MECHANISM OF CYTOCHROME P450 ACTIVATION BY CAFFEINE AND 7,8-BENZOFلافون IN RAT LIVER MICROSOMES

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ABSTRACT:

Caffeine and 7,8-benzoﬂavone activate CYP3A2 in rat liver microsomes. Both activators appear to enhance enzyme activity by an increase in $V_{max}$ and to a lesser extent a decrease in $K_m$. Additive effect studies demonstrated that the two activators oppose one another's effect. Electron transfer steps in the cytochrome P450 cycle are involved in the mechanism of cytochrome P450 activation, as indicated by the lack of effect of caffeine or 7,8-benzoﬂavone on cumene hydroperoxide-supported oxidation of acetaminophen by cytochrome P450. The involvement of cytochrome $b_5$ in the formation of N-acetyl-p-benzoquinone imine (NAPQI) was implicated through a synergistic effect of NADH on the NADPH-supported reaction. Anti-cytochrome $b_5$, but not anti-cytochrome P450 reductase IgG, diminished the activation effect of caffeine on NAPQI formation. Neither antibody altered the effect of 7,8-benzoﬂavone on NAPQI formation. The impairment of NAPQI formation by cytochrome $b_5$ antibody suggests that cytochrome P450 activation by caffeine but not 7,8-benzoﬂavone is mediated in part through enhancement of the transfer of the second electron to cytochrome P450 from cytochrome $b_5$.

Numerous studies have shown that acetaminophen (APAP) $^1$ can undergo oxidative metabolism by cytochrome P450 to form the reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI). In the rat, the reaction is catalyzed by the constitutive forms CYP1A2, 2E1, and 3A2 and the inducible forms CYP3A1 and 1A1, while in the human it is catalyzed by CYP2E1, 1A2, and 3A4 (1–5). Toxicity studies have demonstrated that caffeine can enhance APAP hepatotoxicity in uninduced and phenobarbital-induced adult male rats, but protect against hepatotoxicity in methylcholanthrene-induced adult male rats (6). Increases in both NAPQI formation and the extent of covalent binding of [14C]-APAP in uninduced and phenobarbital-induced rat liver microsomes and uninduced hepatocytes have established that caffeine enhances (activates) cytochrome P450 activity (5, 7, 8). Additional studies have shown that 3- to 4-fold activation of NAPQI formation by 5 mM caffeine occurred in microsomes prepared from juvenile male and female and adult male rats, but activation was completely absent in microsomes prepared from adult females. In microsomes prepared from diabetic adult male rats, in which CYP2E1 was increased approximately 5-fold as shown by Western analysis, the extent of activation by 5 mM caffeine was dampened to 127%. Caffeine was also shown to inhibit NAPQI formation by purified-reconstituted CYP1A1. The age and sex dependent pattern of activation of NAPQI formation in rat liver microsomes, dampened caffeine enhancement of NAPQI formation in CYP2E1 enriched microsomes, inhibition of NAPQI formation by caffeine in reconstituted CYP1A1, and the established pattern of age and sex dependent P450 expression in the rat allowed the conclusion that CYP3A2 was the isozyme activated by caffeine (5).

The mechanism of cytochrome P450 activation has not been explored to the same depth as induction phenomena. Enhancement of aniline para-hydroxylation by acetone was the first reported cytochrome P450 activation interaction (9). Based on studies with liver microsomal fractions of the dog, rabbit, mouse, and rat, it was proposed that acetone affected either the formation of the pterin anion complex of cytochrome P450 or steps beyond this (such as the formation of the oxene complex) because cumene hydroperoxide dependent hydroxylation of aniline was stimulated by acetone (10). Huang et al. (11) demonstrated that the stimulatory effect of 7,8-benzoﬂavone on benzo(a)pyrene metabolism in rabbit liver microsomes was mediated by a different mechanism than that observed with acetone. The effect of 7,8-benzoﬂavone on benzo(a)pyrene metabolism was thought to be a result of enhanced interactions between cytochrome P450 and cytochrome P450 reductase (12). A third mechanism of activation was proposed by Johnson et al. (13), who reported that the stimulatory effect of $\alpha$-naphthoflavone (7,8-benzoﬂavone) on rabbit cytochrome P450 3c (CYP3A6) was a consequence of an allosteric effect, as shown by an increase in the P450 binding affinity for the substrate 22-amino-23, 24-bisnor-5-cholen-3$\beta$-ol (22-ABC). Shou et al. (14) have shown that there is mutual activation between phenanthrene and 7,8-benzoﬂavone and suggest that the two molecules simultaneously occupy the active site, thereby altering active site geometry and oxidation efficiency. In summary, it appears that cytochrome P450 activation may occur by several mechanisms.

