COMPARISON OF THE FORMATION OF N-ALKYLPROTOPORPHYRIN IX AFTER INTERACTION OF PORPHYRINOGENIC XENOBIOTICS WITH SINGLE cDNA-EXPRESSED HUMAN P450 ENZYMES IN MICROSOmes PREPARED FROM BACULOVIRUS-INFECTED INSECT CELLS AND HUMAN LYMPHOBLASTOID CELL LINES

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ABSTRACT:

In a previous study using microsomes from human lymphoblastoid cell lines (HLCL) containing single cDNA-expressed human cytochrome P450 (P450) enzymes, human P450 enzymes were identified that are susceptible to mechanism-based inactivation by the porphyrinogenic xenobiotics, 3-[(arythio)ethyl]sydnone (TTMS), 3,5-diethiocarboxybenzyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine (4-ethylDDC) and allylisopropylacetamide (AIA). In this study, we tested the hypothesis that N-alkylprotoporphyrin IX (N-alkylPP) formation following interaction of porphyrinogenic xenobiotics with single cDNA-expressed human P450 enzymes in microsomes from HLCL would occur only with P450 enzymes that had undergone mechanism-based inactivation. In a previous study, when 4-ethylDDC and NADPH interacted with human liver microsomes possessing elevated levels of CYP1A2 and 2C9, N-ethylprotoporphyrin IX (N-ethylPP) was not formed despite the fact that it was formed in microsomes from baculovirus-infected insect cell lines (BIICL) containing either CYP1A2 or 2C9. In this study, we tested the hypothesis that 4-ethylDDC underwent biotransformation by CYP3A4 present in human liver microsomes, diverting the xenobiotic from CYP1A2 and 2C9. Fluorometry was used to measure N-alkylPP formation following interaction of porphyrinogenic xenobiotics and NADPH with cDNA-expressed human P450 enzymes in microsomes from HLCL or BIICL. With TTMS and 4-ethylDDC but not with AIA, N-alkylPP formation was observed only with human P450 enzymes CYP2D6, 1A2, 3A4, or 2C9 in microsomes from HLCL, which had undergone mechanism-based inactivation. Microsomes from BIICL containing CYP3A4 were added to a mixture of NADPH, 4-ethylDDC, and microsomes from BIICL containing CYP1A2 and 2C9. The addition of CYP3A4 to CYP1A2 and 2C9 did not decrease N-ethylPP formation, providing no support for the hypothesis.
targeted for mechanism-based inactivation also elicited N-alkylPP formation. Since the studies of McNamee et al. (1997) were performed in HLCL, microsomes from baculovirus-infected insect cell lines (BIICL) possessing single cDNA-expressed P450 enzymes (Supersomes) became available. In view of the fact that these microsomes contained considerably higher amounts of P450 than the microsomes from the lymphoblastoid cells, we elected to use Supersomes to enhance the potential for formation of N-alkylPP from the heme moiety of P450. When Supersomes (containing CYP1A2, 2C9, 2D6, or 3A4) were incubated with TTMS, 4-ethylDDC, and AIA, plus NADPH, N-alkylPP formation was quantitated using the sensitive technique of fluorometry (Lavigne et al., 2002). Some of the results obtained were unexpected. Thus, while mechanism-based inactivation of CYP2D6, 2C9, and 3A4 was not observed after interaction of AIA or 3A4 with microsomes prepared from HLCL (McNamee et al., 1997), considerable amounts of N-ethylPP were formed in HLCL microsomes from baculovirus-infected insect cell lines (BIICL) (Correia et al., 1987), and this unexpected result was explained as follows: although human liver microsomes (HG56) contained elevated levels of CYP1A2 and 2C9, CYP3A4 also constitutes 12.5% of the total P450. Correia et al. (1987) have shown that 4-ethylIDDC and AIA of single cDNA-expressed human P450 enzymes in microsomes from HLCL would occur only with P450 enzymes that had undergone mechanism-based inactivation.

