Biochemical and Biophysical Research Communications **287,** 301–304 (2001)
doi:10.1006/bbrc.2001.5581, available online at http://www.idealibrary.com on IDEAL®

Aryl Hydrocarbon Receptor-Mediated Suppression of Expression of the Low-Molecular-Weight Prekininogen Gene in Mice

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Received August 8, 2001

Differential mRNA display showed that a cDNA band disappeared after treatment of mice with 3-methylcholanthrene (MC). The cDNA encoded low-molecular-weight (LMW) prekininogen, known to be the precursor of a potent vasodilator, bradykinin. MC is generally known to bind to aryl hydrocarbon receptor (AhR) as an initial event to cause effects *in vivo*. In accordance with the results, Northern blot analysis for LMW prekininogen mRNA using total RNAs from wild-type and AhR-null mice indicated that the suppression of the mRNA expression by MC was seen in wild-type mice but not in AhR-null mice. The expression of LMW prekininogen mRNA was almost completely lost within 1 h after treatment of mice with MC, while a clear increase of CYP1A2 mRNA, as a positive control, was noted 4 h after the treatment. The plasma concentration of bradykinin released from LMW prekininogen was decreased by MC in wild-type mice, but not in AhR-null mice. Based on these results, we conclude that AhR inhibits bradykinin synthesis in mice via suppression of the expression of LMW prekininogen. Possible mechanism(s) responsible for hypertension caused by treatment of mice with MC is also discussed.

Exposure to halogenated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related polycyclic aromatic hydrocarbons is reported to cause various biological responses including hypertension, epithelial hyperplasia, tumor promotion and the induction of enzymes responsible for the metabolism of xenobiotics (1–7). In addition, teratogenesis such as cleft palate and hydronephrosis is also noted after treatment with TCDD (8). Despite these results, the molecular mechanism(s) responsible for the toxicities seen with TCDD has not yet been well understood.

Most of the biological responses to TCDD and related aromatic hydrocarbons are thought to be mediated by a cytosolic protein termed as AhR (9), which exists as a part of a complex that has a molecular mass of about 280 kDa (10). After the binding of TCDD with AhR, AhR dissociates from the above complex and translocates to the nucleus where it heterodimerizes with Arnt (11). The heterodimer AhR/Arnt complex binds to several copies of 5-bp sequence, named as XRE (5′-GCGTG-3′), located within the 5′-flanking region of the AhR target genes, such as *CYP1A1*, *CYP1A2*, *CYP1B1*, *NQO1*, *GST Ya*, and *UGT1A6* (12).

During the course of our study to search AhR target genes involved in the occurrence of toxicities seen with aromatic hydrocarbons, we found that the expression of LMW prekininogen mRNA was suppressed by MC. LMW prekininogen is known to be a kinin precursor protein that is synthesized in the liver. LMW prekininogen produces lysylbradykinin in plasma by cleavage with kallikrein. Lysylbradykinin is further digested with aminopeptidase to produce a potent vasodilator, bradykinin (13–14), which decreases blood pressure. In the present study, a possible mechanism(s) responsible for hypertension caused TCDD or polycyclic aromatic hydrocarbons seen in rats and humans is also discussed.

EXPERIMENTAL PROCEDURES

Materials. MC was purchased from Sigma Chemical (St. Louis, MO). Markit-bradykinin kit was from Dainippon Pharmaceutical

Abbreviations used: Ah, aryl hydrocarbon; AhR, Ah receptor; Arnt, AhR nuclear translocator; bdw, body weight; C/EBP, CAAT/enhancer binding protein; CYP, cytochrome P450; GST, glutathione *S*-transferase; HMW, high molecular weight; LMW, low molecular weight; MC, 3-methylchoranthrene; NQO_1, NAD(P)H: quinone oxidoreductase$_1$; RT-PCR, reverse transcriptase-polymerase reaction; SDS, sodium dodecyl sulfate; SSC, sodium chloride–sodium citrate solution; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; UGT, UDP-glucuronosyltransferase; XRE, xenobiotic-responsive element.

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0006-291X/01 $35.00

(Osaka, Japan). Other chemicals were of the highest grade commercially available.

Animal treatment. Adult male C57BL6/J mice (7 weeks old; Sankyo Experimental Animals, Tokyo, Japan) and adult male AhR-null mice (7 weeks old) (15) were treated with MC dissolved in corn oil at a dose of 80 mg/kg per bdw intraperitoneally for 2 days. Twenty four hours after the last administration of MC, mice were sacrificed. Livers were removed and were immediately used for following experiments.