In a previous study (15), we observed that 50 mM flavone enhanced NAPQI formation to an extent similar to that observed with 5 mM caffeine in various microsomal preparations. Studies in microsomes prepared from adult and juvenile male and female rats and induced male rats, as described above for caffeine, indicated that flavone also activated NAPQI formation via an effect on CYP3A2. Similar studies with 7,8-benzoﬂavone indicate that it also enhances NAPQI formation

$^1$ Abbreviations used are: APAP, acetaminophen; NAPQI, N-acetyl-p-benzoquinone imine; CHP, cumene hydroperoxide.

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in liver microsomes with a pattern similar to flavone (unpublished observations). The objectives of the present study were to identify the component(s) of the cytochrome P450 cycle affected by caffeine and 7,8-benzoflavone that account for enhanced cytochrome P450 activity in rat liver microsomes. 7,8-Benzoﬂavone was used in these studies because of the mechanistic work previously done with the compound. We speciﬁcally were interested in the role of electron transport from cytochrome P450 reductase and cytochrome b5. We therefore elected to conduct these studies in microsomes rather than puriﬁed reconstituted enzyme or cDNA expression preparations in which the integrity of the association between P450 and the electron donators would be determined by reconstitution or expression conditions. Also, the evidence that a single isoform producing NAPQI is activated by caffeine in microsomes prepared from uninduced adult male rats allows clear interpretation of microsomal studies (5).

Materials and Methods

Chemicals. APAP, NADPH (tetrasodium salt, type IV), reduced glutathione, 7,8-benzoﬂavone, cumene hydroperoxide(CHP), glycine, cytochrome c (horse heart), Triton X-100, rabbit pre-immune IgG, and goat anti-rabbit IgG alkaline phosphatase conjugate were purchased from Sigma Chemical Company (St. Louis, MO). Sodium chloride, sodium phosphate (monobasic), and sodium phosphate (dibasic) were purchased from J.T. Baker (Phillipsburg, NJ). Transfer membrane (nitrocellulose) was obtained from Schleicher and Schuell (Keene, NH). NBT and BCP were purchased from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD). DE-52 was purchased from Whatman Laboratory Division (Maidstone, UK).

Animals. Virus antibody-free male Sprague-Dawley rats (275–300 g) were obtained from Charles River Breeding Laboratories (Wilmington, MA) and were maintained on Wayne Rodent Chow (Animal Specialties, Hubbard, OR). Rats were acclimatized for 3 days before use and were housed in a controlled temperature and lighting environment (12 hr light-dark cycle) on corn cob bedding. Adult female New Zealand white rabbits were purchased from R&R Rabbitry (Stanwood, WA).

Microsomal Incubations. Microsomes were prepared from adult male Sprague-Dawley rats by the standard ultracentrifugation method. The incubation mixture contained APAP (1.0 mM), NADPH (2.0 mM), GSH (5.0 mM), activator (5.0 mM caffeine or 50 μM 7,8-benzoﬂavone), and 2 mg/ml microsomal protein in 100 mM potassium phosphate buffer (pH 7.4). The final incubation volume was 0.5 ml. For determination of kinetic constants for NADPH, APAP concentration was 1 mM and NADPH concentration was varied. In CHP incubations, 50 mM NADPH, APAP concentration was 1 mM and NADPH concentration was incubation volume was 0.5 ml. For determination of kinetic constants for somal protein in 100 mM potassium phosphate buffer (pH 7.4). The final methanol was used to dissolve 7,8-benzoﬂavone because the compound is insoluble in 100 mM potassium buffer (pH 7.4). The final methanol concentration in the incubation mixture was 1% (v/v). Because of the solubility limits of 7,8-benzoﬂavone, the maximum final concentration achieved in the microsomal incubation was 50 μM. Caffeine was easily soluble in the 100 mM potassium phosphate buffer (pH 7.4) without the use of an organic solvent.

