JPP Journal of Pharmacy and Pharmacology

JPP 2005, 57: 459–465

Received September 3, 2004
Accepted November 24, 2004
DOI 10.1211/0022357055588
ISSN 0022-3573

Effect of flavones on rat brain and lung matrix metalloproteinase activity measured by film in-situ zymography

K. Sasaki, N. Tateoka, H. Ando and F. Yoshizaki

Abstract

We have evaluated the inhibitory activity of flavone, nobiletin, and heptamethoxyflavone on matrix metalloproteinase (MMP) activity in the rat. MMP in 9000-*g* supernatant fraction of lung homogenate was activated by *p*-aminophenyl mercuric acetate (APMA), and gelatinolytic activity was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie staining. This activity should be related to MMP-2 and/or MMP-9 and was confirmed by gelatin zymography. Fluorescent-conjugated collagen used as a substrate for collagenolytic activity was investigated by SDS-PAGE also. The film in-situ zymography method was applied to rat brain and lung tissue in the same manner. Flavone and nobiletin inhibited the APMA-stimulated gelatinolytic activity and also the collagenolytic activity by more than 75%. The film in-situ zymography method indicated that these compounds might be potent inhibitors of MMP, suggesting the specific inhibition of localized MMP in brain hippocampus and/or lung terminal bronchioles, which may contribute to the prevention of some types of brain disease or cancer invasion and metastasis.

Introduction

During the past decade, there has been increasing evidence that matrix metalloproteinases (MMPs) play an important role in physiological phenomena. MMPs have been especially correlated with disease progression, due to their ability to depredate the extracellular matrix. They participate in focal cerebral ischaemia (Rosenberg et al 1996; Romanic et al 1998), tumour invasion and metastasis (John & Tuszynski 2001), and human rheumatoid arthritis (Hayakawa et al 1991). Siwik et al (2001) demonstrated that oxidative stress caused by reactive oxygen species (ROS) regulated collagen synthesis and MMP activity in cardiac fibroblasts. It was suggested that ROS regulated collagen metabolism in a variety of non-cardiac cell types, including rat lung (Pardo et al 1998) and human venous endothelial cells (Belkhiri et al 1997).

On the other hand, flavonoid compounds of plant origin are known to have a wide spectrum of biological activities and may prevent the development of chronic diseases (for review, see reference by Rice-Evans et al 2000) with their antioxidant activity. The efficacies of flavonoids on reducing the risk of cardiovascular disease and cancer, after daily consumption (Sun et al 2002) or dietary supplementation (Morrow et al 2001) have been reported. The polymethoxylated flavones are unique and interesting chemical compounds in particular, mainly existing as an ingredient of citrus fruit (Chen et al 1997). They have been reported to exhibit suppression of inflammation-associated phenomena (Murakami et al 2000), and to exert an antiproliferative activity against human cancer cell lines (Manthey & Guthrine 2002). In this study, we have examined the effect of flavone and polymethoxylated flavones (nobiletin and heptamethoxyflavone) on MMPs in rat lung and brain. Electrophoretic methods were adopted to determine the proteolytic activity using both gelatin and collagen as substrates. Film in-situ zymography (FIZ) was used for assessing the net gelatinolytic activity in tissue, and for evaluating its inhibitory activity and/or localization in the ex-vivo model.

Tohoku Pharmaceutical University, 4-1 Komatsushima 4-chome, Aoba-ku, Sendai 981-8558, Japan

K. Sasaki, N. Tateoka, H. Ando, F. Yoshizaki

Correspondence: K. Sasaki, Tohoku Pharmaceutical University, 4-1 Komatsushima 4-chome, Aoba-ku, Sendai 981-8558, Japan. E-mail: kenrs@tohoku-pharm.ac.jp

Materials and Methods

Materials

p-Aminophenylmercuric acetate and collagen–fluorescein (bovine) were purchased from Sigma (St Louis, MO). The reagents used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad Co. Ltd (Hercules, CA). Other drugs (guaranteed Reagent) were purchased from Nacalai Tesque (Kyoto, Japan). MMP in-situ Zymo-Film was purchased from Wako Pure Chemical Ind. Ltd (Osaka, Japan). Nobiletin and heptamethoxyflavone were separated from citrus fruit according to Sasaki & Yoshizaki (2002).

Animals

Male Wistar rats (9–10-weeks old, 200–220 g; Nihon SLC, Hamamatsu, Japan) were housed in groups of two in stainless-steel cages with free access to food (Japan CLEA; CE-2, Tokyo, Japan) and water. They were kept in a room maintained at an ambient temperature and humidity (25 ± 5°C, 55 ± 5%) under a day/night regime (day 0700–1900 h, night 1900–0700 h). All animals were maintained in the laboratory for a minimum of one week before the start of the experiment. Brain and lung were collected from nine naive rats by decapitation, and the tissues were immediately frozen on liquid nitrogen and maintained at −80°C until use. Animal treatment and maintenance were conducted in accordance with the Guidelines for Animal Experimentation of Tohoku Pharmaceutical University, Japan.

