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Down-regulation of 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx)-induced CYP1A2 Expression Is Associated with Bovine Lactoferrin Inhibition of MeIQx-induced Liver and Colon Carcinogenesis in Rats

Ken-ichi Fujita, Takamasa Ohnishi, Kazunori Sekine, Masaaki Iigo and Hiroyuki Tsuda[1]

Experimental Pathology and Chemotherapy Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045

The inhibitory influence of bovine lactoferrin (bLF) on induction of preneoplastic hepatic glutathione *S*-transferase placental form-positive (GST-P$^+$) cell foci and colon aberrant crypt foci (ACF) by diethylnitrosamine (DEN) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) was investigated in F344 rats. Rats were initially treated with DEN, then placed on basal diet containing MeIQx (200 ppm) alone, MeIQx plus 2% bLF, or MeIQx plus 0.2% bLF from week 2 to week 8, with partial hepatectomy performed at week 3. Concomitant administration of 2% or 0.2% bLF with MeIQx caused significant dose-dependent decreases in both number and unit area of GST-P$^+$ cell foci (2% bLF, $P<0.001$; 0.2% bLF, $P<0.01$). Similar results were observed for MeIQx-induced colon ACF in the groups without DEN treatment (2% and 0.2% bLF, $P<0.05$). To investigate the underlying mechanisms, we analyzed the influence of bLF on levels of cytochrome P4501A2 (CYP1A2), a metabolically activating enzyme of MeIQx in the liver. The results demonstrated that combined administration of 2% bLF significantly reduced levels of MeIQx-induced CYP1A2 mRNA ($P<0.05$) and protein ($P<0.05$) to the normal levels, in association with reduced values for MeIQx-DNA adducts ($P<0.05$), liver GST-P$^+$ cell foci and colon ACF. These results suggest that bLF is a chemopreventive agent for DEN alone or DEN plus MeIQx-induced liver, and MeIQx-induced colon carcinogenesis in rats. One possible mechanism is a normalizing down-regulation of CYP1A2 expression by bLF, with consequent reduction of carcinogen activation and adduct formation.

Key words: Lactoferrin — CYP1A2 — MeIQx — Liver carcinogenesis

Lactoferrin, a multifunctional iron-binding glycoprotein, is present in various amounts in mammalian secretions such as tears and milk and its physiological importance might be related to anti-bacterial, anti-viral and immunomodulating effects.[1, 2] Recently, bovine lactoferrin (bLF) was shown to strongly inhibit development of colon aberrant crypt foci (ACF) and tumors induced by azoxymethane in rats without any toxic effects in major organs.[3, 4] Similar effects of bLF were also observed on lung, esophagus, tongue and bladder carcinogenesis in rats.[5–7] The results so far obtained thus indicate that bLF is a promising chemopreventive agent for carcinogenesis, although the underlying mechanisms remain unclear.

The significance of environmental heterocyclic amines in the etiology of human cancers is of growing interest.[8] Most of these formed during cooking of meat products have been shown to be strong mutagens,[9, 10] and heterocyclic amine adduct formation has been considered to be a prerequisite for their initiation of carcinogenesis.[11] One of the heterocyclic amines, 2-amino-3,8-dimethylimidazo [4,5-*f*]quinoxaline (MeIQx) has been demonstrated to be carcinogenic in mice and rats, inducing mainly liver tumors in these animals,[12–14] in line with data for MeIQx-DNA adduct formation.[15, 16] MeIQx is thought to be metabolically activated to a mutagenic/carcinogenic intermediate by initial *N*-oxidation in the liver, mediated primarily by cytochrome P450 (CYP) 1A2 to yield *N*-hydroxy-MeIQx.[17, 18] This is further activated through esterification reactions catalyzed by arylamine *N*-acetyltransferase (NAT) or phenol sulfotransferase (PST) to yield *N*-acetoxy- or *N*-sulfonyloxy-MeIQx, respectively, whose more reactive metabolites can covalently bind to DNA to form MeIQx-DNA adducts.[19, 20] Previous studies have shown that at least two forms of NAT, NAT1 and NAT2, and three forms of PST, ST1A1, ST1B1 and ST1C1, are contained in rat livers.[21, 22] However, the molecular forms responsible for catalyzing *N*-acetoxy- or *N*-sulfonyloxy-MeIQx formation remain to be identified.

For the purpose of determining any chemopreventive effects of bLF on liver carcinogenesis induced by diethylnitrosamine (DEN) and/or MeIQx, the present study was carried out utilizing an established medium-term bioassay protocol for modifying agents of liver carcinogenesis with preneoplastic liver glutathione *S*-transferase placental form-positive (GST-P$^+$) cell foci as the end point

[1] To whom correspondence should be addressed.
E-mail: htsuda@gan2.res.ncc.go.jp

lesion.[23–26] Since MeIQx is carcinogenic to the colon as well, the effects of bLF on preneoplastic ACF induction were also assessed in the same protocol.[27] For the analysis of the underlying mechanisms of action, we also examined the influence of bLF on levels of MeIQx-induced CYP1A2 expression and MeIQx-DNA adduct levels in the liver.