Differential mRNA display. Total RNAs were prepared from the livers of mice by the guanidinium/cesium chloride method (16). RT-PCR-based differential mRNA display was performed by using fluorescence differential display kit (Takara, Kyoto, Japan) essentially according to the manufacturer's instructions (17) with minor modifications. Briefly, total RNA (0.5 μg) and two-base-anchored 5′-fluorescein-labeled oligo(dT) primer (5′-T_{13-16}AC-3′) were used for RT reaction. cDNA was synthesized by means of PCR using an arbitrary primer (5′-GATCATAGTCC-3′ (for clone A67) or 5′-GATCTGACAC-3′ (for clone B20). PCR products were separated on a 6% DNA sequencing gel and analyzed by autofluorography (FLA2000, Fuji Film, Tokyo, Japan). Differentially expressed cDNAs were recovered from a gel, and then amplified using the same PCR primers. Amplified cDNAs were subcloned into pGEM-T Easy Vector (Promega, Madison, WI) and transformed into competent *E. coli* cells. Plasmids which contained inserts were subjected to sequencing. Sequencing was performed with a bigdye primer cycle sequencing kit (PE Applied Biosystems, Foster City, CA) on an ABI 310 automated sequencer (PE Applied Biosystems, Foster City, CA). The sequence similar to the isolated cDNA was sought by using BLAST 2.1 program.

Northern blot analysis. Total RNAs were prepared from the livers of wild-type mice or wild-type mice treated with MC as previously reported (16). A fragment of LMW prekininogen cDNA (18) (from nucleotides 996 to 1317 relative to initiation codon) was used as a probe. A part of CYP1A2 cDNA (19) (from nucleotides 1525 to 1653) was also used as a probe. Total RNA (20 μg) was electrophoresed in a 0.8% agarose gel containing 18% formaldehyde and was transferred to a nylon membrane (Nytran N, Schleicher & Schuell, Dassel, Germany). The membrane was hybridized with ^{32}P-labeled cDNA by using a DNA labeling system (Nippon Gene, Tokyo, Japan). Hybridization was carried out as described previously (20). The membrane was washed twice with 1× SSC containing 0.2% SDS at 50°C for 30 min.

Blood collection. Under ether anesthesia, blood was collected from the inferior vena cava of mice to plastic tubes containing 3.8% sodium citrate. Plasma was prepared by centrifugation at 1500*g* for 15 min at 25°C (21).

Determination of LMW prekininogen level in plasma. The plasma level of LMW prekininogen was determined essentially according to the method of Majima *et al.* (21) with minor modifications. Briefly, plasma was incubated with a glass powder (Wako, Tokyo, Japan) at 37°C for 1 h. The plasma was adjusted to pH 2.0 and was incubated with trypsin (Wako, Tokyo, Japan) (0.1 mg/ml plasma), followed by neutralization with sodium hydroxide. The amounts of kinin which was released from LMW prekininogen by cleavage with trypsin were determined by a bradykinin enzyme immunoassay (20). Protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA) (22).

RESULTS AND DISCUSSION

Using a differential mRNA display technique to detect molecular alteration caused by treatment of mice with MC, we found cDNA bands disappeared (clone A67) (Fig. 1A), or were induced (clone B20) (Fig. 1B) in the livers of wild-type mice treated with MC. These

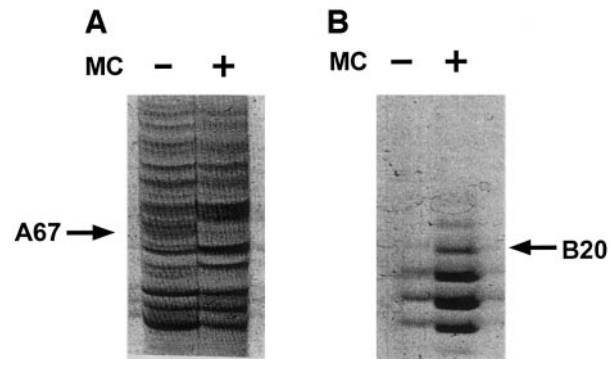

FIG. 1. Identification of differentially expressed cDNAs in the liver of wild-type mice treated with MC. Total RNA (0.5 μg) from the livers of mice treated with MC was converted to cDNA. Differential mRNA display was performed as described under Experimental Procedures. Samples were run on a 6% polyacrilamide/8 M urea gel. (A) Clone A67 was identified as LMW prekininogen mRNA. (B) Clone B20 was identified as CYP1A2 mRNA.

cDNA bands were excised from a gel and were subjected to sequencing. Sequence analysis followed by a homology search with BLAST 2.1 program revealed that the sequences of clone A67 and clone B20 were completely identical to those of LMW prekininogen and CYP1A2 cDNAs, respectively (data not shown). LMW prekininogen is a kinin precursor protein that is synthesized in the liver (13). Bradykinin is known to be a potent vasodilator (13). CYP1A2 is induced by aromatic hydrocarbons including MC and is responsible for the bioactivation of carcinogenic compounds such as heterocyclic amines (23).

