritigenin

and was not cytotoxic. These findings suggested that having a long hydrocarbon chain with moderate hydrophobicity on the A ring is critical for expressing chalcone activity. Orally- or intraperitoneally administered Ashitaba exudate suppresses LPS induces PAI-1 increases in mouse plasma [8]. Therefore, the present results suggested that the inhibition of PAI-1 production in mouse plasma by Ashitaba exudate administered orally or intraperitoneally was mainly due to the effect of XA. The present study also found that the main Ashitaba chalcone XA inhibited TNF α -induced PAI-1 mRNA increases in EA.hy926 cells (data not shown). This indicated that the inhibitory mechanism of the blunted increase of PAI-1 antigen in the medium of EA.hy926 cells proceeds via the inhibition of PAI-1 mRNA expression.

The present results indicated that XA, the main constituent of chalcone, inhibits PAI-1 release from endothelial cells induced by inflammation. High plasma PAI-1 levels disrupt the fibrinolytic system, which results in a prothrombotic state that is associated with the development of thrombotic disorders. Therefore, we considered that maintaining the physiological levels of PAI-1 by XA prevents thrombus formation and avoids thrombotic disorders. The clinical co-administration of XA with tPA might reduce required doses of tPA and prevent adverse reactions.

However, we investigated the effects of chalcones only on endothelial cells *in vitro*. Many types of cells interact and regulate endothelial cell functions. The effect of co-culture endothelial cells with other cells is very important

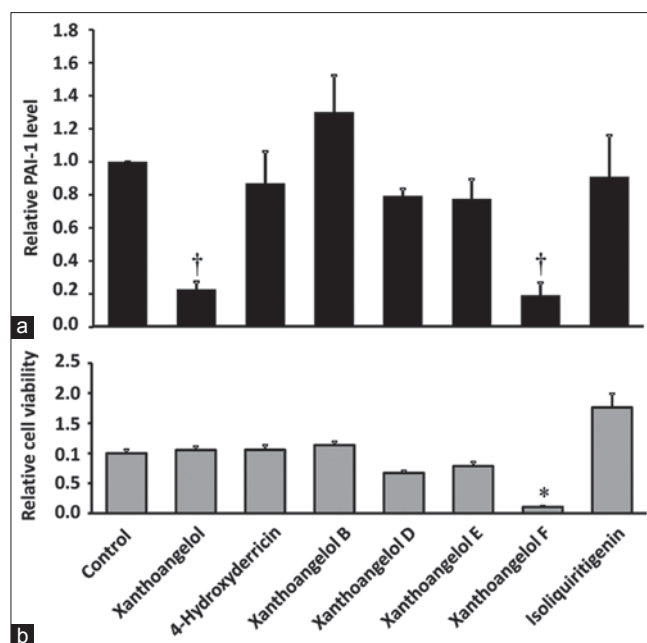


Figure 2: Effect of Ashitaba chalcones on tumor necrosis factors α (TNF α)-induced plasminogen activator inhibitor-1 (PAI-1) increases in medium of EA.hy926 cells. Cells were incubated with 10 μ M chalcones in DMSO for three hours followed by TNF α (10 ng/mL) for 24 h. Thereafter, PAI-1 concentrations were measured using total human PAI-1 ELISA. Longitudinal axis shows relative PAI-1 levels in the medium. Concentration of PAI-1 in medium without TNF α stimulation is expressed as one unit (a). Chalcones cytotoxicity was assessed by cell counting (b). The absorbance of control cells is expressed as one unit. Data are expressed as means \pm SEM ($n = 7$ for xanthoangelol A and 4-hydroxyderricin, $n = 3$ for xanthoangelol F, $n = 4$ for others). * $P < 0.05$; † $P < 0.01$ versus control

for objectively assessing the actions of agents. Indeed, co-culture of endothelial cells with other cells modulates PAI-1 production from endothelial cells [9,10]. Astrocytes that comprise one type of glia cells co-cultured with human brain capillary endothelial cells enhance PAI-1 expression in a blood-brain barrier model [11,12]. Even more complex effects from other cells should modulate endothelial cell function *in vivo* and change PAI-1 production from endothelial cells. Therefore, careful consideration is needed to assess the effect of chalcones on PAI-1 production in endothelial cells *in vitro*.