MATERIALS AND METHODS

Chemicals bLF was kindly provided by Morinaga Milk Industry Co., Ltd. (Zama). DEN and MeIQx were purchased from Tokyo Chemical Industry (Tokyo) and the Nard Institute (Osaka), respectively. ISOGEN and SUPERSCRIPT II were obtained from Nippon Gene, Inc. (Toyama) and Life Technologies, Inc. (Gaithersburg, MD), respectively. Restriction endonucleases, DNA modifying enzymes, T4 polynucleotide kinase and Ex *Taq* were purchased from TaKaRa Shuzo (Kyoto). RNase A, RNase T1, micrococcal nuclease and phosphodiesterase II were purchased from Worthington Biochemical Co. (Freehold, NJ). Apyrase was obtained from Sigma Chemical Co. (St. Louis, MO), [γ-^{32}P]ATP (800 Ci/mmol) and [α-^{32}P]dCTP (3000 mCi/mmol) from ICN Biomedicals, Inc. (Irvine, CA) and Amersham Japan (Tokyo), respectively, rabbit anti-rat glutathione *S*-transferase placental form (GST-P) antibody from Medical and Biological Laboratories (Nagoya), and goat anti-rat CYP1A1, CYP2E1 and CYP3A2 antibodies from Daiichi Pure Chemicals (Tokyo).

Animals and treatments Male F344 rats were obtained from Charles River Japan (Atsugi) at 5 weeks of age and maintained in plastic cages in an air-conditioned room under constant conditions of temperature (23±2°C) and humidity (55±5%) with a 12 h light and dark cycle. The rats were allowed free access to basal diet (Oriental MF; Oriental Yeast Co., Ltd., Tokyo) and tap water and were used in the experiment after acclimatization for 1 week. The experiments were carried out based on an established medium-term liver bioassay for agents modifying carcinogenesis.[23–26] As shown in Fig. 1, rats at 6 weeks of age were treated intraperitoneally with DEN at a dose of 200 mg/kg body weight or 0.9% NaCl. At week 2, the animals were divided into 6 treatment subgroups and administered basal diet containing the following compounds for the next 6 weeks; 0% bLF (basal diet alone), 2% bLF, 0.2% bLF, MeIQx (200 ppm), MeIQx plus 2% bLF, and MeIQx plus 0.2% bLF. All animals were subjected to two-thirds partial hepatectomy (PH) at week 3 and killed at week 8 under deep ether anesthesia. Livers were removed, weighed, and processed for immunohistochemical and biochemical analyses. Colons were also resected and processed for analysis of ACF count as described below. Animals were maintained and treated according to the "Guide for the Care and Use of Laboratory Animals" of the Animal Study Committee of the National Cancer Center, Japan.

GST-P$^+$ cell foci count Liver tissues obtained at week 8 were fixed in ice-cold acetone, routinely embedded in paraffin and immunohistochemically stained for GST-P[23–26] by the avidin-biotin-peroxidase complex method (ABC kit; Vector Laboratories, Burlingame, CA). The numbers and areas of GST-P$^+$ cell foci more than 100 μm in diameter were measured and analyzed with a video image processor (IPAP; Sumika Technos Co., Osaka).

Aberrant crypt (AC) and ACF count For detection of AC and ACF, the colons were inflated with 10% formalin in phosphate-buffered saline, cut longitudinally from the cecum to the anus and spread on filter papers. After fixation, the colons were stained with 0.5% methylene blue in 0.9% NaCl and numbers of ACF comprising more than one crypt were assessed under a light microscope.[27]

Western blot analysis Liver tissue obtained at weeks 3 (PH) and 8 (sacrifice) was homogenized in 10 m*M* sodium phosphate, pH 7.4, containing 1 m*M* DTT and 250 m*M* sucrose. The homogenate was centrifuged at 9000*g* for 20 min, the supernatant was further centrifuged at 105 000*g* for 60 min and the resultant pellet was resuspended in the buffer and used as the microsome fraction. Protein concentration was determined by the method of Bradford using BSA as the standard.[28] The samples were mixed with an equal volume of 62.5 m*M* Tris-HCl buffer, pH 6.8, containing 2% SDS, 20% sucrose, 0.02% pyronin Y and 10% 2-mercaptoethanol, and subjected to SDS-polyacrylamide gel electrophoresis using a 9.0% gel by the method of Laemmli.[29] The separated proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA).[30] CYP1A2, CYP2E1 or CYP3A2 proteins on the membrane were immunostained using the respective antibodies and developed with peroxidase-conjugated rabbit

