Direct inhibition of elastase and matrixmetalloproteinases and stimulation of biosynthesis of fibrillar collagens, elastin, and fibrillins by xanthohumol

NEENA PHILIPS, MATHEW SAMUEL, ROSEMARIE ARENA, YU-JUN CHEN, JENNIFER CONTE, PRASHANTI NATRAJAN, GERHARD HAAS, and SALVADOR GONZALEZ, School of Natural Sciences, Fairleigh Dickinson University, Teaneck, NJ (N.P., M.S., R.A., Y.-J.C., J.C., P.N., G.H.), Industrial Cantabria Farmaceutica, S.A, Madrid, Spain (S.G.), and Dermatology Service, Memorial Sloan-Kettering Cancer Center, New York (S.G.).

Accepted for publication August 24, 2009.

Synopsis

In skin aging there is deterioration of the extracellular matrix's collagen and elastin fibers, from its reduced biosynthesis and increased degradation by elastase and matrixmetalloproteinases (MMPs). Xanthohumol is a flavonoid isolated from the hop plant *Humulus lupulus* L., with anti-microbial, antioxidant, anti-inflammatory, and anti-carcinogenic properties. The goal of this research was to investigate xanthohumol as an anti-skin-aging agent via its beneficial regulation of the extracellular matrix. To this purpose, we examined the direct effect of xanthohumol on the activities of elastase and MMPs (MMPs 1, 2, and 9) and its effect on the expression (protein and/or transcription levels) of collagens (types I, III, and V), elastin, and fibrillins (1 and 2) in dermal fibroblasts. Xanthohumol significantly inhibited elastase and MMP-9 activities from its lowest concentration, and MMP-1 and MMP-2 at its higher concentrations, which implies a greater protective effect on elastin. It dramatically increased the expression of types I, III, and V collagens, and elastin, fibrillin-1, and fibrillin-2 in dermal fibroblasts. The effects were similar to those of ascorbic acid. This is the first report identifying xanthohumol's potential to improve skin structure and firmness: it simultaneously inhibits the activities of elastase/MMPs and stimulates the biosynthesis of fibrillar collagens, elastin, and fibrillins.

INTRODUCTION

Alterations in collagen and elastin, which form the extracellular matrix (ECM), are responsible for the clinical manifestations of skin aging, which are wrinkles, sagging, and laxity (1–6). The fibrillar collagens, types I, III, and V, in the order of predominance, provide structure whereas elastin forms elastic fibers with fibrillin to gives skin firmness and elasticity. The atrophy of collagen and elastin fibers in skin aging is from reduced

Address all correspondence to Neena Philips.

synthesis, and the increased expression of their degradative enzymes, especially collagenases (MMP-1), gelatinases (MMP-2, MMP-9), and elastases (1–14).

The cosmetic industry is active in identifying natural products to counteract skin aging. Many of these products or actives have antioxidant and/or anti-inflammatory properties, and are rich in polyphenols or flavonoids (15–26). A flavonoid that has been extensively investigated for its antimicrobial and anticarcinogenic properties, but not for its anti-skin-aging potential, is xanthohumol from the hop plant *Humulus lupulus* L. (Cannabinaceae) (hops) (27–31). It has potent anti-inflammatory and antioxidant properties, including inhibition of the NF-kB transcription factor and nitric oxide production by inflammatory cytokines (27–31).

Xanthohumol's potential for anti-skin-aging has not been reported. We evaluated the efficacy of xanthohumol in (a) inhibiting elasatase, MMP-1, MMP-2, and MMP-9 activities; (b) stimulating expression of types I, III, and V collagens, elastin, fibrillin-1, and fibrillin-2.

MATERIALS AND METHODS

MATERIALS

The materials used were the following: enzymes [elastase (Elastin Products Co. SE563), MMP-1 (Biomol Se-180), MMP-9 (Biomol Se-244), MMP-2 (Biomol Se-109)]; substrates [elastase (Bachem I-1270), MMP-1 (Bachem, M-2055), MMP-2,9 (Bachem M-1855)]; dermal fibroblasts (Cascade Biologics); escort (Sigma); protein detector kit (KPL Co); antibodies/standards [collagen (Chemicon), elastin (Elastin Products Co)]; plasmids (gifts from Dr. Joel Rosenbloom, School of Dental Medicine, University of Pennsylvania, PA); dual luciferase reporter assay (Promega); xanthohumol (gift from Dr. Gearhaard Haas, Fairleigh Dickinson University, NJ); and positive controls [ascorbic acid (AA, Sigma), EDTA (Sigma), PMSF (Sigma), protease inhibitor (Roche)]