Preparation of tissue homogenate

Lung tissue was homogenized with five volumes of homogenate-medium composed of 50 mM Tris-HCl buffer, pH 7.5 at 4°C, followed by centrifugation (9000 *g*, 4°C, 20 min) to obtain supernatant fractions to be used as the enzyme source for repeat experiments. The samples were maintained at −80°C until use.

Preparation of cryostat sections

The cryostat sections (16-μm thick for brain or 20-μm thick for lung) were made from the frozen blocks of tissues embedded in cryomold O.C.T. Compound (Sakura Finetek Inc., Tokyo, Japan). The frozen blocks were sliced sequentially using a cryostat microtome (MICROM HM505N, MICROM International GmbH, Walldorf, Germany) to prepare serial frozen-thin sections. These sections were then mounted on 3-amino-propyltriethoxy-silane (APS)-coated micro slide glass (Matsunami Glass Ind. Ltd, Tokyo, Japan) for assay of the anatomical regions, or mounted on a polyethylene terephthalate base-film coated with cross-linked gelatin 7-μm thick (MMP in-situ Zymo-Film; Wako Pure Chemical Ind. Ltd, Osaka, Japan). Anatomical brain regions and brain areas were identified using the rat brain atlas (Paxinos & Watson 1997). The respective sections were maintained at −80°C until use.

Assay of gelatinolytic or collagenolytic activity in-vitro

The gelatinolytic or collagenolytic activities of rat lung homogenates were performed with modification to the literature (Gendron et al 1999; Demeule et al 2000). Briefly, a lung 9000-*g* supernatant fraction was activated by incubation at 37°C for 1 h with 0.5 mM *p*-aminophenyl mercuric acetate (APMA) in a buffer containing 50 mM Tris-HCl, pH 7.5 and 50 mM NaCl. The protein content was measured by the method of Bradford (1976) and equal amounts (2.5 mg mL^{-1}) of protein were used. The effect of flavones on gelatinolytic activity was tested as follows. An activated supernatant fraction was incubated with 10 μL of a flavone solution and 5 μL Tris-HCl, pH 7.5 for 30 min at 37°C. Final concentration of each flavone was 0.1–1.0 mM prepared in dimethyl sulfoxide (DMSO) solution (5%). Thereafter, 10 μL gelatin at 0.5 mg mL^{-1} was added, and the assay mixtures were further incubated at 37°C for 18 h. The final pH of the assay mixtures was 7.5. EDTA (10 mM) was used as a control for inhibition of gelatinolytic and collagenolytic activity. Following incubation, the assay mixture for determination of gelatinase activity was re-suspended in a sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.00625% (w/v %) bromophenol blue, boiled for 4 min followed by loading onto 7.5% acrylamide/bisacrylamide (29:1) separating gel and applied to SDS-PAGE performed with a Mini-Protein III apparatus (Bio-Rad, Hercules, CA). Electrophoresis was carried out with molecular mass makers of myosin (200 kDa), ß-galactosidase (116 kDa), bovine serum albumin (66 kDa), at a constant voltage of 200 V for 40 min. Proteins were stained using a Rapid Stain Coomassie Brilliant Blue kit (Nacalai Tesque, Kyoto, Japan). The collagenolytic activity was assessed by measuring the fluorescence (excitation: 473, Y520 nm filter) using fluorescein-conjugated collagen as the substrate (Baici et al 1980), and quantified using a fluorescein-image analyser; FLA-3000 (Fuji Photofilm Co, Ltd, Tokyo, Japan).

SDS-PAGE gelatin zymography

Substrate gelatin-gel zymography was performed using a non-reducible SDS-PAGE condition. The lung 9000-*g* supernatant fraction was resuspended in Laemmli sample buffer, with the exception of 2-mercaptoethanol, and boiled followed by separation with SDS-PAGE gel containing 1.0 mg mL^{-1} gelatin. The electrophoresis was carried out using the same conditions described above. After electrophoresis, the gel was soaked in 0.25% Triton X-100 (30 min, twice) at room temperature and rinsed in water following incubation at 37°C for 18 h in the incubation buffer containing 50 mM Tris-HCl (pH 7.6), 20 mM NaCl, 5 mM $CaCl_2$ and 0.02% Brij-58 with or without 10 mM EDTA or 0.1 mM flavone. The gel was then stained using a Rapid Stain Coomassie Brilliant Blue kit.

Film in-situ zymography

Thin sections were placed on MMP in-situ Zymo-Film. The films with sections were incubated in a moist chamber (P-BOX; SANPLATEC Corp., Osaka, Japan) at 37°C for 1 h with 0.5 mM APMA to activate the MMP. After activation of MMP, the films were incubated for 18 h with sample solutions followed by staining with Biebrich Scarlet Stain Solution (Wako Pure Chemical Ind. Ltd, Osaka, Japan) over 5 min. These stained films were then decolorized with H_2O for 10 min twice. The degraded gelatin was not stained with Biebrich Scarlet Stain Solution, and areas of gelatinolytic activity were visualized as white to pale pink areas on the red background.