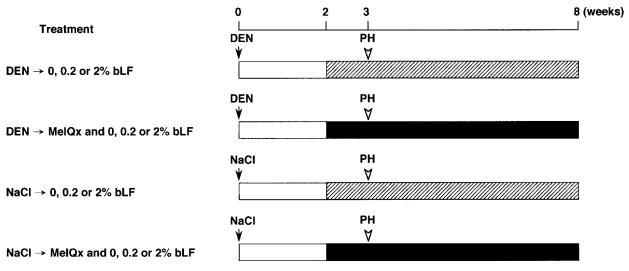

Fig. 1. Experimental protocol. Rats were initially treated with DEN (200 mg/kg, i.p.) at week 0. From week 2, animals were administered the basal diet with (solid columns) or without (hatched columns) MeIQx and 0, 0.2 or 2% bLF, and subjected to PH at week 3. Liver samples were obtained at PH and sacrifice at week 8. Results were evaluated in terms of quantitative values of liver GST-P$^+$ cell foci and colon ACF and ACs.

anti-goat IgG, diaminobenzidine and hydrogen peroxide. Quantitation was achieved using standard microsomes with known protein concentrations, supplied with the antibodies. Blots were scanned and intensities were calculated using NIH Image (Version 1.58) software.

Isolation of CYP, NAT and PST cDNAs Total RNAs were isolated from rat liver using ISOGEN and CYP1A2, CYP2E1, CYP3A2, ST1A1, ST1B1, ST1C1, NAT1, NAT2 and β-actin cDNAs were generated by reverse transcription-PCR. Using 5 μg of total RNAs as templates, reverse transcription was carried out for 1 h at 42°C in a reaction mixture (20 μl) containing 50 ng of oligo$(dT)_{12-18}$ primer, 0.5 m*M* each of dATP, dGTP, dCTP and dTTP, 200 units of SUPERSCRIPT II and the first strand buffer. PCR was performed in a reaction mixture (30 μl) containing 1 μl of the reverse transcription mixture, 20 pmol of each primer, 0.2 m*M* each of dATP, dGTP, dCTP and dTTP, 1.5 units of Ex *Taq* and the Ex *Taq* buffer. The sequences of sense and antisense primers for PCR for the respective cDNAs are shown in Table I. After initial denaturation for 3 min at 94°C, amplification was performed for 30 cycles of 45 s at 94°C, 1.5 min at 55°C, 3 min at 72°C, and a final extension period of 5 min at 72°C. The amplification products were purified, digested with *Bam*HI (CYP1A2, ST1A1, ST1B1, ST1C1, NAT1 and NAT2), *Eco*RI (CYP2E1) or *Bgl*II (CYP3A2) and *Hin*dIII, then subcloned into *Bam*HI or *Eco*RI and *Hin*dIII sites of pUC118 and sequenced using a dideoxynucleotide chain termination method.[31] The nucleotide sequences of the cDNAs obtained by reverse transcription-PCR completely matched those described previously.[32–38] The subcloned cDNA fragments were used for northern blotting as probes with the sizes listed in Table I.

Northern blot analysis Five micrograms of total RNA was denatured for 20 min at 65°C, subjected to electrophoresis on 1.0% formaldehyde-agarose gel, transferred to nylon membranes (Hybond-XL; Amersham) by capillary force in 20× SSC (0.3 *M* sodium citrate, pH 7.0 and 3 *M* NaCl) and fixed with a UV cross-linker. The membranes were hybridized for 16 h at 42°C in ULTRAhyb (Ambion, Inc., St. Austin, TX) containing ^{32}P-labeled probe prepared by the random primer labeling method, then washed twice for 15 min at 42°C with 2× SSC containing 0.1% SDS. Radioactive bands were assessed by use of a Bio-Imaging Analyzer (BAS2000; Fuji Photo Film Co., Tokyo). All transcripts were detected at almost the same position as 18S rRNA (28S»CYP1A2≠CYP2E1≠CYP3A2>18S>ST1A1≠ST1B1≠ST1C1). NAT1 and NAT2 mRNAs were not detected in the current study, though they could be detected (18S>NAT1≠NAT2) in colon tissue in other experiments. Therefore, both probes could be applied for the detection of NAT mRNAs. Quantification of each mRNA was normalized to β-actin mRNA levels.

^{32}P-Postlabeling Liver tissue obtained at weeks 3 and 8 was homogenized in 3 ml of 10 m*M* Tris-HCl, pH 8.0, containing 1% SDS and 1 m*M* EDTA, to which 20 μl each of 20 mg/ml RNase A and 10 000 units/ml RNase T_1 were added, and the mixture was incubated for 1 h at 37°C. Then 100 μl of 20 mg/ml proteinase K was introduced and incubation was continued for 1 h at 37°C. DNA was extracted with phenol and chloroform/isoamyl alcohol (24:1, v/v), precipitated with ethanol and resuspended in 10 m*M* Tris-HCl, pH 8.0, containing 0.1 m*M* EDTA. DNA (10 μg) was hydrolyzed to 3′-monophosphate nucleotides by incubation in 10 μl of 20 m*M* sodium succinate, 10 m*M* $CaCl_2$, pH 6.0, containing 3.0 units of micrococcal nuclease and 0.03 units of phosphodiesterase II for 3 h at 37°C. The hydrolyzed DNA was labeled at the 5′ position under adduct-intensification and standard conditions by the method of Randerath *et al.*[39] Reactions were performed for 1 h at 37°C in a reaction mixture (15 μl) containing 5 μg of hydrolyzed DNA, 2.3 μl of [γ-^{32}P]ATP and 5 units of T4 polynucleotide kinase (adduct-intensification condition) or 0.17 μg of hydrolyzed DNA, 2.9 μl of [γ-^{32}P]ATP, 0.1 m*M* ATP and 9.5 units of T4 polynucleotide kinase (standard condition) and the reaction buffer (30 m*M* Tris-HCl, pH 9.5, 10 m*M* DTT, 10 m*M* $MgCl_2$ and 1 m*M* spermidine). Subsequently, samples were treated with 3 μl of 13.3 units/ml apyrase for 45 min at 37°C. Aliquots