Studies using antibodies were conducted in the following manner: the antibody was mixed with microsomal protein and allowed to sit for 25 min at room temperature. GSH and activators were added to the microsomal/antibody mixture, followed by the addition of APAP to start the 5 min pre-incubation at 37°C. The reaction was initiated by the addition of NADPH and proceeded for 10 min. The reaction was terminated with 200 μl 2.0 M perchloric acid and vortexed briefly. Approximately 600 μl of the suspension was transferred to an Eppendorf tube (1.5 ml) and centrifuged at 12,000 × g for 2 min. A 400 μl aliquot was then neutralized by the addition of an equal volume of 1.0 M K2HPO4, centrifuged, and 20 μl of the supernatant was analyzed by HPLC.

HPLC separation was carried out on a Rainin microsorb column (5μm: 4.6 × 250 mm) with a guard column on a Hewlett-Packard 1050 HPLC system with a model 3396 recording integrator. The solvents used in the gradient elution of APAP-3-GSH conjugate were: A, 2% methanol in 50 mM ammonium phosphate buffer (pH 6.0) and B, 35% acetonitrile in 50 mM ammonium phosphate buffer (pH 6.0). The sample was eluted isocratically with solvent A for 2 min, followed by a linear increase to 25% B between 2 and 15 min. Solvent B was further increased linearly to 100% between 15 and 19 min, maintained for 2 min, and then decreased to 0% for 1 min. The eluent was monitored at 254 nm at a flow rate of 1 ml/min. The retention times for the glutathione conjugate and APAP were 13.5 and 15.8 min, respectively. Quantitation of APAP-3-GSH was conducted using an external standard of 14C-APAP-3-GSH (5).

Cytochrome P450 Reductase Purification. NADPH-cytochrome P450 reductase was purified from liver microsomes isolated from PB-treated adult male rats as described previously (5, 16). Freshly purified reductase had a speciﬁc activity of 34,000 nmol cytochrome c reduced/min/mg protein at room temperature (22°C) in 300 mM potassium phosphate buffer (pH 7.7).

Cytochrome b5 Purification. Cytochrome b5 was purified from adult rat liver as described previously for preparation of rabbit cytochrome b5 (17). Preparative of Cytochrome P450 Reductase and Cytochrome b5 Antibodies. The administration of antigen (cytochrome P450 reductase or cytochrome b5) and collection of blood were performed by technicians in the Department of Comparative Medicine at the University of Washington. Primaty antigens were used in microsomes isolated from PB-treated adult male rats as described previously (5, 16). Freshly purified reductase had a speciﬁc activity of 34,000 nmol cytochrome c reduced/min/mg protein at room temperature (22°C) in 300 mM potassium phosphate buffer (pH 7.7). The dialyzed protein was centrifuged at 12,000 × g for 20 min at 4°C and then washed three times with PBS. The supernatant was subjected to SDS-PAGE electrophoresis and transferred to nitrocellulose membranes using a Trans-Blot apparatus. The nitrocellulose membranes were probed with either mouse monoclonal antibody against cytochrome P450 or goat polyclonal antibody against cytochrome b5. Western blots analyses were conducted to determine the immunoreactivity of the antibodies for the respective primary antigens after each bleed. The primary antibody recovered from all bleeds was immunoreactive with cytochrome P450 reductase protein (0.5 pmol) or cytochrome b5 (1 pmol), respectively, at dilutions of 1 to 5000 or 1 to 1000, respectively.