Quantification of gelatinolytic activity by SDS-PAGE, and collagenolytic activity by fluorescence and FIZ

The protein band identified with a molecular weight of 211 kDa in the gel separated by SDS-PAGE was quantified using PC-associated image analysis. The SDS-PAGE gel and FIZ-film were digitized using a PC-scanner (Epson ES-2200, Epson Co. Ltd, Nagano, Japan), or FLA-3000 for collagen-fluorescent assay, operating on the image acquisition and analysis program, L Process V2.0 and Image Gauge V4.0 (Fuji Photofilm Co, Ltd, Tokyo, Japan).

Statistical analysis

The results were expressed as means ± standard error of the mean (s.e.m.). All statistical analysis was performed with Sigma Stat statistical software ver. 2.03 (SPSS Inc., IL). Data were tested for significance using the Kruskal–Wallis analysis of variance by rank test with Dann's post hoc comparison when comparing multiple groups, Mann–Whitney rank sum test when comparing two experimental groups. Significance was defined as $P < 0.05$.

Results

The gelatinolytic activity of rat lung homogenate 9000-*g* supernatant fraction was activated with 0.5 mM APMA and its activity was measured using gelatin or fluorescein-conjugated collagen as the substrate. As indicated in Figure 1, APMA activated gelatinolytic activity, as seen by the disappearance of the specific band for gelatin, identified by the molecular weight 211 kDa, whereas no degradation occurred when the chelating agent EDTA (10 mM) was included during the incubation period. SDS-PAGE gelatin zymography was carried out to confirm that this gelatinolytic activity was induced by MMP. After the staining of this gel, the clear bands observed (Figure 2A) correspond to the gelatinolytic activity of MMP. These clear bands were identified from their molecular weights as 66 kDa for activated MMP-2, 72 kDa for pro MMP-2 and 87 kDa for activated MMP-9. Each band was completely diminished by incubation with EDTA or

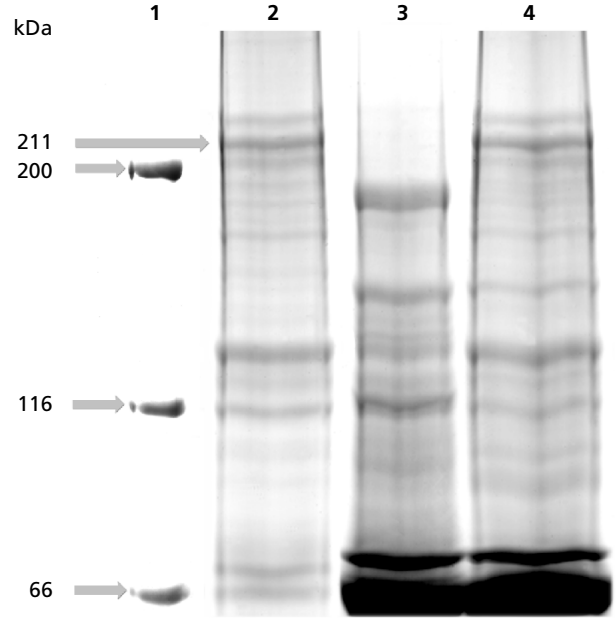


Figure 1 Gelatinolytic activity in lung 9000-*g* supernatant fraction activated by APMA. Activation, incubation and electrophoresis were carried out as described in Materials and Methods. Lane: 1, molecular mass marker; 2, gelatin alone; 3, gelatin, 9000-*g* supernatant fraction activated by APMA; 4, gelatin, 9000-*g* supernatant fraction activated by APMA with 10 mM EDTA.

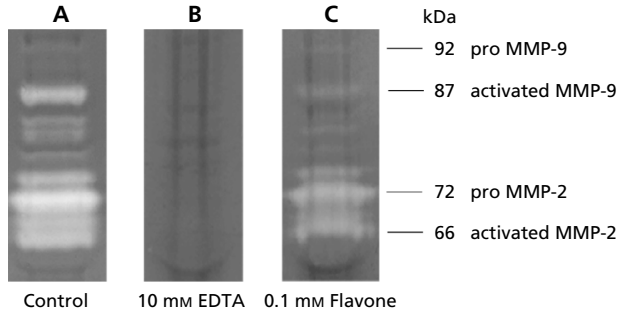


Figure 2 SDS-PAGE gelatin-gel zymography of lung 9000-*g* supernatant fraction. Augmentation of gelatinolytic activity and other methods were performed as described in Figure 1. The assay mixtures were applied to non-reducible SDS-PAGE condition for gelatin (1.0 mg mL^{-1}) containing gel, followed by incubation at 18 h with or without EDTA. A. Control. The clear bands correspond to MMP-2 and MMP-9. B. With EDTA. C. With flavone.

partially inhibited by flavone (Figure 2B, C). Thus this gelatinolytic activity induced by APMA was expected to involve MMP-2 and MMP-9 activity. The effects of flavone, nobiletin and heptamethoxyflavone at 0.01–1.0 mM were investigated using the previous gelatinolytic assay conditions, and quantified using image analysis.