Table I. Oligonucleotide Primers Used for PCR

Target cDNA	Forward primer (5′→3′)	Reverse primer (5′→3′)	Product size (bp)	Accession number
CYP1A2	GCGGATCCATGGCGTTCTCCCAGTATATC	GCAAGCTTCACTTGGAGAAGCGTGGCC	1560	K02422
CYP2E1	GCGAATTCATGGCGGTTCTTGGCATCACC	GCAAGCTTCTCATGAACGGGGAATGACAC	1501	J02627
CYP3A2	GCAGATCTATGGACCTGCTTTCAGCTCTC	GCAAGCTTCTTTAACCATGCATCAAGAGC	1666	M13646
ST1A1	GCGGATCCATGGAGTTCTCCCGTCCACCGC	GCAAGCTTCCACTCATAGTTCACAACG	898	X52883
ST1B1	GCGGATCCATGGGTACTGCAGAAGATGT	GCAAGCTTGTTTAGGCACTCTGAATATC	920	D89375
ST1C1	GCGGATCCATGTCCTTGGAAAAAATGAAAG	GCAAGCTTGCTCTCAGATCTCTGTGCGG	937	L22339
NAT1	GCGGATCCATGGACATCGAAGCATACTTCG	GCAAGCTTAAGACTGAGATAATAATGG	923	U01343
NAT2	GCGGATCCATGGACATTGAAGCATACTTTG	GCAAGCTTCGCATACATCAACATGGTC	988	U01348
β-actin	GCGGATCCATGGATGACGATATCGCTGCGC	GCAAGCTTCTAGAAGCATTTGCGGTGCACG	1144	V01217

(15 μl) of each sample were applied to a polyethyleneimine cellulose plate (POLYGRAM CEL 300 PEI; Mathery-Nagel, Duren, Germany) and developed in 2.3 *M* sodium phosphate buffer (pH 6.0). The plates were rinsed, and each origin was excised and transferred to separate 9.5×10 cm plates using a magnet-transfer technique. Plates were developed for 1.5 h in 2.0 *M* lithium formate, 5.5 *M* urea, pH 3.5 from bottom to top and for 1.5 h in 1.0 *M* lithium chloride, 0.5 *M* Tris, 8.1 *M* urea, pH 8.0 followed by 1.7 *M* sodium phosphate buffer (pH 6.0) from left to right. The plates were exposed to a Fuji imaging plate (Fuji Photo Film Co.) and the radioactive spots were analyzed by use of a Bio-Imaging Analyzer (BAS2000; Fuji Photo Film Co.). Relative adduct labeling was estimated from the radioactivity of each adduct spot and of the total nucleotides by the method of Gupta.[40]

Statistical analysis Data were analyzed statistically using the Dunnett's multiple comparison *t* test with a JMP software package (Version 3.1; SAS Institute Japan, Tokyo) on a Macintosh computer.

RESULTS

Effects of bLF on development of GST-P$^+$ cell foci in liver The average body, liver and kidney weights of rats at week 8 did not significantly differ among the groups (data not shown). Data for numbers and areas of GST-P$^+$ cell foci are summarized in Table II. The numbers of GST-P$^+$ cell foci in the group receiving DEN followed by 2% bLF were significantly lower than for DEN followed by the basal diet ($P<0.05$). Furthermore, in the DEN-treated groups, numbers of GST-P$^+$ cell foci developing under the enhancing influence of MeIQx were significantly and dose-dependently reduced by the concomitant administration of 2% bLF ($P<0.001$) and of 0.2% bLF ($P<0.01$). Similarly, the areas of foci were also significantly and dose-dependently decreased by the addition of 2% bLF ($P<0.001$) and of 0.2% bLF ($P<0.05$). In the DEN-untreated groups, the values for GST-P$^+$ cell foci in groups receiving MeIQx plus 2% or 0.2% bLF were lower, but not significantly so, than that for the group receiving MeIQx alone, with a tendency for dose-dependence.

Table III. Inhibitory Effect of bLF on MeIQx-induced ACF Development

Treatment	No. of rats	No. of ACF[a)]	No. of AC[a)]
DEN→MeIQx	18	10.9±4.0	20.9±7.0
DEN→MeIQx+2% bLF	18	9.9±2.5	16.5±4.7
DEN→MeIQx+0.2% bLF	16	10.4±3.8	16.9±6.3
NaCl→MeIQx	5	17.4±5.3	26.0±9.1
NaCl→MeIQx+2% bLF	5	10.0±1.4[b)]	18.0±1.4
NaCl→MeIQx+0.2% bLF	5	11.2±1.3[b)]	18.4±2.3

a) Data are mean±SD value.
b) Significantly different ($P<0.05$) from the NaCl→MeIQx.