Cytochrome P450 Reductase and Cytochrome b5 Antibody Purification. A volume of 20–40 ml of unhemolyzed rabbit antiserum was thawed. 2 M saturated ammonium sulfate solution was added to the serum (2.3 v/v%) dropwise. The suspension was stirred for 30 min at room temperature (4°C) to form a rouleau and then centrifuged at 12,000 × g for 20 min at 4°C. The supernatant was collected and stored at −20°C. The supernatant was subjected to DE-52 chromatography as described by Thomas et al. (18). Isolated IgG was dialyzed against 100 mM potassium phosphate buffer (pH 7.4) and stored at −70°C. Western blots showed that neither antibody reacted with rat liver microsomal P450 under conditions optimal for detection of cytochrome P450 reductase or cytochrome b5.

Inhibition of NADH-dependent cytochrome c reductase activity by the purified cytochrome b5 was assessed in rat liver microsomes with ratios of IgG to microsomal protein of 10 and 40 (w/w). Conditions were: 0.5 mg/ml cytochrome c, 12.5 μM/ml microsomal protein, 300 μM K2HPO4 (pH 7.7), final volume 1 ml. All components other than NADH were preincubated at room temperature for 15 min. The tubes were transferred to a shaking water bath at 37°C, and the reaction was initiated with the addition of NADH and continued for 60 sec, with absorbance recorded at 550–538 nm. The antibody inhibited the reaction by 45% (in comparison with preimmune control) when the microsomal protein: IgG protein concentration ratio was 1:10 and by 45% when the ratio was 1:40. Inhibition of NAPQI formation in adult male rat liver microsomes incubated with 1 mM APAP was assessed with microsomal protein:IgG ratios of 1:1, 1:5, and 1:10 for the anti-cytochrome P450 reductase antibody. The extent of inhibition from the lowest to highest ratio was 2%, 13%, and 30%.

Results

Effects of 50 μM 7,8-Benzoflavone and 5 mM Caffeine on Formation Kinetics of NAPQI. To determine whether 7,8-benzoﬂavone and caffeine activate the same cytochrome P450 isozyme, an additive effect study was conducted. The concentration of caffeine

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was optimized for the formation of NAPQI. Fig. 1 shows that maximum product formation occurred at 10 mM and that at a caffeine concentration of 20 mM the degree of activation began to diminish. Table 1 shows that the individual activation effects of caffeine and 7,8-benzoflavone on NAPQI formation in adult male rat liver microsomes were 5-fold and 4-fold, respectively. The addition of both activators simultaneously resulted in a 3-fold degree of cytochrome P450 activation, which was lower than that observed with either activator alone. This result suggested that caffeine and benzoflavone mutually antagonized one another’s actions, indicating different mechanisms of activation.

The Eadie-Hofstee plots shown in fig. 2 are concave in the absence of activators, suggesting the involvement of more than one cytochrome P450 isoform in NAPQI formation. This result is consistent with the finding of Harvison et al. (19) that multiple rat cytochrome P450 isoforms are capable of forming NAPQI. The plot became linear when 5 mM caffeine or 50 μM 7,8-benzoflavone was added. This result indicates that the activatable P450 isoform dominates NAPQI formation in the presence of either activator. Furthermore, the similarity of the slopes suggests that the $K_m$ value(s) of APAP in microsomes was not markedly affected by either activator; however, the $V_{\text{max}}$ (y-intercept) was obviously changed to a great extent. Data from control and activator (low concentration) incubations were best fit by a two enzyme (two $K_m$) equation, and the data from the high activator concentration were best fit by a single enzyme (one $K_m$) equation. A 4-fold increase in $V_{\text{max}}$ was observed for both caffeine and 7,8-benzoflavone in comparison with control (table 2). $K_{\text{m1}}$ did not change substantially relative to control at the low activator concentration and decreased to one-half of the control value at the high activator concentration. Thus, it appears that activation is accompanied by both an increase in $V_{\text{max}}$ and a decrease in $K_{\text{m1}}$, but the effect on $V_{\text{max}}$ apparently predominates and is observed at the lower concentration of activator.