ELASTASE OR MMP ACTIVITY CALIBRATION AND INHIBITION

The enzymes (elastase, MMP-1, MMP-9, and MMP-2) were calibrated by reacting twofold serial dilutions of each of the enzymes (starting concentration of 1 μ g/ μ l) with respective substrates (0.5 mM) in incubation buffer (elastase: 0.09 M Tris-0.5 M NaCl buffer; MMPs: 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM CaCl₂). The reaction kinetics was measured fluorometrically (355 excitation/450 emission) every five minutes for a total length of 1.5 hours. The optimal enzyme concentrations (linear dose response) were determined to be 0.1 μ g/ml for elastase, 0.77 μ g/ μ l for MMP-1, 0.5 μ g/ μ l for MMP-2, and 0.1 μ g/ μ l for MMP-9.

Xanthohumol (0.001%, 0.01%, 0.1%, and 1% of a stock of 50 mg/ml) (Xan) or positive controls (10 mM PMSF, 5m MEDTA, 0.5 mM ascorbic acid or 1XProtease inhibitor) were incubated with the optimal concentration of each of the enzymes in incubation buffer for ten minutes followed by the addition of the respective substrate. The activities of elastase and each of the MMPs were measured fluorometrically at 355 excitation/450 emission.

EXPRESSION OF FIBRILLAR COLLAGENS, ELASTIN, AND FIBRILLINS

Dermal fibroblasts were cultured in complete Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin/ streptomycin, and 1% L-glutamine (Sigma). For the experiments, cells were trypsinized, seeded in 33-mm dishes, transfected with COL1A1 promoter (or elastin promoter), firefly luciferase (pGL4 vector), and TK-hRenilla luciferase plasmids with escort, and dosed with or without (control) xanthohumol (0.01%, 0.1%, 1%) or 2.5 mM ascorbic acid for 24 hours in basal media containing 1% serum replacement (Sigma) (19–21).

The cells were examined for viability by MTS assay (Promega). The media were examined for collagen (types I, III, and V) and elastin fiber components (tropoelastin, fibrillin-1, and fibrillin-2) with respective antibodies by indirect ELISA (19–21). For ELISA, aliquots of media (from experiments) or respective standards were added to 96-well plates and incubated overnight at 4°C. The wells were blocked with bovine serum albumin and then incubated with the respective antibodies for one hour at room temperature. The plates were washed thoroughly with wash buffer, incubated with secondary antibody linked to peroxidase for one hour at room temperature, washed with wash buffer thoroughly, and subsequently incubated with peroxidase substrate until the development of color, which was measured spectrophotometrically at 405 nm. The data were normalized for cell viability.

The cells were lysed and measured for luminescence from firefly and renilla luciferase activities with specific substrates (Promega). The luminescence from firefly luciferase was normalized with that from renilla luciferase.

DATA ANALYSIS

The effects of xanthohumol or positive controls are represented as a percent of control (no additives) represented at 100%. The data were statistically analyzed for significant differences by ANOVA and Student *t*-tests at the 95% confidence interval. Significant effects of xanthohumol, relative to control, are represented by * in the figures.

RESULTS AND DISCUSSION

INHIBITION OF ELASTASE AND MMP-1, 2, AND 9 ACTIVITIES BY XANTHOHUMOL

The ECM damaging enzymes are produced by epidermal keratinocytes, dermal fibroblasts, and neutrophils, which are the predominant source for photoaged skin (9–14). While collagen is degraded by MMP-1 and MMP-2, the elastic fibers are degraded by elastase, MMP-9, and MMP-2 (7–9). Xanthohumol inhibited the activities of elastase and MMP-9 at all concentrations (0.001-1%), and MMP-1 and MMP-2 at the higher concentrations (0.1% and 1%), suggesting a greater benefit to the elastin fibers (Figure 1a–d). There was complete inhibition of each of the enzyme activities by 1% xanthohumol. The concentrations of xanthohumol that inhibited elastase, MMP-9, MMP-1, and MMP-2 activities by 50% were extrapolated as 0.001%, 0.05%, 0.25%, and 0.5%, respectively.



Figure 1. Inhibition of elastase (a), MMP-1 (b), MMP-2 (c), and MMP-9 (d) activities by 0.001-1% xanthohumol (xan within arrows, a-d), PMSF (a), EDTA (b-d), ascorbic acid (AA, a-d), or protease inhibitor (PI, a, b, d). The effect of additives is represented as % of control (0, no additives), represented at 100%; *=p<0.05, relative to control. Error bars represent standard deviation (n=4).