Table II. Inhibitory Effect of bLF on MeIQx-induced GST-P$^+$ Cell Foci Development

Treatment	No. of rats	GST-P$^+$ cell foci[a)]	
		Number (/cm^2)	Area (mm^2/cm^2)
DEN→basal diet	19	17.3±3.6	0.72±0.18
DEN→2% bLF	20	14.5±4.3[b)]	0.66±0.21
DEN→0.2% bLF	20	15.4±4.3	0.64±0.25
DEN→MeIQx	18	40.4±12.9	2.33±0.95
DEN→MeIQx+2% bLF	18	28.1±5.8[c)]	1.27±0.38[c)]
DEN→MeIQx+0.2% bLF	16	37.6±9.2[d)]	1.73±0.47[e)]
NaCl→basal diet	5	0	0
NaCl→2% bLF	5	0	0
NaCl→0.2% bLF	4	0	0
NaCl→MeIQx	5	1.9±0.8	2.70±1.40[f)]
NaCl→MeIQx+2% bLF	5	0.8±1.1	1.20±1.70[f)]
NaCl→MeIQx+0.2% bLF	5	1.4±1.0	1.60±1.30[f)]

a) Data are mean±SD value.
b) Significantly different ($P<0.05$) from the DEN→basal diet.
c) Significantly different ($P<0.001$) from the DEN→MeIQx.

Effects of bLF on AC and ACF count in the colon The findings for AC and ACF are summarized in Table III. AC and ACF were not induced in groups not given MeIQx. In the DEN-untreated groups, significant and dose-dependent reduction in the numbers of ACF was noted with concomitant administration of 2% bLF ($P<0.05$) or 0.2% bLF ($P<0.05$) as compared with the group receiving MeIQx alone. On the other hand, no significant change was observed in the numbers of ACF among groups receiving DEN followed by MeIQx, MeIQx plus 2% and 0.2% bLF. Although not significant, a similar trend was observed for AC count.

Effects of bLF on levels of liver CYP1A2 protein and mRNA The influence of bLF on protein and mRNA levels of CYP1A2 in the liver was investigated by western and northern blot analyses, respectively. CYP1A2 protein and mRNA expressed constitutively in the livers were detected (Fig. 2). No effects of treatment with bLF alone were observed, as shown in Fig. 2. Both protein and mRNA levels were increased about 2-fold at weeks 3 and 8 by treatment with MeIQx (Figs. 3 and 4), but this was significantly reduced by combined administration of 2% bLF at weeks 3 and 8 ($P<0.05$), but not 0.2% bLF, irrespective of the initial DEN treatment (Fig. 3). Levels of CYP1A2 mRNA were increased about 1.5–2-fold by treatment with MeIQx as compared to the control group, and the rise was again blocked by 2% bLF (Fig. 4). Analysis of other CYP family enzymes indicated that expression of CYP2E1 and CYP3A2 protein and mRNA did not show any detectable alterations (Figs. 3 and 4).

Effects of bLF on expression of NAT and PST mRNAs were also analyzed by northern blot analysis (data not shown). ST1A1, ST1B1 and ST1C1, but not NAT1 and NAT2, mRNAs were detected in livers of all groups at

