The *Ah* domain of the mouse

Induction of proteins by the carcinogen 3-methylcholanthrene

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The effect of the carcinogen 3-methylcholanthrene (MCA) on protein accumulation in mouse tissues was examined. Administration of the hydrocarbon (250 mg/kg, intraperitoneally) to high-responder C57BL/6 (B6) mice resulted in the induction of five proteins in liver tissue. Quantitative analysis revealed that maximal induction of all five proteins occurred 2 days after MCA administration, with liver protein concentrations returning close to control values by 7 days after the treatment. No consistent effects on the concentrations of other liver proteins were seen. Cell-fractionation studies demonstrate that these proteins appear to be non-microsomal in origin. The induction of the five proteins was tissue-specific, since MCA had no effect on protein accumulation in the B6-mouse kidney, spleen or heart. In addition, their induction appeared to be correlated with the *Ah* locus, since MCA administration had no effect on the concentrations of the five proteins in the liver of the low-responder DBA/2 (D2) mouse strain. Comparing the extent and time course of this induction with that of previously characterized MCA-induced enzymes, we suggest that these five proteins may represent a new, previously unidentified, set of MCA-induced proteins.

INTRODUCTION

Organisms are constantly exposed to a variety of drugs, chemical carcinogens and other pollutants that exist in the environment. Many of these xenobiotics are hydrophobic and would remain in the organism indefinitely, if not for the action of Phase I and Phase II enzyme systems that act to metabolize and thus detoxify these chemical pollutants [1]. The Phase I enzymes, of which the cytochromes *P*-450 comprise the largest class, introduce polar groups into the substrate. This provides a hydrophilic site by which Phase II enzymes, such as UDP-glucuronosyltransferase, glutathione *S*-transferase and menadione oxidoreductase, can act upon the compound, ultimately leading to the excretion of the substance from the cell or organism. In addition to the production of innocuous compounds, the metabolism of some substrates, i.e. aryl hydrocarbons, by the cytochromes *P*-450 can also result in the production of oxygenated compounds that are chemically more active than the substances from which they were derived. These metabolites are capable of producing damage to important cellular macromolecules, and have thus been correlated with increases in cancer, drug toxicity and developmental mutations [2].

In mouse systems the induction of cytochromes *P*₁-450 and *P*₃-450 can be effected by polycyclic aromatic compounds {e.g. 3-methylcholanthrene (MCA), benzo[*a*]pyrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin} [3]. This process appears to be mediated by a cytosolic receptor encoded by the *Ah* (aryl hydrocarbon) locus [1]. By genetic analysis, the *Ah* locus appears to be complex, consisting of at least two genes with six different alleles, but in crosses between some mouse strains [e.g. C57BL/6 (B6) and DBA/2 (D2)] the locus behaves as a single Mendelian trait [4]. B6 mice are high responders

and possess high-affinity aryl hydrocarbon receptors, whereas D2 mice are low responders and have low amounts of detectable receptor and/or low-affinity receptor-binding sites [5]. The mechanism by which the aryl hydrocarbon receptor mediates expression of the induced genes is still unclear, but appears to be similar to that seen in hormonally inducible systems [6]. The aryl hydrocarbon receptor with its bound inducer appears to translocate to the nucleus, where it interacts with chromatin [5,6].

Treatment of aryl hydrocarbon-responsive animals with MCA induces not only the cytochromes *P*-450 but also several other enzyme activities. Among these are microsomal UDP-glucuronosyltransferase, cytosolic menadione oxidoreductase, cytosolic ornithine decarboxylase and glutathione *S*-transferase [7–11].

In the present investigation we have studied the regulation of protein induction by MCA in responder (B6) and non-responder (D2) mouse strains, in several tissues. We have found that a number of proteins are induced in liver to high concentrations following the administration of the hydrocarbon in B6 but not in D2 mice. Comparing the extent, time course and tissue-specificity of these proteins with that of previously characterized enzymes, we suggest that these proteins may represent a previously uncharacterized set of MCA-induced proteins.