To further confirm that the expression of LMW prekininogen mRNA is suppressed by MC, Northern blot analysis was performed using total RNAs from wild-type mice or wild-type mice treated with MC (Fig. 2). In agreement with the results shown in Fig. 1, the expression of LMW prekininogen mRNA was clearly decreased by treatment of mice with MC. In addition, to examine whether or not the decreased expression of LMW prekininogen by MC was mediated by AhR, total RNAs from AhR-null mice or AhR-null mice treated with MC were also analyzed by Northern blot analysis. As expected, the expression of LMW prekininogen mRNA in AhR-null mice was unaffected by the treatment of the mice with MC. The inducibility of CYP1A2 expression by MC was monitored as a positive control (Fig. 2). The MC-induced expression of CYP1A2 was seen in wild-type mice but not in AhR-null mice. Thus, it appeared that the suppression of LMW prekininogen mRNA expression by MC as well as the induction of CYP1A2 occurred dependent on AhR.

Northern blot analysis of total RNAs showed that the expression of LMW prekininogen mRNA was lost 1 h after treatment with MC (Fig. 3). The expression of LMW prekininogen mRNA apparently tended to restore 4 h after treatment with MC. On the other hand,

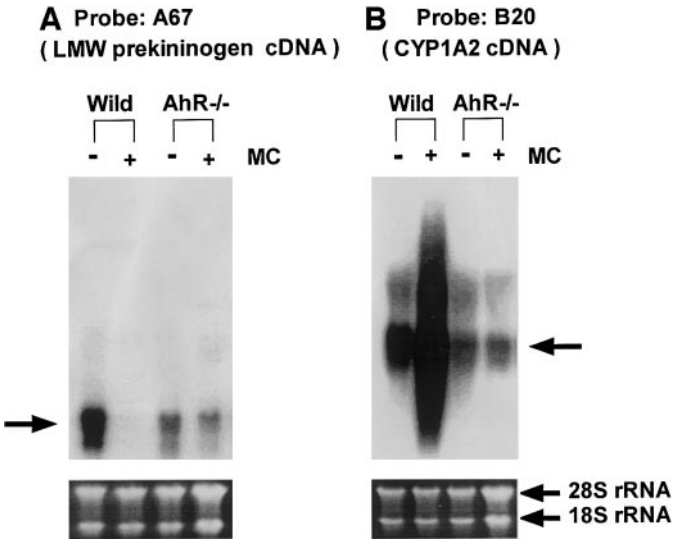

FIG. 2. Influence of MC treatment on the expression of LMW prekininogen and CYP1A2 mRNAs. A portion (20 μg) of total RNA was allocated to Northern blot analysis as described under Experimental Procedures. (A) The expression of LMW prekininogen mRNA is indicated by an arrow. (B) CYP1A2 mRNA expression is indicated as control. A 28S and a 18S ribosomal RNA were stained with ethidium bromide to verify the amounts of RNA loaded (lower panel).