Relative to the control (0 at 100%), xanthohumol at 0.001%, 0.01%, 0.1%, and 1%, respectively, significantly inhibited elastase activity to 50%, 48%, 23%, and 0.2% of control and inhibited MMP-9 activity to 76%, 68%, 26%, and 3% of control (p<0.05) (Figure 1a,d). Xanthohumol at 0.1%, and 1% significantly inhibited MMP-1 activity to 84% and 4% of control, and MMP-2 activity to 70% and -17% of control, respectively (p<0.05) (Figure 1b,c). The effects of xanthohumol at the higher concentrations were similar to those of the positive controls ascorbic acid, PMSF or EDTA, and protease

129



Figure 2. Stimulation of expression of types I, III, and V collagens (a) and elastin, fibrillin-1, and fibrillin-2 (b) by 0.01%, 0.1%, and 1% xanthohumol or ascorbic acid (AA). Effect of xanthohumol or ascorbic acid is represented as % of control (0, no additives), represented at 100%; *=p<0.05, relative to control. Error bars represent standard deviation (n=4).

inhibitor, which significantly inhibited the activities of elastase and each of the MMPs (Figure 1a-d).

STIMULATION OF EXPRESSION OF TYPES I, III, AND V COLLAGENS, ELASTIN, FIBRILLIN-1, AND FIBRILLIN-2

The identification of natural products that inhibit MMPs and elastase, and simultaneously stimulate collagen and elastin fiber formation, is ideal to counteract skin aging, which is

associated with drastic deterioration of the structural ECM (1,2,12,13,15,18). The dermal fibroblasts are the predominant cells responsible for the alterations in the ECM. Hence the effects of xanthohumol on the expression of ECM proteins were examined in these cells.

Xanthohumol drastically increased expression of fibrillar collagen and elastin fiber components, especially at 0.1%, where the effect was similar to that of ascorbic acid for collagens and about fourfold greater than the effect of ascorbic acid on elastin and fibrillin expression (Figure 2).

Xanthohumol significantly increased type I collagen expression transcriptionally in fibroblasts at 1% (protein: 224% of control; promoter: 449% of control) and 0.1% (protein: 578% of control; promoter: 1347% of control) (p<0.05) (Figure 2a). Relative to the control (0 at 100%), xanthohumol at 1% and 0.1%, respectively, significantly stimulated the protein levels of type III collagen to 745% and 1390% of the control and type V collagen to 304% and 893% of the control (p<0.05) (Figure 2a).

Elastin expression was significantly stimulated at the transcriptional level by xanthohumol at 1% (protein to 218% and promoter to 367%) and 0.1% (protein to 692% and promoter to 1288% relative to control) (Figure 2b). Xanthohumol at 0.1% and 1% significantly increased the expression of fibrillin-1 to 1823% and 603%, respectively, relative to the control, and fibrillin-2 expression to 1713% and 373% of the control, respectively (p<0.05) (Figure 2b).

CONCLUSION

Xanthohumol has antioxidant and anti-inflammatory properties, and thereby its potential for anti-skin-aging was investigated in this research. The targets examined were the extracellular matrix remodeling and structural proteins: elastase, MMP-1, MMP-2, MMP-9, types I, III, and V collagens, elastin, fibrillin-1, and fibrillin-2. Xanthohumol demonstrated dual protective effects on the extracellular matrix via the inhibition of the ECM proteolytic enzymes and the stimulation of the structural ECM components, collagens, elastin, and fibrillins. We identify xanthohumol as an anti-skin-aging agent via its beneficial regulation of the ECM. The intake or topical application of xanthohumol may be beneficial to skin health and preferred over hormones with similar effects (19).

ACKNOWLEDGMENTS

The authors thank Tiffany Chang, Joshua Givant, Sujini Yarlagadda, Marvin Tuason, and May Taw for their assistance.

REFERENCES

- M. H. Shin, G. E. Rhie, C. H. Park, K. H. Kim, K. H. Cho, H. C. Eun, and J. H. Chung, Modulation of collagen metabolism by the topical application of dehydroepiandrosterone to human skin, *J. Invest. Dermatol.*, 124, 315–323 (2005).
- (2) J. Uitto, M. J. Fazio, and D. Olsen, Molecular mechanisms of cutaneous aging, J. Am. Acad. Dermatol., 21, 614–622 (1989).