As indicated in Figure 3, all flavones inhibited the gelatinolytic activity in a significant dose-dependent manner (Kruskal–Wallis test; $P < 0.05$). High concentrations

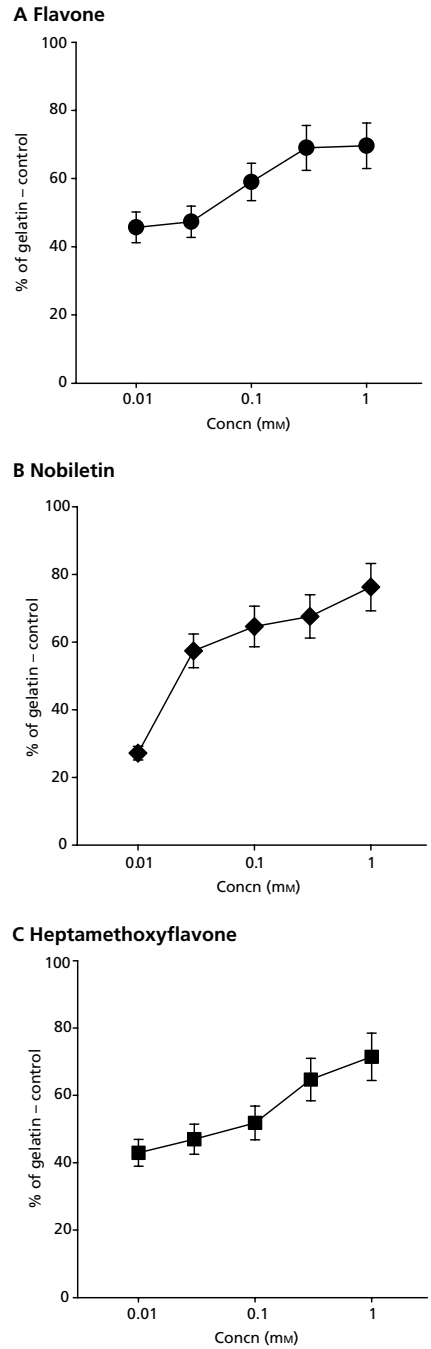


Figure 3 Effects of flavones on gelatinolytic activity in lung 9000-*g* supernatant fraction. Quantification for inhibitory activity was performed by determination of the disappearance of gelatin-peculiar protein band (211 kDa). The inhibitory activity is shown as the % of gelatin-control values. Each experiment was performed independently in quadruplet, and the data are shown as mean ± s.e.m. (n = 9).

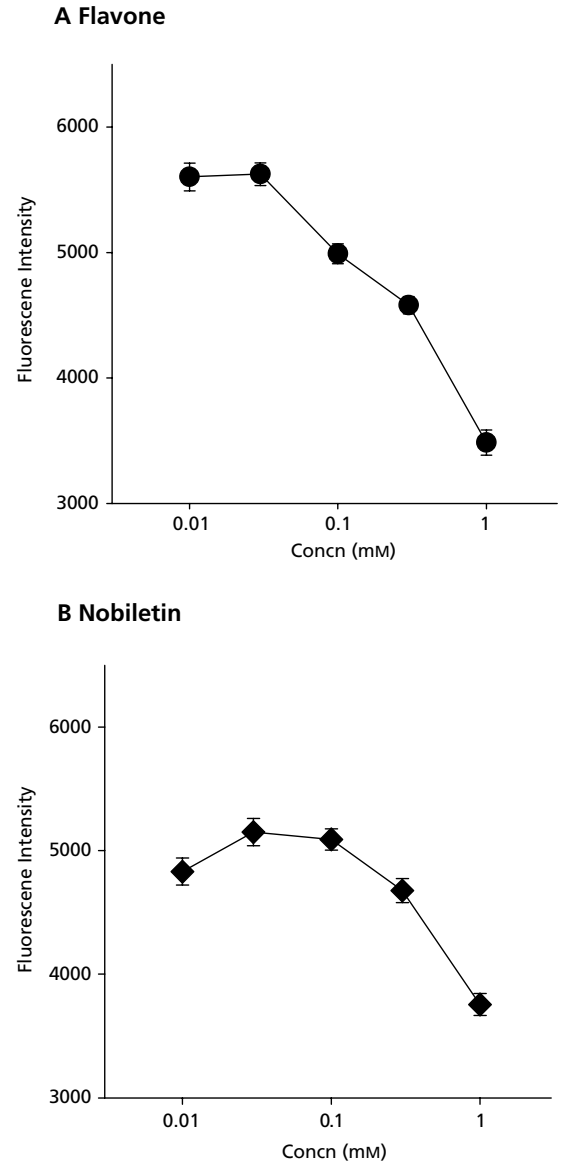
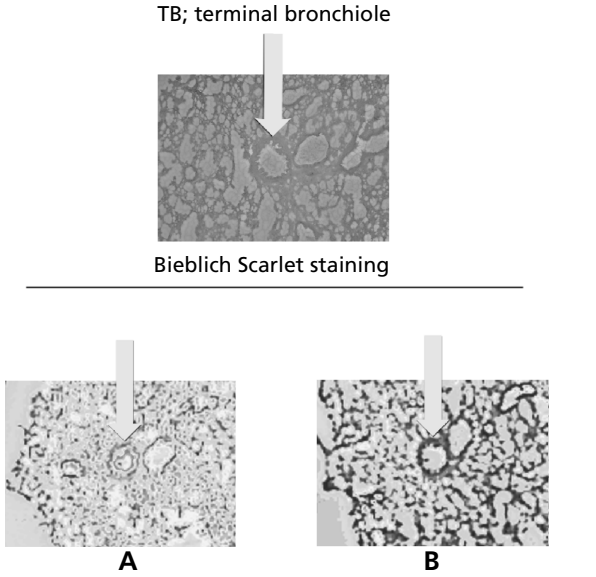