CONCLUSIONS

The main xanthoangelol subtype in Ashitaba was the most potent inhibitor of a TNF α stimulated PAI-1 increase in the culture medium of human EA.hy926 endothelial cells. The side hydrocarbon chain played an important role in this process and small modifications to the hydrocarbon chain or the addition of a small functional group to the A ring of XA influenced the inhibitory activity. The present findings indicated that Ashitaba could serve as an antithrombotic agent and that more novel antithrombotic agents could be developed to suppress PAI-1. Further investigation into the antithrombotic action of Ashitaba is warranted.

ACKNOWLEDGMENTS

This study was supported in part by a Grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan 25350144 (to N Ohkura) and by Japan Bio Science Laboratory Co. Ltd.

**Naoki Ohkura¹, Hiroaki Oiwa¹,
Katsunori Ohnishi², Masahiko Taniguchi³,
Kimiye Baba³, Gen-ichi Atsumi¹**

¹Department of Molecular Physiology and Pathology, School of Pharma-Sciences, Teikyo University, Itabashi, Tokyo, Japan,

²Japan Bio Science Laboratory Co. Ltd., Fukushima, Osaka,

³Division of Pharmacognosy, Osaka University of Pharmaceutical Sciences, Takatsuki, Osaka, Japan

Address for correspondence:

Naoki Ohkura, Department of Molecular Physiology and Pathology,
School of Pharma-Sciences, Teikyo University, 2-11-1 Kaga,
Itabashi, Tokyo 173-8605, Japan.

E-mail: n-ohkura@pharm.teikyo-u.ac.jp

Received: July 23, 2015

Accepted: August 25, 2015

Published: November 04, 2015

REFERENCES

1. Rajendran P, Rengarajan T, Thangavel J, Nishigaki Y, Sakthisekaran D, Sethi G, *et al.* The vascular endothelium and human diseases. *Int J Biol Sci* 2013;9:1057-69.
2. Endemann DH, Schiffrin EL. Endothelial dysfunction. *J Am Soc Nephrol* 2004;15:1983-92.

3. Gils A, Declerck PJ. Plasminogen activator inhibitor-1. *Curr Med Chem* 2004;11:2323-34.
4. Declerck PJ, Gils A. Three decades of research on plasminogen activator inhibitor-1: A multifaceted serpin. *Semin Thromb Hemost* 2013;39:356-64.
5. Van De Craen B, Declerck PJ, Gils A. The biochemistry, physiology and pathological roles of PAI-1 and the requirements for PAI-1 inhibition *in vivo*. *Thromb Res* 2012;130:576-85.
6. Yasueda A, Ito T, Maeda K. Review: Evidence-based clinical research of anti-obesity supplements in Japan. *Immunol Endocr Metab Agents Med Chem* 2013;13:185-195.
7. Baba K, Nakata K, Taniguchi M, Kido T, Kozawa K. Chalcones from *Angelica keiskei*. *Phytochemistry* 1990;29:3907-10.
8. Ohkura N, Nakakuki Y, Taniguchi M, Kanai S, Nakayama A, Ohnishi K, *et al.* Xanthoangelols isolated from *Angelica keiskei* inhibit inflammatory-induced plasminogen activator inhibitor 1 (PAI-1) production. *Biofactors* 2011;37:455-61.
9. Zhang JC, Fabry A, Paucz L, Wojta J, Binder BR. Human fibroblasts downregulate plasminogen activator inhibitor type-1 in cultured human macrovascular and microvascular endothelial cells. *Blood* 1996;88:3880-6.
10. Funayama H, Sakata Y, Kitagawa S, Ikeda U, Takahashi M, Masuyama J, *et al.* Monocytes modulate the fibrinolytic balance of endothelial cells. *Thromb Res* 1997;85:377-85.
11. Kim JA, Tran ND, Wang SJ, Fisher MJ. Astrocyte regulation of human brain capillary endothelial fibrinolysis. *Thromb Res* 2003;112:159-65.
12. Tran ND, Schreiber SS, Fisher M. Astrocyte regulation of endothelial tissue plasminogen activator in a blood-brain barrier model. *J Cereb Blood Flow Metab* 1998;18:1316-24.

© SAGEYA. This is an open access article licensed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>) which permits unrestricted, noncommercial use, distribution and reproduction in any medium, provided the work is properly cited.

Source of Support: Nil, Conflict of Interest: None declared.