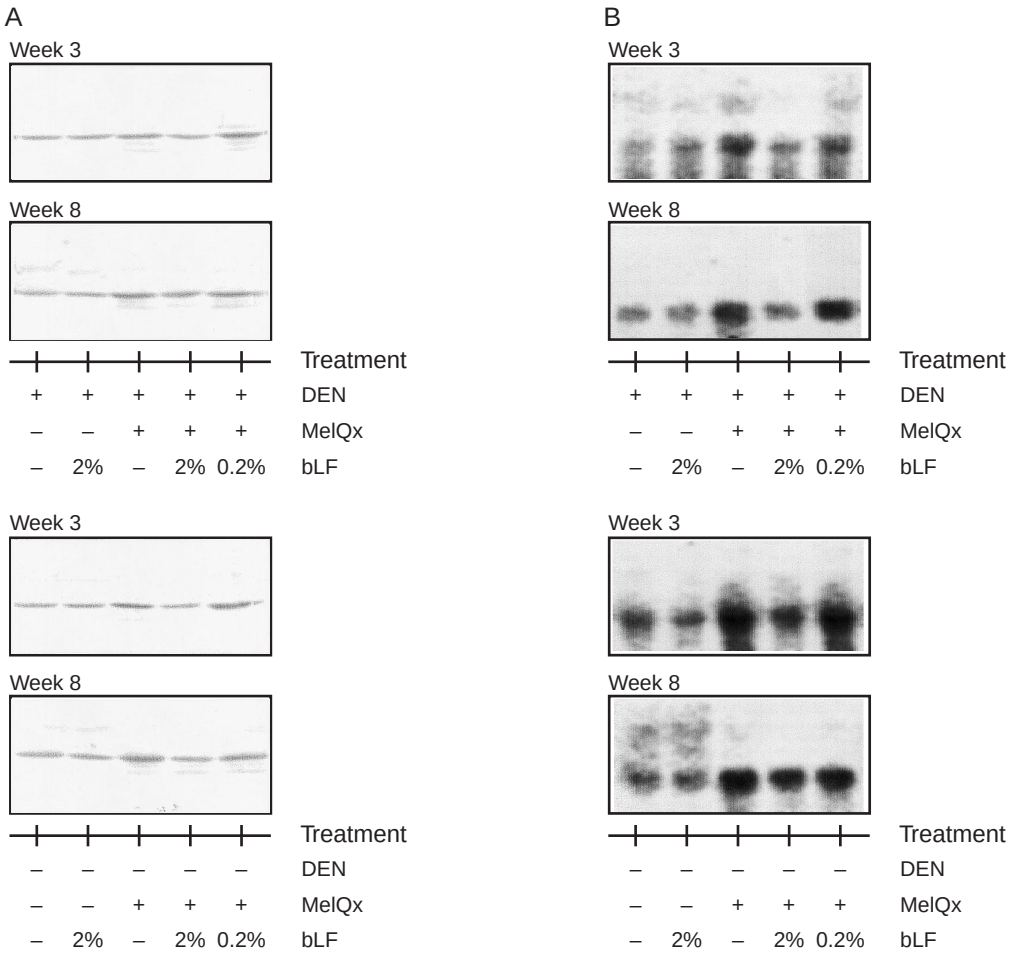

Fig. 2. Changes in the level of CYP1A2 expression in livers of rats treated with DEN, MeIQx and/or bLF. (A) Western blot analyses of CYP1A2 protein levels. (B) Northern blot analyses of CYP1A2 mRNA levels. Treatment patterns with MeIQx and/or bLF with or without DEN initial treatment are illustrated at the bottom. Whether liver samples were obtained at weeks 3 or 8 is shown at the top of each panel.

both weeks 3 and 8, though the levels of NAT and PST mRNAs did not show significant inter-group differences.

Effects of bLF on the MeIQx-DNA adduct formation in liver The influence of bLF on MeIQx-DNA adduct formation in the liver was studied by ^{32}P-postlabeling. As shown in Fig. 5A, no spots were detected in the livers of rats without MeIQx treatment, whereas ^{32}P-postlabeling products were clearly observed for all groups receiving MeIQx at both weeks 3 and 8. In the DEN-treated groups, the total adduct level for the group receiving MeIQx alone at week 3 was significantly higher than that with combined administration of 2% bLF (P<0.05), but not 0.2% bLF (Fig. 5B). In the DEN-untreated groups, total adduct levels were also significantly decreased by the administra-

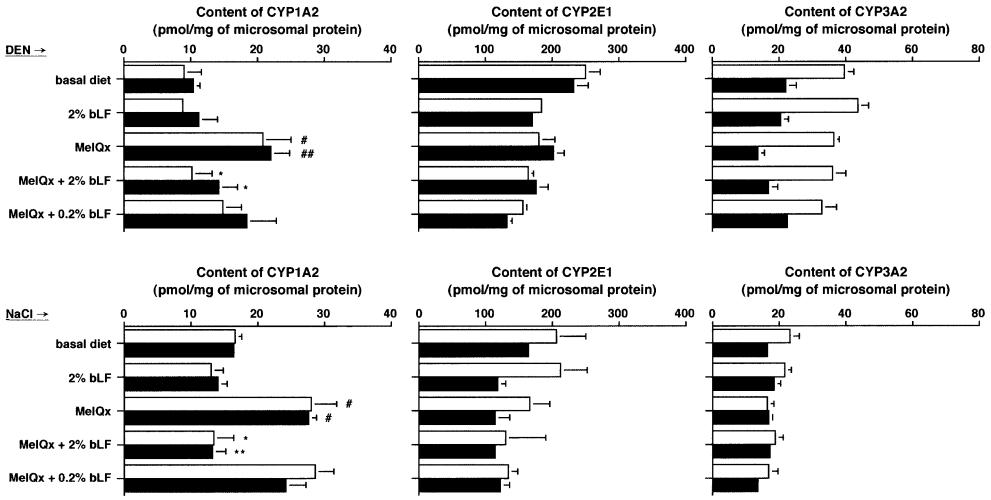

Fig. 3. Inhibitory effects of bLF on MeIQx-induced CYP1A2 protein expression in rat livers. Western blot analyses of CYP1A2 (left), CYP2E1 (middle) and CYP3A2 (right) were performed using the respective antibodies. Data (pmol of CYP/mg of microsomal protein) are mean±SD values (n=3). # P<0.05, ## P<0.005 compared with the basal diet group. ∗ P<0.05, ∗∗ P<0.005 compared with the MeIQx alone group. □, week 3; ■, week 8.