Figure 4 Effects of flavonoids on collagenolytic activity in lung 9000-*g* supernatant fraction. Quantification for inhibitory activity was performed by determination of fluorescent intensity (ex: 473 nm, Y520 nm filter) of fluorescein-conjugated collagen. The inhibitory activity is shown as the % of control values. Each experiment was performed independently in quadruplet, and the data are shown as mean ± s.e.m. (n = 9).

showed significant inhibition by more than 75% compared with the control value. IC50 values (μM) were as follows: flavone 25.29, nobiletin 40.22, heptamethoxyflavone 40.77. Collagenolytic activity, using fluorescein-conjugated collagen as the substrate, was investigated. Nobiletin and flavone significantly (Kruskal–Wallis test; $P < 0.05$) inhibited collagenase activity in a dose-dependent manner (Figure 4).

The inhibition of lung gelatinolytic activity by nobiletin was examined by FIZ. The lysis indicated that MMP was able to produce lysis zones on the gelatin-coated film. When the gelatin-coated film was incubated with EDTA, no lysis zone was detected on the film, showing that the lysis zones were most likely the result of gelatinolytic

activity. Light halo-like lysis in native rat tissue sections indicated MMP activity. A specific zone of strong lysis activity was found in the area surrounding the lung terminal bronchioles (Figure 5A). In the area surrounding the lung terminal bronchioles nobiletin (425 μM) significantly (Mann–Whitney test; $P < 0.05$) inhibited MMP activity by 34%; in other areas of lung tissue the inhibition was insignificant (Figure 5B). The same methodology was used to evaluate the inhibition of brain MMPs by flavones. The coronal brain sections were separated in to right and left hemispheres and mounted for MMP in-situ Zymo-Film. After activation of MMPs by APMA, heterogeneous brain MMP activity-distribution was observed. Figure 6 shows the typical FIZ image. The selected coronal sections of the rat brain from rostral to caudal brain regions included forebrain sections (corresponding to approximately bregma +1.20 mm), diencephalon sections (corresponding to approximately bregma −3.80 mm), and midbrain sections (corresponding to approximately bregma −5.8 mm). The neuroanatomical patterns of MMP were visualized by image analysis. The results showed distinct anatomical distributions in the brain. A tendency for inhibition of MMP activity was seen in the flavone-treated (30–300 μM) left-brain hemisphere compared with the untreated right hemisphere. The change in activity was compared in six brain regions, as presented in Table 1. The numerical units of the controls were from 3300 to 9400; those for flavone-treated were from 1700 to 8500 approximately. Flavone (300 μM) inhibited MMP activity significantly in the nucleus accumbens, hippocampus, and posteromedial cortical amygdaloid nucleus. The strongest inhibition, 47%, was observed in the hippocampus area in a concentration-dependent manner. Moreover, the lowest numerical unit was observed in the flavone-treated hippocampus.



Nobiletin (425μM)	
–	+
Inhibition (%)	
0	33.7±2.5*

Figure 5 Visualization of MMP activity in rat lung tissue in FIZ. Serial cryosections of lung tissues were incubated with APMA to activate MMP, and subjected to FIZ in the absence (A) or presence (B) of nobiletin (425 μM) as mentioned in Materials and Methods. One of the representative pictures has been depicted. The inhibitory activities are shown as the % of control values. Each experiment was performed independently in quadruplet with three different sections, and the data are shown as mean ± s.e.m. (n = 9). *$P < 0.05$ compared with control.