MATERIALS AND METHODS

Isolation and separation of proteins

The 6–8-week-old male C57BL/6 and DBA/2 mice were treated with MCA (250 mg/kg, intraperitoneally, dissolved in corn oil) or with corn oil alone. Control and treated mice were housed in separate cages, and fed and

Abbreviations used: MCA, 3-methylcholanthrene; PAGE, polyacrylamide-gel electrophoresis; IEF, isoelectric focusing; NEF, non-equilibrium focusing.

watered *ad libitum*. After various periods of time, animals were killed and organs were immediately removed, washed free of blood and homogenized, with a tight-fitting Teflon/glass homogenizer, at 4 °C in 0.1 M-KCl/2 mM-MgCl₂/0.5 mM-dithiothreitol/0.1 M-Tris/HCl buffer, pH 7.0, containing the proteinase inhibitors leupeptin and pepstatin (each at 5 µg/ml). DNAase I (100 µg/ml) and RNAase (20 µg/ml) were added, and homogenates were incubated at 37 °C for 20 min. Protein was concentrated by means of an acetone precipitation and then solubilized in lysis buffer [9.5 M-urea/2 % (w/v) Nonidet P-40/2 % (v/v) Ampholines (consisting of 1.6 % pH range 5–7 and 0.4 % pH range 3.5–10) and 5 % (v/v) 2-mercaptoethanol] [12].

Proteins were fractionated on two-dimensional gels, according to the methods of O'Farrell [12,13] except that the first-dimension isoelectric-focusing gel contained Ampholines in the pH range 3.5–10 (2 %) and that the second-dimension separating gel consisted of 10 % polyacrylamide. Equal amounts of protein were loaded on to each gel as determined by the Bradford assay [14].

Quantification of proteins resolved on two-dimensional gels

Protein concentrations were quantified by scanning the two-dimensional gels with the use of a computerized densitometer, the Quantimet 970 Image Analysis System. The background of each gel was subtracted from the spot density.

Preparation of microsomal fractions

Liver tissue was homogenized with a tight-fitting Teflon/glass homogenizer at 4 °C in 0.15 M-KCl/0.25 M-potassium phosphate buffer, pH 7.3, containing the proteinase inhibitors leupeptin and pepstatin (each at 5 µg/ml). The homogenate was centrifuged at 12000 *g* for 20 min at 4 °C. The resulting supernatant was centrifuged at 100000 *g* for 1 h at 4 °C. The pellet was resuspended in lysis buffer. One-dimensional SDS/PAGE was performed according to the methods of Laemmli & King [15]. Equal amounts of protein were loaded on to each lane, as determined by the Bradford assay [14].

Materials

Animals were obtained from Jackson Laboratories (Bar Harbor, ME, U.S.A.). RNAase was obtained from Calbiochem-Behring Corp. (La Jolla, CA, U.S.A.). Leupeptin, pepstatin, MCA and DNAase I were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All chemicals for electrophoresis were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

RESULTS

Effect of MCA on protein accumulation in C57BL/6 mouse liver

To determine the scope of gene regulation by MCA, an analysis of steady-state protein accumulation was performed in mice treated with the hydrocarbon. Briefly, male C57BL/6 mice were injected intraperitoneally with MCA, the animals were killed and protein from various tissues was solubilized as described in the Materials and methods section. Proteins were resolved by two-dimensional gel electrophoresis, utilizing IEF in the first

Fig. 1. Effect of MCA on protein accumulation in B6-mouse liver: IEF gel analysis

Two-dimensional PAGE of total liver lysate from control (a) and MCA-treated (b) C57BL/6 mice was performed as described in the Materials and methods section. Proteins were stained with Coomassie Blue. I1, induced protein I1; I2, induced protein I2; I3, induced protein I3; N1, non-induced protein N1.

dimension. Ampholines in the pH range 3.5–10 were used in order to resolve the greatest possible number of acidic proteins. We chose Coomassie Blue staining as our detection system since it is quantitative, moderately sensitive and eliminates background problems due to contaminating nucleic acids. By this method we were able to resolve more than 300 abundant and moderately abundant proteins.

At 2 days after the administration of MCA there was increased accumulation of three proteins in the liver (Fig. 1). Protein I1 has a molecular mass of 158 500 Da and a pI of 6.05, protein I2 has a molecular mass of 83 100 Da and a pI of 5.25, and protein I3 has a molecular mass of 80 500 Da and pI of 5.35. This effect appeared to be selective, since neither differences in total protein content nor differences in amounts of several individual proteins could be detected between control and MCA-treated tissues (i.e. protein N1). Irreproducible differences that were occasionally observed in the gel patterns and were not correlated with the experimental conditions were rejected. These may be due to undetected differences in the nutritional or health status of the animals or small effects of the xenobiotic on the accumulation of these proteins.