- (3) J. Varani, R. L. Warner, and M. Gharaee-Kermani, Vitamin A antagonizes decreased cell growth and elevated collagen-degrading matrix metalloproteinases and stimulates collagen accumulation in naturally aged human skin, *J. Invest. Dermatol.*, 114, 480–486 (2000).
- (4) C. Lee, H. Choi, H. Kim, D. Kim, I. Chang, H. T. Moon, S. Lee, W. K. Oh, and E. Woo, Biflavonoids isolated from *Selaginella tamariscina* regulate the expression of matrix metalloproteinase in human skin fibroblasts, *Bioorg. Med. Chem.*, 16, 732–738 (2008).
- (5) M. R. Khorramizadeh, E. E. Tredget, C. Telasky, Q. Shen, and A. Ghahary, Aging differentially modulates the expression of collagen and collagenase in dermal fibroblasts, *Mol. Cell. Biochem.*, 194, 99–108 (1999).
- (6) A. J. Millis, M. Hoyle, H. M. McCue, and H. Martini, Differential expression of metalloproteinase and tissue inhibitor of metalloproteinase genes in aged human fibroblasts, *Exp. Cell. Res.*, 201, 373–379 (1992).
- (7) A. Berton, G. Godeau, H. Emonard, K. Baba, P. Bellon, W. Hornebeck, and G. Bellon, Analysis of the ex vivo specificity of human gelatinases A and B towards skin collagen and elastic fibers by computerized morphometry, *Matrix Biology*, 19, 139–148 (2000).
- (8) M. Brennan, H. Bhatti, K. C. Nerusu, N. Bhagavathula, S. Kang, G. J. Fisher, J. Varani, and J. J. Voorhees, Matrix metalloproteinase-1 is the major collagenolytic enzyme responsible for collagen damage in UV-irradiated human skin, *Photochem. Photobiol.*, 78, 43–48 (2003).
- (9) N. Tsuji, S. Moriwaki, Y. Suzuki, Y. Takema, and G. Imokawa, The role of elastases secreted by fibroblasts in wrinkle formation: Implication through selective inhibition of elastase activity, *Photochem Photobiol.*, 71, 283–290 (2001).
- (10) F. Rijken, R. C. Kiekens, E. van den Worm, P. L. Lee, H. van Weelden, and P. L. Bruijnzeek, Pathophysiology of photoaging of human skin: Focus on neutrophils, *Photochem. Photobiol. Sci.*, 5, 184–189 (2006).
- (11) F. Rijken, R. C. Kiekens, and P. L. Bruijnzeek, Skin-infiltrating neutrophils following exposure to solarsimulated radiation could play an important role in photoageing of human skin, *Br. J. Dermatol.*, 152, 321–328 (2005).
- (12) E. Jung, J. Lee, J. Baek, K. Jung, J. Lee, S. Huh, S. Kim, J. Koh, and D. Park, Effect of *Camellia japon-ica* oil on human type I procollagen production and skin barrier function, *J. Ethnopharmacol.*, 112, 127–131 (2007).
- (13) H. S. Cho, M. H. Lee, J. W. Lee, K. O. No, S. K. Park, H. S. Lee, S. Kang, W. G. Cho, H. J. Park, K. W. Oh, and J. T. Hong, Anti-wrinkling effects of the mixture of vitamin C, vitamin E, pycnogenol and evening primrose oil, and molecular mechanisms on hairless mouse skin caused by chronic ultraviolet B irradiation, *Photodermatol. Photoimmunol. Photomed.*, 23, 155–162 (2007).
- (14) J. Labat-Robert, A. Fourtanier, B. Boyer-Lafargue, and L. Robert, Age dependent increase of elastase type protease activity in mouse skin. Effect of UV-irradiation, *J. Photochem. Photobiol B*, 57, 113–118 (2000).
- (15) K. K. Lee and J. D. Choi, The effects of Areca catechu L extract on anti-aging, *Int. J. Cosmet. Sci.*, 21, 285–295 (1999).
- (16) Y. H. Kim, K. S. Kim, C. S. Han, H. C. Yang, S. H. Park, K. I. Ko, S. H. Lee, K. H. Kim, N. H. Lee, J. M. Kim, and K. H. Son, Inhibitory effects of natural plants of Jeju Island on elastase and MMP-1 expression, *J. Cosmet. Sci.*, 58, 19–33 (2007).
- (17) K. Tsukahara, H. Nakagawa, S. Moriwaki, Y. Takema, T. Fujimura, and G. Imokawa, Inhibition of ultraviolet-B-induced wrinkle formation by an elastase-inhibiting herbal extract: Implication for the mechanism underlying elastase-associated wrinkles, *Int. J. Dermatol.*, 45, 460–468 (2006).
- (18) F. Jimenez, T. F. Mitts, K. Liu, Y. Wang, and A. Hinek, Ellagic and tannic acids protect newly synthesized elastic fibers from premature enzymatic degradation in dermal fibroblast cultures, *J. Invest. Derma*tol., 126, 1272–1280 (2006).
- (19) N. Philips and J. Devaney, Beneficial regulation of type I collagen and matrixmetalloproteinase-1 expression by estrogen, progesterone and its combination in skin fibroblasts, *J. Am. Aging Assoc.*, 26, 59–62 (2004).
- (20) N. Philips, J. Smith, T. Keller, and S. Gonzalez, Predominant effects of *Polypodium leucotomos* on membrane integrity, lipid peroxidation, and expression of elastin and matrixmetalloproteinase-1 in ultraviolet radiation exposed fibroblasts, and keratinocytes, *J. Dermatol. Sci.*, 32, 1–9 (2003).
- (21) N. Philips, T. Keller, C. Hendrix, S. Hamilton, R. Arena, M. Tuason, and S. Gonzalez, Regulation of the extracellular matrix remodeling by lutein in dermal fibroblasts, melanoma cells, and ultraviolet radiation exposed fibroblasts, *Arch. Dermatol. Res.*, 299, 373–379 (2007).