The apparent decrease in $K_{\text{m2}}$ with the addition of 1.5 mM caffeine is insignificant in comparison with the standard deviation.

### Table 1

<table>
<thead>
<tr>
<th>Caffeine (mM)</th>
<th>0</th>
<th>10</th>
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<tbody>
<tr>
<td>(nmol APAP-3-GSH formed/min/mg prot)</td>
<td>0.32 ± 0.02$^a$</td>
<td>1.32 ± 0.02</td>
</tr>
<tr>
<td>50 μM (nmol APAP-3-GSH formed/min/mg prot)</td>
<td>1.65 ± 0.03$^b$</td>
<td>0.96 ± 0.03$^c$</td>
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The concentration of acetaminophen was 1.0 mM. Values are mean ± SD, N = 3.

$^a$ Control vs. 50 μM 7,8-benzoflavone, $p < 0.05$.  
$^b$ Control vs. 10 mM caffeine, $p < 0.05$.  
$^c$ 10 mM caffeine vs combination of 10 mM caffeine and 7,8-benzoflavone, $p < 0.05$.

**Role of electron transport.** Organic hydroperoxides support aromatic hydroxylation in liver microsomes at reaction rates comparable to NADPH-supported hydroxylations (20), and CHP has been shown to mediate the formation of NAPQI at rates comparable to the NADPH-supported reaction (3). This property was exploited to evaluate the role of electron transfer from cytochrome P450 P450 reductase to cytochrome $b_5$ in the mechanism of P450 activation by caffeine or 7,8-benzoflavone. The results (table 3) confirm the previous observation that CHP supports the reaction (3). The results also show that the formation of NAPQI was not stimulated by 5 mM caffeine or 50 μM 7,8-benzoflavone in liver microsomes when supported with CHP, while approximately 3-fold activation was observed for both caffeine and 7,8-benzoflavone in NADPH-supported incubations.

To assess the possibility that caffeine might facilitate the transfer of electrons from NADPH, the effect of caffeine on NAPQI formation was determined at varying concentrations of NADPH. The results (fig. 3) show that both $V_{\text{max}}$ and $K_m$ for NAPQI increased in the presence of caffeine: $V_{\text{max}}$ by 4.5-fold and $K_m$ by 2.6-fold. The catalytic efficiency ($V_{\text{max}}/K_m$) was 2.45 and 3.77 μmol/min/mg protein for control and caffeine, respectively. The fact that $V_{\text{max}}$ changed indicated that caffeine affected the electron flow pathway, the rate-limiting step in product formation. The increase in $K_m$ suggested that caffeine either increased $k_{\text{cat}}$ or decreased the affinity of cytochrome P450 reductase for NADPH.

**Involvement of Cytochrome $b_5$ in NAPQI Formation.** Enhancement of NADPH-dependent cytochrome P450 oxidation by NADH has been suggested to be the result of the participation of cytochrome $b_5$ in the transfer of the second electron to cytochrome P450 (21,22). In table 4, it is shown that NADH-mediated cytochrome P450 dependent formation of NAPQI proceeded at one-third the rate of NADPH-supported oxidation velocity at dinucleotide concentrations of 3.000 μM; NAPQI formation was not detectable at NADH concentrations of 100 μM and 500 μM. The combination of 400 μM NADH and 100 μM NADPH resulted in a greater NAPQI formation rate than 100 μM NADPH alone (0.225 ± 0.005 versus 0.129 ± 0.003 nmol/min/mg protein), consistent with the synergistic phenomena reported by Sato and Marumo (23). The synergistic effect of NADH on the NADPH-dependent cytochrome P450 formation of NAPQI suggests that the reduction of cytochrome $b_5$ supports NAPQI formation in this microsomal system.

**Roles of Cytochrome P450 Reductase and Cytochrome $b_5$ in Cytochrome P450 activation.** Antibodies to cytochrome P450 reductase and cytochrome $b_5$ were used to investigate further the role of these two proteins in cytochrome P450 activation. The antibodies were respectively shown by Western blot analysis to react with the
immunizing antigen only, and not with opposite coenzyme or rat liver microsomal cytochrome P450 under conditions optimal for the immunizing antigen.