In a previous study, 4-ethylIDDC and NADPH were allowed to interact with human liver microsomes possessing elevated levels of CYP1A2 and 2C9 (P450-NADPH-P450 reductase, 1:0.34; BD Gentest Donor HG56). It was anticipated that N-ethylPP would be formed since N-ethylPP formation was observed with Supersomes containing either CYP1A2 (ratio of P450:NADPH-P450 reductase, 1:10.4) or 2C9 (ratio of P450:NADPH-P450 reductase, 1:0.7) after 4-ethylIDDC treatment. However, following incubation less than 0.04 nmol of N-ethylPP was formed where 0.04 nmol is the lower limit of detection (LLD) (Lavigne et al., 2002), and this unexpected result was explained as follows: although human liver microsomes (HG56) contained elevated levels of CYP1A2 and 2C9, CYP3A4 also constitutes 12.5% of the total P450. Correia et al. (1987) have shown that 4-ethylIDDC may have undergone biotransformation by CYP3A4 diverting the xenobiotic from CYP1A2 and 2C9. The second objective of this study was to test this hypothesis by comparing N-ethylPP formation in a mixture containing NADPH-P450 reductase and human cDNA-expressed CYP1A2 and 2C9 with N-ethylPP formation in a mixture of NADPH-P450 reductase and human cDNA-expressed CYP1A2, 2C9, and 3A4.

Materials and Methods

Source of Chemicals. TTMS and 4-ethylIDDC were obtained from Color Your Enzyme (Bath, Ontario, Canada). AIA was obtained as a gift from F. Hoffmann-La Roche (Vaudreuil, Quebec, Canada). Solvents were purchased from WVR Canada (Mississauga, Ontario, Canada). NADPH was purchased from Sigma-Aldrich (St. Louis, MO). Microsomes containing human lymphoblast-expressed P450 enzymes and Supersomes were purchased from BD Gentest Corporation (Woburn, MA).

Mechanism-based Inactivation of Single Human P450 Enzymes Expressed in Microsomes from Human Lymphoblastoid Cell Lines with Porphyrinogenic Xenobiotics. Microsomes containing single cDNA-expressed P450 enzymes in microsomes from HLCL, prepared in Microsomes from Human Lymphoblastoid Cell Lines with Porphyrinogenic Xenobiotics, and human cDNA-expressed CYP1A2, 2C9, and 3A4.

Partial Purification and Measurement of N-alkylPP Formation. After the incubation period, the sample mixture was combined in a ratio corresponding approximately to the ratio of these enzymes in human microsomes from Donor HG56 (BD Gentest Corporation). The mixture of enzymes was incubated with NADPH (2.0 mM) and 4-ethylIDDC (1.0 mM) for 30 min at 37°C. The experiment was repeated with the omission of NADPH or the porphyrinogenic xenobiotic.

Results and Discussion

The first objective of this study was to determine by fluorometry the formation of N-alkylPPs after mechanism-based inactivation by TTMS, 4-ethylIDDC, or AIA of single cDNA-expressed human P450 enzymes in microsomes prepared from HLCL to compare the results with those previously obtained in microsomes from BIICL. The hypothesis to be tested was that N-alkylPP formation following interaction of porphyrinogenic xenobiotics with single cDNA-expressed human P450 enzymes in microsomes from HLCL would be accompanied by formation of N-ethylPP.
occur only with P450 enzymes that had undergone mechanism-based inactivation. When TTMS (0.5 mM), 4-ethylDDC (1.0 mM), or AIA (1.0 mM) were incubated in the presence of NADPH with microsomes from HLCL possessing cDNA-expressed human CYP2D6, no significant amount of N-alkylIPP was detected (Table 1). The LLD for N-alkylIPP detection by fluorometry was found previously in our laboratory to be 0.04 nmol/2 ml of DCM (Lavigne et al., 2002). This LLD would apply equally to studies of laboratory to be 0.04 nmol/2 ml of DCM (Lavigne et al., 2002). This