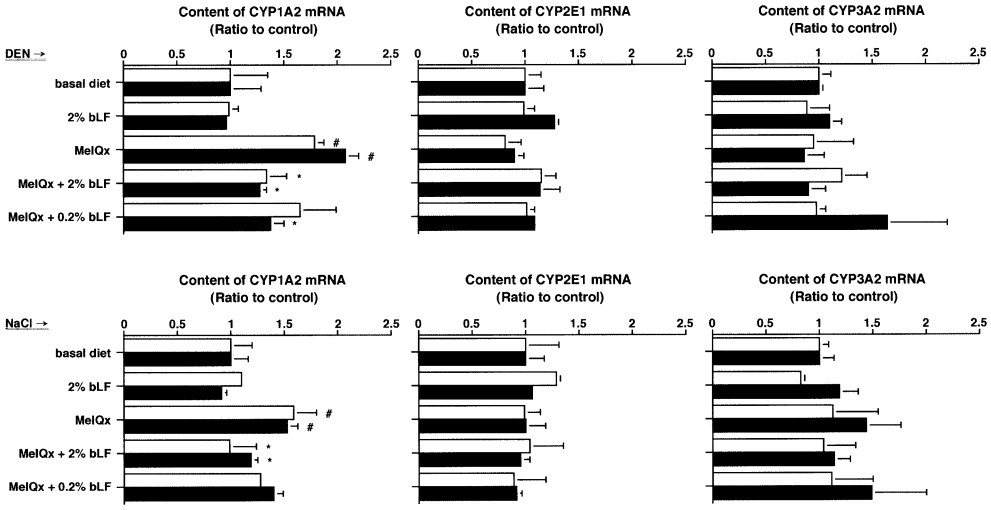

Fig. 4. Inhibitory effects of bLF on MeIQx-induced CYP1A2 mRNA expression in rat livers. Northern blot analyses of CYP1A2 (left), CYP2E1 (middle) and CYP3A2 (right) were performed using the respective cDNAs as probes. Data (ratio to control) are mean±SD values (n=3). # P<0.05 compared with the basal diet group. ∗ P<0.05 compared with the MeIQx alone group. □, week 3; ■, week 8.

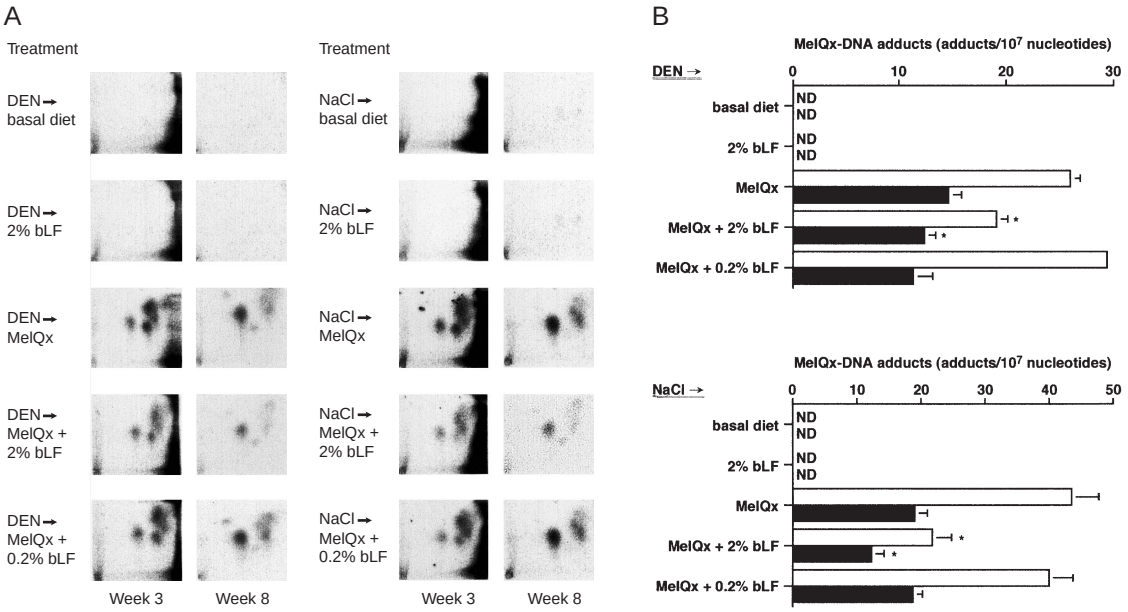

Fig. 5. Inhibitory effects of bLF on the MeIQx-DNA adduct formation in rat livers. (A) Autoradiograms of two-dimensional thin layer chromatographs of MeIQx-DNA adducts. Treatment patterns of MeIQx and/or bLF with (DEN→) or without (NaCl→) DEN initial treatment are illustrated on the left. Weeks 3 and 8 at the bottom indicate liver samples obtained at partial hepatectomy and sacrifice, respectively. (B) Change of MeIQx-DNA adduct levels in rats fed bLF. The top and bottom graphs illustrate MeIQx-DNA adduct levels (adducts/10^7 nucleotides), with (DEN→) or without (NaCl→) the initial DEN treatment, respectively. Data are mean±SD values (n=3). ND, not detected. ∗ $P<0.05$ compared with the MeIQx alone group. □, week 3; ■, week 8.

tion of 2% bLF ($P<0.05$) as compared with the group receiving MeIQx alone.