Fig. 4 shows that microsomal protein-to-antibody ratios of 1:30 and 1:10 for anti-cytochrome b₅ and anti-cytochrome P450 reductase, respectively, resulted in a 40–50% decrease in NAPQI formation in the absence of caffeine (top panel). In the presence of anti-cytochrome P450 reductase, the addition of 5 mM caffeine to the incubation mixture resulted in an approximately 280% activation of NAPQI formation, similar to the degree observed in the absence of IgG or with the addition of pre-immune IgG (bottom panel). In contrast, in the presence of anti-cytochrome b₅, a 40–50% attenuation of cytochrome P450 activation by caffeine was observed (bottom panel). Fig. 5 shows that none of the IgG proteins, preimmune IgG, anti-cytochrome P450 reductase, or anti-cytochrome b₅ diminished the activation of NAPQI formation by 7,8-benzoflavone (bottom panel), although the concentration of both of the antibodies was sufficient to inhibit the formation of NAPQI in the absence of 7,8-benzoflavone (top panel).

**Discussion**

The major findings are: 1) that caffeine and 7,8-benzoflavone activate NAPQI formation by increasing $V_{\text{max}}$ and to a lesser extent by decreasing the apparent microsomal $K_{\text{m}}$, 2) that caffeine and 7,8-benzoflavone antagonize one another’s activation, 3) that activation...
by either caffeine or 7,8-benzoflavone is blocked when NADPH is replaced by CHP, and 4) that an inhibitory cytochrome b\(_5\) antibody significantly diminished the activation of NAPQI formation by caffeine. It was also observed that the formation of NAPQI was enhanced by the inclusion of NADH in incubations containing NADPH, consistent with the participation of cytochrome b\(_5\) in the formation of NAPQI.

Previous studies with microsomes prepared from adult and juvenile male and female rat liver have shown that caffeine activates CYP3A2 (5). In the present study, the Eadie-Hofstee plots clearly indicate the involvement of more than one enzyme in the absence of activators. Under such conditions, \(K_m\) values for the different enzymes are difficult to estimate unambiguously. Thus, the finding that the activators increased \(V_{max}\) is unambiguous, but the observation of a decrease in \(K_m\) could result from a secondary enzyme becoming predominant upon activation rather than a change in the actual \(K_m\) of a single enzyme. Our mechanistic studies therefore focused on the increase \(V_{max}\).

The finding that the combination of caffeine and 7,8-benzoflavone (table 1) resulted in less activation of NAPQI formation than when either activator was included separately is consistent with the interpretation that the activators operate by different and apparently antagonistic mechanisms on the same enzyme. Alternatively, simultaneous binding of the two activators may prevent optimal interaction of either molecule with the enzyme system. It must also be recognized that the additive effect studies were conducted in microsomes that contain both CYP1A2 and 3A2. Caffeine activates the latter and...
inhibits the former (5). Since Raucy and Johnson (24) reported that rabbit CYP1A2 activity [using benzo(a)pyrene as substrate] was inhibited by 7,8-benzoflavone, it is plausible that the rat ortholog also may be inhibited. If so, the apparently diminished extent of activation in the presence of caffeine and 7,8-benzoflavone could be because the activation effect on CYP3A2 was masked by a concomitant additive inhibition of CYP1A1. Under these conditions, 7,8-benzoflavone might contribute to the inhibition of the formation of NAPQI by CYP1A1, but would not be able to enhance the maximal activation of CYP3A2 produced by 10 mM caffeine.

The absence of activation of NAPQI formation in incubations supported with CHP is consistent with at least two mechanisms of activation in the absence of CHP. CHP acts by formation of a peroxide anion-cytochrome P450 complex essentially as a second substrate. Although binding of substrate to the low-spin ferric complex occurs before the formation of the peroxide anion-cytochrome P450 complex by CYP, the requirement that CHP be present at the active site is likely to preclude the simultaneous occupation of the active site by the two additional molecules, APAP and 7,8-benzoflavone, according to the mechanism of activation of CYP3A4 described by Shou et al. (14). CHP would also interfere with activation by enhanced electron flow from cytochrome bs, as this step is not required in CHP-supported incubations.