In order to extend our analysis, we also separated homogenate proteins on a different two-dimensional gel system, using NEF in the first dimension. This system improves resolution of the basic proteins that are excluded or poorly resolved by two-dimensional gel

Fig. 2. Effect of MCA on protein accumulation in B6-mouse liver: NEF gel analysis

Two-dimensional non-equilibrium pH-gradient electrophoresis of total liver lysate from control (a) and MCA-treated (b) C57BL/6 mice was performed as described in the Materials and methods section. Proteins were stained with Coomassie Blue. I4, induced protein I4; I5, induced protein I5; N2, non-induced protein N2.

electrophoresis with IEF in the first dimension [13]. By using this system we detected two additional proteins that were induced 2 days after MCA administration (Fig. 2). Protein I4 has a molecular mass of 21 200 Da and a pI of 6.85, and protein I5 has the same molecular mass and a pI of 6.68. It is unlikely that these proteins represent modified forms of the same peptide, since there is no obvious loss of protein of similar size in treated compared with untreated samples.

The quantitative extent of induction of proteins I1–I5 was determined by using a computerized densitometric scanning system. At 2 days after MCA treatment proteins I1, I2 and I3 were induced 29-fold, 21-fold and 22-fold respectively, and proteins I4 and I5 were induced 7.5-fold and 6.0-fold respectively, relative to control proteins N1 and N2 (Fig. 3). In all cases the extent of induction declined by 4 days, the concentrations returning close to control values by 7 days after administration of the hydrocarbon.

Characterization of induced proteins

We considered the possibility that proteins I4 and I5 were isoforms of glutathione *S*-transferase. These enzymes comprise a family of abundant drug-metabolizing proteins, with molecular masses in the range

Fig. 3. Quantification of protein induction by MCA in C57BL/6-mouse liver

Two-dimensional gels were scanned with the use of a computerized densitometric scanning system as described in the Materials and methods section. Each point represents the average for two determinations, which agreed within 5%. Similar results were obtained in at least three independent experiments. ●—●, Protein I1; ○—○, protein I2; △—△, protein I3; ●—●, protein I4; ○—○, protein I5; ■—■, protein N1; □—□, protein N2.

26000–29000 Da [16]. In addition, several members of this family are induced 5–8-fold by MCA treatment [11,17]. In order to rule out the possibility that proteins I4 and I5 were members of this family, the glutathione *S*-transferases were first purified by affinity chromatography from MCA-treated and control C57BL/6 mouse livers [18]. The transferases were then resolved on one- and two-dimensional gels. Two dimensional PAGE resolved seven isoforms with pI values ranging from 8.0 to 8.75 and molecular masses ranging from 25000 to 30000 Da. None of these proteins co-electrophoresed with protein I4 or protein I5 (results not shown), demonstrating that these two MCA-induced proteins were not members of the glutathione *S*-transferase family. No evidence of MCA induction of any of the resolved glutathione *S*-transferase proteins could be seen. This is likely to be due to the fact that the MCA-induced isoforms have pI values substantially higher than 8.9, which is the limit of resolution of our gel system. On one-dimensional SDS/PAGE multiple forms co-electrophoresed, which obscured the MCA-inducible forms.

We analysed total cellular protein for all of the studies described here. The gel systems were chosen in order to provide high resolution of most cellular proteins. However, it has been reported that many microsomal proteins, especially the cytochromes *P*-450, cannot be resolved by the systems that we have utilized owing to their extreme insolubility in the urea buffer system [19]. It remained possible that cytochromes *P*-450 that were partially soluble in the first-dimension urea buffer could aggregate and migrate anomalously in the gels. To test

Fig. 4. Effect of MCA on the accumulation of liver microsomal proteins

One-dimensional SDS/PAGE of microsomal proteins prepared from livers of control and 2-days-previously MCA-treated C57BL/6 mice was performed as described in the Materials and methods section. The arrow indicates the position of cytochromes *P*-450, as described in the text.

this possibility, we purified the microsomal components from homogenates of control and MCA-treated livers and subjected the proteins to one- and two-dimensional electrophoresis. One-dimensional SDS/PAGE of the microsomal fraction revealed that, of the large number of proteins resolved on the gel, only one was clearly induced by MCA (Fig. 4, see arrow). This band probably contains both cytochromes *P*₁-450 and *P*₃-450, which have very similar molecular masses [20].

When two-dimensional analysis was performed for the microsomal proteins, few proteins were resolved. Of these few, no differences could be detected between control and MCA-treated samples (results not shown). No evidence could be seen of induced cytochromes *P*-450 in the microsomal fraction, although one-dimensional analysis clearly demonstrated their presence. In addition, none of the microsomal proteins co-electrophoresed with the induced proteins resolved with either two-dimensional gel technique (results not shown). This suggests that the induced polypeptides seen in total cell extracts are cytosolic rather than microsomal in origin, and in addition that proteins I1–I5 are not aggregates of the cytochromes *P*-450 or of other microsomal proteins.