Discussion

The study demonstrated that some flavones prevented regionally-specific proteolysis activity in lung and brain. The effects of these flavones on MMP activity were confirmed by gelatin-zymography using tissue homogenates. The autocatalytic activation by APMA for pro MMP-2 and MMP-9 in lung and brain tissue was used to assess the activity. The activation mechanism had been described previously (Fridman et al 1995; Itoh et al 1995). We adopted the APMA-activated tissue proteolysis activity for the model of in-vitro or ex-vivo artificial disease model corresponding to extracellular matrix-degeneration associated diseases. The expression of MMP in tissue had been investigated at the transcriptional level using in-situ hybridization and at the protein level by immunohistochemical analysis. However, the immunohistochemical method did not provide information regarding the functional state of MMP. Biological study of tissue extracts or homogenates precluded localization of cell type exhibiting activity of MMP. Thus, conventional biochemical techniques such as zymography were expected to have limited availability for assessing the net functional activity of MMP in tissue. A new method, film in-situ zymography (FIZ), which could identify gelatinolytic activity in normal and pathological tissue, allowed preservation of the tissue structure and quantitation of its activity (Ikeda et al 2000).

Numerous studies have been performed to assess the mechanisms controlling MMP expression in lung tissue and lung-derived cells, and to localize the sites of enzyme expression in various diseases. Our results indicated that flavone was a potent inhibitor of gelatinolytic activity and of collagenolytic activity in-vitro using lung homogenate. The FIZ technique revealed its localization and net functional activity in tissue. The strongest activity was localized in the area surrounding the lung terminal bronchioles, and flavone inhibited its net activity by more than 34%. Moreover, a strong inhibition (47%) of proteolysis by flavones was observed in the hippocampus area of the brain. Recently, Szklarczyk et al (2002) indicated that MMP-9 but not MMP-2 was highly expressed by neurons in adult rat brain. MMP-9 protein is enriched in neuronal cell bodies and is present in dendrites suggesting this enzyme may be located within dendrites of the extracellular matrix. It was clear that the MMP was expressed by adult CNS neurons, and its level or cellular localization might be regulated

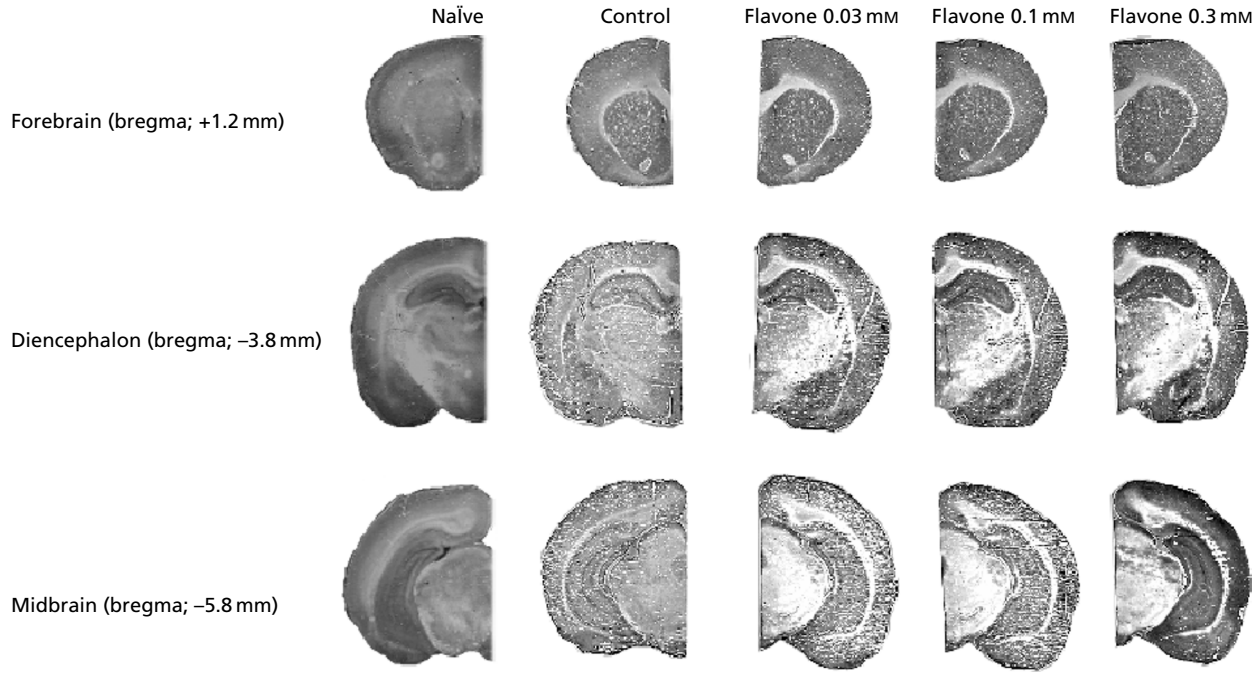


Figure 6 Visualization of brain MMP activity by FIZ. Serial cryosections of rat brain were incubated with APMA to activate MMP, and subjected to FIZ in the absence (control) or presence of flavone (30–300 μM) as mentioned in Materials and Methods. One of the representative pictures has been depicted.