<table>
<thead>
<tr>
<th>Xenobiotic</th>
<th>P450 Enzyme</th>
<th>Mechanism-based Inactivation in Microsomes from HLCL*</th>
<th>N-AlkylIPP Formation in Microsomes from HLCL nmol</th>
<th>N-AlkylIPP/mmol P450</th>
<th>N-AlkylIPP Formation in Microsomes from BIICL nmol</th>
<th>N-AlkylIPP/mmol P450</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTMS</td>
<td>2D6</td>
<td>—</td>
<td>0.01 (0.01, 0.0)</td>
<td>0.08 (0.08)</td>
<td>N.D.</td>
<td>0.05 (0.0, 0.0)</td>
</tr>
<tr>
<td>4-EthylDDC</td>
<td>2D6</td>
<td>—</td>
<td>0.03 (0.04, 0.01)</td>
<td>0.08 (0.08)</td>
<td>N.D.</td>
<td>0.02 (0.0, 0.04)</td>
</tr>
<tr>
<td>AIA</td>
<td>2D6</td>
<td>+</td>
<td>0.02 (0.0, 0.04)</td>
<td>0.23 (0.24, 0.22)</td>
<td>N.D.</td>
<td>0.06 (0.07, 0.05)</td>
</tr>
<tr>
<td>TTMS</td>
<td>1A2</td>
<td>+</td>
<td>0.06 (0.07, 0.05)</td>
<td>N.D.</td>
<td>0.60 (0.94, 0.25)</td>
<td>0.22 (0.22, 0.22)</td>
</tr>
<tr>
<td>4-EthylDDC</td>
<td>1A2</td>
<td>+</td>
<td>0.22 (0.22, 0.22)</td>
<td>N.D.</td>
<td>0.94 (0.94, 0.25)</td>
<td>0.05 (0.06, 0.04)</td>
</tr>
<tr>
<td>AIA</td>
<td>1A2</td>
<td>+</td>
<td>0.24 (0.29, 0.20)</td>
<td>0.87 (0.85, 0.89)</td>
<td>N.D.</td>
<td>0.04 (0.06, 0.03)</td>
</tr>
<tr>
<td>TTMS</td>
<td>3A4</td>
<td>+</td>
<td>0.04 (0.06, 0.03)</td>
<td>N.D.</td>
<td>0.30 (0.28, 0.32)</td>
<td>0.04 (0.11, 0.06)</td>
</tr>
<tr>
<td>4-EthylDDC</td>
<td>3A4</td>
<td>+</td>
<td>0.04 (0.11, 0.06)</td>
<td>N.D.</td>
<td>0.30 (0.28, 0.32)</td>
<td>0.14 (0.13, 0.15)</td>
</tr>
<tr>
<td>AIA</td>
<td>3A4</td>
<td>—</td>
<td>0.01 (0.0, 0.02)</td>
<td>N.D.</td>
<td>0.29 (0.39, 0.18)</td>
<td>0.02 (0.02, 0.02)</td>
</tr>
<tr>
<td>TTMS</td>
<td>2C9</td>
<td>+</td>
<td>0.02 (0.02, 0.02)</td>
<td>N.D.</td>
<td>0.29 (0.39, 0.18)</td>
<td>0.14 (0.13, 0.15)</td>
</tr>
<tr>
<td>4-EthylDDC</td>
<td>2C9</td>
<td>+</td>
<td>0.02 (0.02, 0.02)</td>
<td>N.D.</td>
<td>0.29 (0.39, 0.18)</td>
<td>0.14 (0.13, 0.15)</td>
</tr>
<tr>
<td>AIA</td>
<td>2C9</td>
<td>—</td>
<td>0.02 (0.02, 0.02)</td>
<td>N.D.</td>
<td>0.29 (0.39, 0.18)</td>
<td>0.14 (0.13, 0.15)</td>
</tr>
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</table>

N.D., Not Detected.

* +, denotes mechanism-based inactivation; −, denotes no mechanism-based inactivation.

TABLE 1

**Relationship between mechanism-based inactivation of cDNA-expressed human P450 enzymes by porphyrinogen xenobiotics in microsomes from HLCL and N-alkylIPP formation in cDNA-expressed human P450 enzymes in microsomes from HLCL and BIICL (Lavigne et al., 2002).**

Each value given is the average of the values obtained in two experiments (individual values shown in parentheses). Values below 0.08 were not considered significant whereas a value of 0.08 was considered borderline (Lavigne et al., 2002).