DISCUSSION

It is important to identify chemopreventive agents active against environmental carcinogens such as heterocyclic amines, since epidemiological surveys have shown that there may be a positive correlation between ingestion of contaminated cooked foods and risk of cancer.[8] In the present study, we demonstrated that bLF significantly reduced MeIQx-induced liver and colon carcinogenesis, the effects being much greater at a dose of 2% than at 0.2% (Tables II and III). Similar results were earlier obtained for reduction of azoxymethane-induced colon, 4-nitroquinoline 1-oxide-induced tongue and *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-induced bladder carcinogenesis,[3, 6, 7] indicating that bLF, particularly at a dose of 2%, is capable of exerting chemopreventive potential in many organs.

To clarify the underlying mechanisms, the influence of bLF on the levels of MeIQx-induced expression of CYP1A2 in rat livers was investigated since this enzyme catalyzes the *N*-oxidation of the amino group of MeIQx to form *N*-hydroxy-MeIQx.[17, 18] Levels of CYP1A2 protein induced by MeIQx were significantly decreased to almost normal levels by the concomitant administration of 2% bLF at weeks 3 and 8 in the liver, independently of initial DEN treatment (Fig. 3). Strong correlations were observed between levels of CYP1A2 protein and mRNA among groups receiving DEN or saline followed by basal diet, 2% bLF, MeIQx, MeIQx plus 2% bLF and MeIQx plus 0.2% bLF (DEN, r=0.89; saline, r=0.85). In contrast, levels of CYP2E1 and CYP3A2 mRNAs and protein, which were up-regulated by MeIQx, were not altered by concomitant administration of bLF. These results suggested that bLF may specifically down-regulate only CYP1A2 expression in the liver which was selectively up-regulated by MeIQx at the mRNA transcriptional level through undetermined mechanisms. On the other hand, MeIQx did not up-regulate CYP2E1 and CYP3A2 with bLF, thus exerting no influence on the expression of these CYPs.

Concomitant administration of 2% bLF significantly reduced MeIQx-DNA adduct formation at both weeks 3 and 8 in the liver in the presence or absence of initial DEN treatment, as shown in Fig. 5. Strong correlations were observed between levels of CYP1A2 protein and MeIQx-DNA adduct levels among groups receiving DEN or saline followed by MeIQx, MeIQx plus 2% bLF and MeIQx plus 0.2% bLF at week 3 (r=0.93) and week 8 (r=0.87). To

form MeIQx-DNA adducts, further metabolic activation of *N*-hydroxy-MeIQx through esterification reactions catalyzed by NAT or PST is required.[19, 20] In this experiment, however, no detectable changes were observed with regard to NAT or PST mRNAs. In addition, Davis *et al.* reported that only very low levels of activation of *N*-hydroxy-MeIQx were observed in rat hepatic cytosol mediated by NAT or PST.[20] Therefore, *N*-oxidation by CYP1A2 may play a more important role in MeIQx-DNA adduct formation than esterification by NATs or PSTs in rats.

The strong correlation observed between MeIQx-DNA adduct levels at week 3 and the numbers of MeIQx-induced GST-P$^+$ cell foci for groups receiving DEN or saline followed by MeIQx, MeIQx plus 2% bLF or MeIQx plus 0.2% bLF (DEN, r=0.86; saline, r=0.95) is in line with the dependence on activation potential. Similarly, a strong correlation was also observed between levels of CYP1A2 protein at week 8 and ACF for groups receiving DEN or saline followed by MeIQx, MeIQx plus 2% bLF or MeIQx plus 0.2% bLF (DEN, r=0.99; saline, r=0.78). The results suggest that down-regulation of CYP1A2 expression by bLF, followed by reduction of MeIQx-DNA adduct formation, may contribute to the inhibitory effects of bLF on MeIQx-induced liver and colon carcinogenesis. Therefore, bLF may be applicable for inhibition of carcinogenesis by other heterocyclic amines with similar activation processes by CYP1A2. However, as administration of 2% bLF alone slightly, but significantly, reduced DEN/PH-induced liver carcinogenesis (Table II), the observed up-regulation of immune reactions in the intestine with stimulation of cytotoxic T cells and induction of interleukin-18 in mice,[41] may also play a role. Further studies are required to clarify this point.

In conclusion, the present study demonstrated that bLF exerts chemopreventive effects on MeIQx-induced liver and colon carcinogenesis in the rat. The mechanisms of the observed inhibition may involve down-regulation of MeIQx-induced CYP1A2 expression, to the normal, untreated levels, subsequent decrease of metabolic activation and reduction of the MeIQx-DNA adduct formation. Although bLF does not exist in commercially available milk due to degeneration after ultra high temperature sterilization, it could have practical clinical application.

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