Tissue-specific effect of MCA on protein accumulation in the C57BL/6 mouse

The induction of the cytochromes *P*-450 by MCA in the mouse has been shown to exhibit tissue-specificity. Both cytochromes *P*₁-450 and *P*₃-450 are induced by the drug in the liver. However, cytochrome *P*₁-450 and not cytochrome *P*₃-450 is induced by MCA in the kidney, lung, intestine and spleen [21–23]. Aryl hydrocarbon hydroxylase activity, associated with cytochrome *P*₁-450,

Fig. 5. Effect of MCA on protein accumulation in B6-mouse kidney

Two-dimensional PAGE of total kidney lysate from control (a) and MCA-treated (b) C57BL/6 mice was performed as described in the legend to Fig. 1.

has been shown to be inducible in nearly every tissue examined [2]. To determine if the induction of proteins I1–I5 demonstrated tissue-specificity, we performed two-dimensional gel electrophoresis of proteins solubilized from C57BL/6-mouse kidney, spleen and heart 2 days after MCA administration, exactly as was done for liver. No consistent differences were detected in the accumulation of proteins between control and drug-treated tissues (see Fig. 5 for kidney; other results not shown).

Because of tissue-specific protein expression among the tissues examined, it was virtually impossible to locate proteins I1–I5 in the non-hepatic tissue samples. However, since no differences were seen, it can be inferred that these proteins are not induced in the three tissues examined. It is also unlikely that the failure to induce proteins in these tissues is simply a function of low concentration of MCA exposure to these tissues. Intraperitoneal injection of the relatively high doses of MCA used in this study is sufficient for induction of cytochrome *P*₁-450 in most tissues examined [21–23]. In addition, Fig. 4 clearly demonstrates that the dose of MCA administered is sufficient to result in cytochrome *P*-450 induction in liver.

Effect of MCA on protein accumulation in the DBA/2 mouse

Induction of the cytochromes *P*-450 in mouse systems appears to be mediated by a cytosolic receptor encoded

Fig. 6. Effect of MCA on protein accumulation in D2-mouse liver: IEF gel analysis

Two-dimensional PAGE of total liver lysate from control (a) and MCA-treated (b) DBA/2 mice was performed as described in the legend to Fig. 1.

Fig. 7. Effect of MCA on protein accumulation in D2-mouse liver: NEF gel analysis

Two-dimensional non-equilibrium pH-gradient electrophoresis of total liver lysate of control (a) and MCA-treated (b) DBA/2 mice was performed as described in the legend to Fig. 2.

Fig. 8. Comparison of protein induction by MCA in B6-mouse and D2-mouse liver

Two-dimensional gels were scanned as described in the legend to Fig. 3. B6-mouse data were derived from Fig. 3. Data represent the average for two determinations, which agreed within 5%. Similar results were obtained in at least three independent experiments. □, Induction in B6-mouse liver; ■, induction in D2-mouse liver.

at the *Ah* locus [1]. B6 mice, which were used in all the experiments described above, are high responders and possess high-affinity aryl hydrocarbon receptors [5]. Another mouse strain, D2, is a low responder and has low amounts of detectable receptor and/or low-affinity receptor binding sites [5]. In D2 mice, there is no induction of cytochrome *P*₁-450 or cytochrome *P*₃-450 by MCA [24]. In order to determine if the induction of proteins I1–I5 could be correlated with the *Ah* locus, we repeated the above experiments with D2 mice, using the same high dosage as that given to B6 animals.

At 2 days after MCA administration, there were no detectable differences in the accumulation of proteins I1–I5 in hydrocarbon-treated animals, as compared with controls (Figs. 6 and 7). The results of densitometric scanning of these proteins are shown in Fig. 8. There is virtually no induction of these proteins in D2 animals treated with the hydrocarbon, as compared with the large induction of these same proteins by MCA in the B6 mice. Although it is possible that failure to induce proteins I1–I5 is due to strain-specific differences that are unrelated to the *Ah* locus, these findings are consistent with the hypothesis that the induction of these proteins by MCA in the B6 mouse is correlated with the *Ah* locus.

DISCUSSION

We have shown that the administration of MCA to B6 mice resulted in a 6–29-fold induction of five specific proteins in the liver. By combining IEF and NEF techniques we estimate that we could examine around 600 proteins or roughly 5–10% of the total number of liver proteins. Since we could resolve five inducible proteins and know of at least two more (i.e. cytochromes *P*₁-450 and *P*₃-450), MCA appears to induce about 1% of identifiable moderately abundant proteins in liver.