Table 1 Effect of flavone on net matrix metalloproteinase activity in different brain regions of the rat

Brain region	Inhibition of activity (%)		
	Flavone 300 μM	Flavone 30 μM	Flavone 3 μM
Caudate putaman	21.7 ± 2.75	16.96 ± 2.33	9.05 ± 0.22
Nucleus accumbens	25.09 ± 1.98*	21.09 ± 0.47	10.47 ± 1.09
Hippocampus	46.88 ± 1.11*	42.63 ± 2.82*	36.75 ± 0.58*
Posteromedial cortical amygdaloid nucleus	34.71 ± 0.76*	15.16 ± 1.01	3.11 ± 0.63
Superficial gray layer of superior colliculus	20.16 ± 3.56	14.03 ± 0.56	12.52 ± 1.96

The inhibitory activity is shown as the % of control values. Each experiment was performed independently in quadruplet with three different sections for each brain region. The data shown are mean ± s.e.m. (n = 9). *$P < 0.05$ compared with control.

according to the developmental and/or functional status of neurons. Interactions between MMPs and brain function injury, such as kinate-induced seizures (Zang et al 1998; Kashihara et al 2000; Jourquin et al 2003) or brain ischaemia (Rosenberg et al 1996; Romanic et al 1998) have been reported. The hippocampus has been well characterized with respect to spatial temporal expression on plasticity-related genes and is known to undergo significant structural plasticity, associated with the function of learning and memory. Our data were in agreement with evidence of flavonoid bioactivity, and demonstrated for the first time that flavones inhibited MMP activity in the lung and brain tissues, suggesting specific inhibition in localized areas in the brain hippocampus and/or lung terminal bronchioles.

The MMP family share several common characteristics. Each degrades at least one component of the basement membrane. They are active at physiological pH and require two Zn^{2+} ions/molecules to be active. They are inhibited by metal chelators and tissue inhibitors, and they are re-secreted as zymogens and require activation extracellularly (John & Tuszynski 2001). Since flavonoids are known potent metal-chelating agents (Pietta 2000), zinc ions/molecules might be affected by the flavones contributing the inhibitory activity. The effects of metal-chelating activity on production of tissue inhibitors of MMP

(TIMP) and on membrane fluidity requires further study to clarify the molecular mechanisms underlying the inhibitory effect of the flavones on MMP activity.

Conclusion

Our results demonstrated that flavones might be possible inhibitors of MMPs and suggested that they could contribute to the prevention of some kinds of brain disease or cancer invasion and metastasis. Most notably flavones are found in citrus fruits, which worldwide are consumed on a daily basis either as food or as a dietary supplement. Further studies are needed to clarify the inhibition mechanism in detail, or the interaction between other flavonoids and other MMP families. We have established a simple method to assess tissue MMP activity that would be useful for other tissues and/or biological evaluation of other inhibitors. In-vivo experiments, such as using the brain-ischaemia rat model or kinate-stimulated rat seizure model would be of interest.

References

Baici, A., Cohen, G., Fehr, K., Boni, A. (1980) A handy assay for collagen using reconstituted fluorescein-labeled collagen fibrils. *Anal. Biochem.* **108**: 230–232

Belkhiri, A., Richards, C., Whaley, M., McQueen, S. A., Orr, F. W. (1997) Increased expression of activated matrix metalloproteinase-2 by human endothelial cells after sublethal H_2O_2 exposure. *Lab. Invest.* **77**: 533–539

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248–254

Chen, J., Montanari, A. M., Widmer, W. W. (1997) Two new polymethoxylated flavonoids, a class of compounds with potential anticancer activity, isolated from cold pressed Dancy tangerine peel oil solids. *J. Agricultural Food Chem.* **45**: 364–368

Demeule, M., Brossard, M., Page, M., Gingras, D., Beliveau, R. (2000) Matrix metalloproteinase inhibition by green tea catechins. *Biochim. Biophys. Acta* **1478**: 51–60

Fridman, R., Toth, M., Pena, D., Mobashery, S. (1995) Activation of progelatinase B (MMP-9) by gelatinase A (MMP-2). *Cancer Res.* **55**: 2548–2555

Gendron, R., Grenier, D., Sorsa, T., Mayrand, D. (1999) Inhibition of the activities of matrix metalloproteinases 2, 8, and 9 by chlorhexidine. *Clin. Diagnostic Lab. Immunol.* **6**: 437–439

Hayakawa, T., Yamashita, K., Kodama, S., Iwata, H., Iwata, K. (1991) Tissue inhibitor of metalloproteinase and collagenase activity in synovial fluid of human rheumatoid arthritis. *Biomed. Res.* **12**: 169–173