- (22) X. Z. Song, J. P. Xia, and Z. G. Bi, Effects of (-)-epigallocatechin-3-gallate on expression of matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 in fibroblasts irradiated with ultraviolet A, *Chin. Med. J. (Engl.)*, 117, 1838–1841 (2004).
- (23) C. Ende and R. Gebhardt, Inhibition of matrix metalloproteinase-2 and -9 activities by selected flavonoids, *Planta Med.*, **70**, 1006–1008 (2004).
- (24) S. K. Katiyar, Matrix metalloproteinases in cancer metastasis: Molecular targets for prostate cancer prevention by green tea polyphenols and grape seed proanthocyanidins, *Endocr. Metab. Immune Disord.* Drug Targets, 6, 17–24 (2006).
- (25) M. A. Zaid, F. Afaq, D. N. Syed, M. Dreher, and H. Mukhtar, Inhibition of UVB-mediated oxidative stress and markers of photoaging in immortalized HaCaT keratinocytes by pomegranate polyphenol extract POMx, *Photochem. Photobiol.*, 83, 882–888 (2007).
- (26) J. H. Lee, J. H. Chung, and K. H. Cho, The effects of epigallocatechin-3-gallate on extracellular matrix metabolism, J. Dermatol. Sci., 40, 195–204 (2005).
- (27) C. Gerhauser, A. Alt, E. Heiss, A. Gamal-Eldeen, K. Klimo, J. Knauft, I. Neumann, H. R. Scherf, N. Frank, H. Bartsch, and H. Becker, Cancer chemopreventive activity of xanthohumol, a natural product derived from hops, *Molec. Cancer Ther.*, 1, 959–969 (2002).
- (28) E. C. Colgate, C. L. Miranda, J. F. Stevens, T. M. Bray, and E. Ho, Xanthohumol, a prenylflavonoid derived from hops induces apoptosis and inhibits NF-kappaB activation in prostate epithelial cells, *Cancer Lett.*, 246, 201–209 (2007).
- (29) F. Zhao, H. Nozawa, A. Daikonnya, K. Kondo, and S. Kitanaka, Inhibitors of nitric oxide production from hops (Humulus lupulus L), *Biol. Pharm. Bull.*, 26, 61–65 (2003).
- (30) M. Tripp, G. Darland, R. Lerman, D. Lukaczer, J. Bland, and J. Babish, Hop and modified hop extracts have potent in vitro anti-inflammatory properties, *Acta Hort.*, 668, 217–228 (2005).
- (31) C. R. Overk, P. Yao, L. R. Chadwick, D. Nikolic, Y. Sun, M. A. Cuendet, Y. Deng, A. S. Hedayat, G. F. Pauli, N. R. Farnsworth, R. B. van Breemen, and J. L. Bolton, Comparison of the in vitro estrogenic activities of compounds from hops (Humulus lupulus) and red clover (Trifolium pretense), J. Agric. Food Chem., 53, 6246–6253 (2005).