WhenTTMS (0.5 mM), 4-ethylDDC (1.0 mM), or AIA (1.0 mM) were incubated in vitro with microsomes from HLCL possessing cDNA-expressed human CYP3A4, TTMS elicited N-ethylIPP formation in a NADPH-dependent manner. The average ratio of N-ethylIPP formation to the amount of CYP3A4 present was 0.24 (Table 1). On the other hand, N-ethylIPP formation was not significant with 4-ethylDDC and was borderline with AIA. In the previous results of McNamee et al. (1997), TTMS and 4-ethylDDC caused mechanism-based inactivation of CYP3A4 in microsomes from human lymphoblastoid cell lines. The detection of N-ethylIPP after TTMS administration shows that mechanism-based inactivation of CYP3A4 proceeds by a route that includes N-ethylIPP formation. The fact that no significant amount of N-ethylIPP formation was detected shows that mechanism-based inactivation of CYP3A4 by 4-ethylDDC occurs either by direct alkylation of the apoprotein or destruction of the heme moiety. This result is consistent with a report by Correia et al. (1987) that mechanism-based inactivation of rat CYP3A2 by 4-ethylDDC involves fragmentation of the heme moiety to reactive metabolites that irreversibly bind to the P450 apoprotein. In the previous study of McNamee et al. (1997), AIA (1 mM) caused 19% mechanism-based inactivation of CYP3A4, which, however, did not reach statistical significance. In the present study the amount of N-AIAPP detected was at the borderline of significance. Our new N-ethylIPP results contrast with those previously obtained with AIA in microsomes from BIICL in which 0.30 nmol of N-AIAPP were formed per nanomole of CYP3A4 (Lavigne et al., 2002).

WhenTTMS (0.5 mM), 4-ethylDDC (1.0 mM), or AIA (1.0 mM) were incubated in vitro with microsomes from HLCL possessing cDNA-expressed human CYP2C9, only AIA elicited N-alkylIPP formation in a NADPH-dependent manner (Table 1). With TTMS and 4-ethylDDC, no significant amounts of N-alkylIPP were detected. In previous studies of McNamee et al. (1997), 4-ethylDDC but neither TTMS nor AIA caused mechanism-based inactivation of CYP2C9 in microsomes derived from HLCL. Thus, the inability to detect N-ethylIPP formation after TTMS administration accorded with its inability to cause mechanism-based inactivation of CYP2C9. The fact that no N-ethylIPP was detected shows that mechanism-based inactivation of CYP2C9 by 4-ethylDDC occurs either by direct alkylation of the apoprotein or destruction of the heme moiety. The fact that the average ratio of N-AIAPP formation to the amount of P450 was 0.14 nmol/nmol was unexpected since no significant mechanism-based inactivation of CYP2C9 by AIA had been previously detected, and
N-alkylPP formation is believed to depend on mechanism-based inactivation of P450. A comparison of our results in HLCL with those previously obtained in microsomes from BIICL reveals similar results with TTMS and AIA (Table 1). However, in contrast to insignificant amounts of N-ethylPP detected after 4-ethylIDDC interaction with microsomes of HLCL, the average ratio was found to be 0.29 nmol of N-ethylPP/nmol CYP2C9 in microsomes from BIICL.