Ikeda, M., Maekawa, R., Tanaka, H., Matsumoto, M., Takeda, Y., Tamura, Y., Nemori, R., Yoshikawa, T. (2000) Inhibition of gelatinolytic activity in tumor tissue by synthetic matrix metalloproteinase inhibitor: application of Film in-situ zymography. *Clin. Cancer Res.* **6**: 3290–3296

Itoh, Y., Binner, S., Nagase, H. (1995) Steps involved in activation of the complex of pro-matrix metalloproteinase 2 (progelatinase A) and tissue inhibitor of metalloproteinases (TIMP)-2 by 4-aminophenylmercuric acetate. *Biochem. J.* **308**: 645–651

John, A., Tuszynski, G. (2001) The role of matrix metalloproteinases in tumor angiogenesis and tumor metastasis. *Pathol. Oncol. Res.* **7**: 14–23

Jourquin, J., Tremblay, E., Decanis, N., Charton, G., Hanessian, S., Chollet, A. M., Diguardher, T. L., Khrestchatisky, M., Rivera, S. (2003) Neuronal activity-dependent increase of net matrix metalloproteinase activity is associated with MMP-9 neurotoxicity after kainite. *Eur. J. Neurosci.* **18**: 1507–1517

Kashihara, K., Akiyama, K., Komada, M., Kohira, I., Abe, K. (2000) Temporal changes in expression of neuronal nitric oxide synthase mRNA in the rat hippocampus associated with kainite-induced seizures. *Neurol. Res.* **22**: 409–412

Manthey, J. A., Guthrine, N. (2002) Antiproliferative activities of citrus flavonoids against six human cancer cell lines. *J. Agricultural Food Chem.* **51**: 5837–5843

Morrow, D. M., Fitzsimmons, P. E., Chopra, M., McGlynn, H. (2001) Dietary supplementation with the anti-tumor promoter quercetin: its effects on matrix metalloproteinase gene regulation. *Mutat. Res.* **480**: 269–276

Murakami, A., Nakamura, Y., Torikai, K., Tanaka, T., Koshiba, T., Koshimizu, K., Kuwahara, S., Takahashi, Y., Ogawa, K., Yano, M., Tokuda, H., Nishino, H., Mimaki, Y., Sashida, Y., Kitanaka, S., Ohigashi, H. (2000) Inhibitory effect of citrus nobiletin on phorbol ester-induced skin inflammation, oxidative stress, and tumor promotion in mice. *Cancer Res.* **60**: 5059–5066

Pardo, A., Barrios, R., Maldonado, V., Melendez, J., Perez, J., Ruiz, V., Segura-Valdez, L., Sznajder, J. I., Selman, M. (1998) Gelatinase A and B are up-regulated in rat lungs by subacute hyperoxia: pathogenic implications. *Am. J. Pathol.* **153**: 833–844

Paxinos, G., Watson, C. (1997) *The rat brain in stereotaxic coordinates*. 4th edn, Academic Press, New York

Pietta, P. G. (2000) Flavonoids as antioxidants. *J. Natural Products* **63**: 1035–1042

Rice-Evans, C., Spencer, J. P., Schroeter, H., Rechner, A. R. (2000) Bioavailability of flavonoids and potential bioactive forms in vivo. *Drug Metab. Drug Interact.* **17**: 291–310

Romanic, A. M., White, R. F., Arleth, A. J., Ohlstein, E. H., Barone, F. C. (1998) Matrix metalloproteinase expression increases after cerebral focal ischemia in rats. *Stroke* **29**: 1020–1030

Rosenberg, G. A., Navratil, M., Barone, F., Feuerstein, G. (1996) Proteolytic cascade enzymes increase in focal cerebral ischemia in rat. *J. Cerebral Blood Flow Metab.* **16**: 360–366

Sasaki, K., Yoshizaki, F. (2002) Nobiletin as a tyrosinase inhibitor from the peel of *Citrus* fruit. *Biol. Pharmaceut. Bullet.* **25**: 806–808

Siwik, D. A., Pagano, P. J., Colucci, W. S. (2001) Oxidative stress regulates collagen synthesis and matrix metalloproteinase activity in cardiac fibroblasts. *Am. J. Physiol. – Cell Physiol.* **280**: c53–c60

Sun, J., Chu, Y. F., Wu, X., Liu, R. H. (2002) Antioxidant and antiproliferative activities of common fruits. *J. Agriculture Food Chem.* **50**: 7449–7454

Szklarczyk, A., Lapinska, J., Rylski, M., McKay, R. D. G., Kaczmarek, L. (2002) Matrix metalloproteinase-9 undergoes expression and activation during dendritic remodeling in adult hippocampus. *J. Neurosci.* **22**: 920–930

Zang, J. W., Deb, S., Gottschall, P. E. (1998) Regional and differential expression of gelatinase in rat brain after systemic kainic acid or bicuculline administration. *Eur. J. Neurosci.* **10**: 3358–3368