The accumulation of induced proteins was maximal at approx. 2 days after MCA treatment. All of the proteins

began to decrease in abundance by 4 days and returned to control concentrations by 7 days after the treatment. The rates of decay of the individual proteins are clearly different from one another and most probably reflect differing protein stability after synthesis. The similarity of the time course of induction may indicate that these five proteins are regulated co-ordinately.

The increased accumulation of these five proteins induced by MCA was apparently tissue-specific, since this induction seemed to occur only in the liver. The five detected proteins were not inducible in the kidney, the heart or the spleen of B6 mice. This was probably not the result of lack of exposure of these tissues to the carcinogen, but more probably reflected an inability of these genes to respond to MCA induction in a non-hepatic environment. Previous work by others [2] has demonstrated that intraperitoneal administration of MCA, similar to the method used here, resulted in the induction of aryl hydrocarbon hydroxylase activity in essentially every tissue examined. This activity has been shown to be predominantly mediated by cytochrome P_1 -450, an observation that implies that these tissues are exposed to sufficiently high doses of MCA to achieve an induction response. Interestingly, the induction of cytochrome- P_3 -450 displays a tissue-specific distribution similar to that of the five induced proteins. For example, administration of MCA resulted in the induction of cytochrome P_3 -450 in B6-mouse liver, but not in B6-mouse kidney [16–18]. The similarity of tissue-specific inducibility of the five proteins and cytochrome P_3 -450 suggests that the induction of these proteins may be controlled by the same factors.

The induction of cytochromes P -450 by MCA is mediated by the aryl hydrocarbon receptor in the mouse. Although the mechanism of induction of the five identified proteins is unknown, our data are consistent with the hypothesis that a functional aryl hydrocarbon receptor is required for their induction. Thus induction of the five proteins was seen in B6 mice but not in D2 mice, which lack high-affinity aryl hydrocarbon receptors. This hypothesis can be proven either by demonstrating co-segregation of the gene for inducibility of the five proteins with the *Ah* gene among members of the segregative back-cross population (D2 $\times$ B6) F1 $\times$ D2, or by studying the response in strains of mice congenic with respect to the *Ah* locus. In this regard it may be noted that strain-specific differences are seen in the basal concentration of one of the induced proteins. Protein I2 was expressed at relatively high concentrations in both MCA-induced and control D2 mice, whereas B6 mice express high concentrations of protein I2 only after MCA treatment. A basis for this difference cannot be explained by the available data.

A number of enzyme activities have been shown to be increased in response to MCA treatment. Although it is possible that proteins I1–I5 correspond to some of these enzymes, we consider that it is unlikely. As noted above, the cytochrome P -450 proteins, although induced by MCA administration, were not resolved by the two-dimensional gel system used. The molecular masses of menadione oxidoreductase (31 000 Da) [10], UDP-glucuronosyltransferase(s) (60 000 Da) [25] and ornithine decarboxylase (subunit molecular mass 53 000 Da) [26] do not correspond to the molecular masses of any of the induced proteins observed here. In addition, menadione oxidoreductase and UDP-glucuronosyltransferase are

induced maximally 2–3-fold at 5 days after MCA induction, and ornithine decarboxylase is only transiently induced by the hydrocarbon [7–9]. This is strikingly different from the extent and time course of induction observed for proteins I1–I5. The molecular masses of proteins I4 and I5 are similar to that of the glutathione S -transferase(s) (25 000–29 000 Da) [16]. However, we have demonstrated that proteins I4 and I5 are not members of the glutathione S -transferase family. We have used affinity chromatography to purify all proteins that bind to GSH [18], then resolved the highly purified proteins on the same two-dimensional gel systems that resolved proteins I4 and I5. No GSH-binding species migrated in the region of the two induced proteins. This rules out the possibility that these proteins are members of the glutathione S -transferase family. In addition, examination of microsomal fractions from MCA-treated and control livers has demonstrated that proteins I1–I5 are not aggregates of the cytochromes P -450 or of other microsomal proteins. Our failure to locate these other known MCA-induced enzymes on our gels is almost surely due to their relatively low abundance after induction and the relative insensitivity of our detection system. Therefore proteins I1–I5 might represent a previously unappreciated moderately abundant class of proteins that appear to be induced by MCA and whose regulation is mediated by the aryl hydrocarbon receptor.

Until molecular hybridization probes are available, it is not possible to determine definitively the level of regulation of accumulation for the five proteins. However, if their accumulation is in fact regulated by the aryl hydrocarbon receptor, it is likely to be transcriptional. It will be of considerable interest to compare cis-acting elements upstream of this set of five putative aryl hydrocarbon-responsive proteins with those already described for the cytochrome P_1 -450 gene [27–30].

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