In summary, N-alkylPP formation with TTMS and 4-ethylIDDC in microsomes from HLCL was observed only with human P450 that had undergone mechanism-based inactivation. The converse was not the case and in some cases mechanism-based inactivation was not accompanied by N-alkylPP formation. This was to be expected since mechanism-based inactivation can occur by pathways that do not include N-alkylPP formation. In the case of AIA, despite the fact that mechanism-based inactivation was not observed with either CYP2D6, 1A2, 3A4, or 2C9, N-alkylPP formation was significant with CYP2C9 and at the borderline of significance with CYP3A4. A possible explanation for this result is that significant mechanism-based inactivation may not be detected due to relatively large standard deviations in the NADPH-treated and NADPH-un-treated groups. On the other hand, detection of an N-alkylPP is by direct measurement and does not depend on group differences. In contrast to the results obtained with N-alkylPP formation in microsomes from HLCL, significant N-alkylPP formation was found with AIA in microsomes from BIICL for three of four P450 enzymes, 2D6, 3A4, and 2C9, in which mechanism-based inactivation had not been observed. We conclude that mechanism-based inactivation and N-alkylPP formation after porphyrinogenic xenobiotic administration should be compared in microsomes from the same cell system. The P450:NADPH-P450 reductase ratios for microsomes from HLCL were 1:0.11 (CYP1A2), 1:4.30 (CYP2C9), 1:0.60 (CYP2D6), and 1:0.77 (CYP3A4). These values differ in BIICL where the ratios were 1:10.4 (CYP1A2), 1:0.70 (CYP2C9), 1:13.28 (CYP2D6), and 1:0.31 (CYP3A4). However, there is no obvious correlation between differences in these ratios and N-alkylPP formation (Table 1). It is of interest that these ratios in general are considerably higher than the P450:NADPH-P450 reductase ratio in normal human liver microsomes (1:0.1) (Benet et al., 1996).

We had previously observed that when 4-ethylIDDC and NADPH interacted with human liver microsomes containing 4.98 nmol of total P450 of which 25% was CYP1A2 and 29% was CYP2C9 (BD Gentest Donor HG56), N-alkylPP was not formed. This was a surprising finding since N-ethylPP formation was observed in microsomes from BIICL containing either CYP1A2 or 2C9. This unexpected finding was explained as follows: human liver microsomal preparation HG56 also contains 12.5% of CYP3A4. Correia et al. (1987) have shown that 4-ethylIDDC causes mechanism-based inactivation of rat CYP3A2, which is not accompanied by N-ethylPP formation. It was therefore suggested that a major portion of 4-ethylIDDC may have undergone biotransformation by CYP3A4 thus diverting the xenobiotic from CYP1A2 and 2C9.

The second objective of this study was to test this explanation by comparing N-ethylPP formation after administration of 4-ethylIDDC to a mixture containing human cDNA-expressed CYP1A2 and 2C9 with formation of N-ethylPP in a mixture of human cDNA-expressed CYP1A2, 2C9, and 3A4. When a mixture of CYP1A2 (0.5 nmol) and 2C9 (0.5 nmol) was incubated with NADPH and 4-ethylIDDC (1.0 mM), an average of 0.10 nmol of N-ethylPP/nmol P450 was formed (first experiment 0.01 nmol, second experiment 0.09 nmol). On the other hand, when a mixture of CYP1A2 (0.5 nmol), 2C9 (0.5 nmol), and 3A4 (0.25 nmol) was incubated with NADPH and 4-ethylIDDC (1.0 mM) an average of 0.11 nmol of N-ethylPP/nmol P450 was formed (first experiment 0.09 nmol, second experiment 0.13 nmol). Thus, no significant difference was observed in the formation of N-ethylPP from 4-ethylIDDC when CYP3A4 was added to a mixture of CYP1A2 and 2C9. Therefore, it is necessary to consider other possible explanations for the discrepancy between the ability of 4-ethylIDDC to cause N-ethylPP formation when interacting with cDNA-expressed human CYP1A2 or 2C9, and the inability to cause N-ethylPP formation when interacting with human liver microsomes possessing elevated levels of CYP1A2 and 2C9. One possibility is that the human liver microsomes contained, in addition to CYP1A2, 2C9, and 3A4, 11.6% CYP2A6, 5% CYP2C19, 2.2% CYP2D6, and 13.2% CYP2E1, and results obtained with expression systems may not reflect the overall in vivo effects of these compounds, which is dependent on the specific activity and relative abundance of each P450 enzyme. In addition, the phospholipid environment, lipid to protein ratio and the P450:NADPH-P450 reductase ratio can differ between native microsomal systems and cDNA-expressed single P450 enzyme systems (Rodrigues, 1999). It is of interest that the P450: NADPH-P450 reductase ratio in the human microsomes (BD Gentest Donor HG56) was 1:0.34. This value contrasts with the P450: NADPH-P450 reductase ratios for microsomes from BIICL, which were 1:10.4 (CYP1A2), 1:0.70 (CYP2C9), and 1:0.31 (CYP3A4).

References