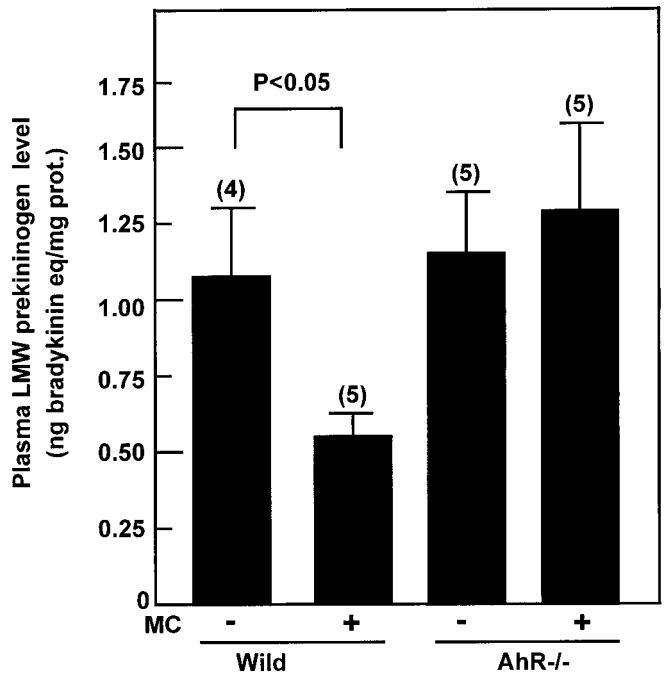

FIG. 4. Effect of treatment of wild-type and AhR-null mice with MC on the plasma level of bradykinin. The measurement of bradykinin concentration in plasma was performed as described under Experimental Procedures. Plasma was collected 24 h after the last administration. Values are means ± SD of at least five mice.

the expression of CYP1A2 mRNA was clearly induced by MC 4 h after treatment with MC. Thus, it seemed that the responsiveness of the LMW prekininogen gene to MC occurred faster than that of the *CYP1A2* gene.

LMW prekininogen produce bradykinin (13). Thus, the functional consequence of the down-regulation of LMW prekininogen expression by MC was examined. It was found that the plasma level of bradykinin was reduced by MC in wild-type mice, but not in AhR-null mice (Fig. 4). These results confirmed that the rapid loss of the expression of the LMW prekininogen mRNA caused the reduced plasma level of bradykinin in the liver by a mechanism(s) dependent on AhR.

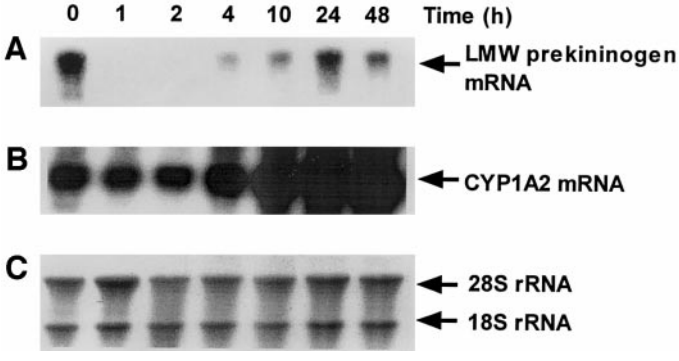

FIG. 3. Time course of the suppression of LMW prekininogen expression by MC. Total RNAs were prepared from the livers of mice 1, 2, 4, 10, 24, or 48 h after treatment with MC. A portion (20 μg) of total RNA was allocated to Northern blot analysis as described under Experimental Procedures. (A) The expression of LMW prekininogen mRNA as indicated by an arrow. (B) CYP1A2 mRNA expression as a positive control. (C) The same blots were stained with ethidium bromide to verify the amounts of RNA loaded.

Prekininogen gene encodes HMW and LMW prekininogens. These prekininogens were reportedly produced by alternative splicing (14). In the present study, we found that the expression of HMW prekininogen was also decreased by the treatment of mice with MC, indicating that the down-regulation of LMW prekininogen expression by MC was not caused by the abnormal alternative splicing. An AhR/Arnt complex binds to several copies of XRE (5′-GCGTG-3′), located within the 5′-flanking region of the AhR target genes (12). Although we could not find any XREs within the promoter regions up to −427 of the rat K-kininogen gene (24) and up to −2 kb of the human kininogen gene (GenBank accession number NT005962), the expression of LMW prekininogen was decreased by MC. Detailed mechanism(s) accounting for the discrepancy are not known at present. It has been reported that the expression of rat K-kininogen is regulated by C/EBP family (24–25). The C/EBP binding site is present within the K-kininogen promoter region. Interestingly, the C/EBP binding site is also conserved in the 5′-upstream region (−181 to −190) of the human kininogen gene. The expression of C/EBPα is known to be suppressed by TCDD (26). Thus, the down-regulation of the expression of LMW prekininogen by MC may be caused by the suppression of C/EBPα expression.

Exposure to aromatic hydrocarbons causes hypertension (7). In the present study, we found that the treatment of mice with MC reduced the plasma level of bradykinin that functions as a potent vasodilator. Thus, the hypertension caused by an exposure to aromatic hydrocarbons can be accounted for, at least in part, by the AhR-mediated suppression of LMW prekininogen and bradykinin levels.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan, a grant (No. 99-2) from the Organization for Pharmaceutical Safety and Research (OPSR), and the Core Research for Evolutional Science and Technology.

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