The enhancement of catalysis by NADH when added in addition to NADPH suggests a role for cytochrome bs in the formation of NAPQI (23, 25). Enhanced electron flow via NADPH-dependent cytochrome P450 reductase or NADH-cytochrome bs reductase/cytochrome bs are two possible pathways by which caffeine or 7,8-benzoflavone can increase the V_max of NAPQI formation. It has been proposed that the first of the two electrons donated to the cytochrome P450 cycle is provided only by cytochrome P450 reductase, with either NADPH or NADH serving as the electron donor, although the electron transfer rate with NADH is substantially slower than with NADPH (23, 25).

The second electron for the cytochrome P450 cycle can be supplied by cytochrome P450 reductase or by cytochrome bs via NADPH-dependent cytochrome bs reductase. Cytochrome bs can also interact with the P450-cytochrome P450 reductase complex (in the absence of NADH) to facilitate drug oxidation. The transfer of the second electron has been postulated to be rate limiting in cytochrome P450-catalyzed oxidations in which cytochrome bs participates (26). Since the transfer of the second electron is both rate limiting when cytochrome bs participates and can be initiated by NADH in the presence of NADPH (the first electron would still come from NADPH in the presence of NADH), acceleration of catalysis by NADH suggests a role for cytochrome bs as an electron donor to cytochrome P450. Sato and Marumo (23) have observed this synergistic effect of NADH on the NADPH-dependent oxidation of APAP to NAPQI as well.

The role of cytochrome bs in reconstituted cytochrome P450 systems has been extensively studied. Enhanced activity in reconstituted systems containing cytochrome bs has been observed with CYP2B4, 1A2, 2C6, 2C11, 2E1, 3A1, and 3A4 (27–31). Substrate and cytochrome bs apparently bind to different and nonoverlapping sites on cytochrome P450 (32,33). The binding of substrate to cytochrome P450 putatively induces a structural change that increases the proportion of high spin cytochrome P450; these two factors appear to increase the affinity of the cytochrome P450-substrate complex for cytochrome bs (32–34). The close proximity of cytochrome bs in the ternary complex (substrate-cytochrome P450-cytochrome P450 reductase) has been suggested to improve coupling of cytochrome P450 reductase to cytochrome P450, resulting in a decreased lag time between the introduction of the first and second electrons, thereby increasing cytochrome P450 activity (34). Studies of the role of cytochrome bs in the cytochrome P450 oxidation processes have been performed entirely with purified reconstituted enzymes. The relevance of this mechanistic work for enzymes in the intact membrane of the endoplasmic reticulum remains to be established.

Studies conducted with antibodies directed against cytochrome P450 reductase and cytochrome bs indicated that the latter protein is involved in microsomal cytochrome P450 activation by caffeine (fig. 4). Anti-cytochrome bs caused a 50% decrease in the extent of caffeine-mediated cytochrome P450 activation. This antibody had no effect on 7,8-benzoflavone-mediated cytochrome P450 activation, confirming that the two activators operate by different mechanisms. The data presented in table 3 provide compelling evidence that the mechanism of cytochrome P450 activation by caffeine is mediated at least in part through cytochrome bs-enhanced transfer of the second electron to the cytochrome P450 cycle. It is most likely that this effect is exerted on cytochrome bs support of CYP3A2. It is possible that anti-cytochrome bs diminished the competitive inhibition of caffeine on CYP1A2, but this seems unlikely. At 5 mM, caffeine causes approximately a 2.5-fold increase in total microsomal formation of NAPQI (fig. 4). Thus, in the presence of caffeine, the contribution of other isofoms of P450 to total microsomal formation of NAPQI becomes minor, an interpretation supported by the monophasic Eadie-Hofstee plots in fig. 2. Under these conditions, an effect of anti-cytochrome bs on the CYP3A2 reaction is the most likely explanation for the effect observed.

The activation of P450-dependent metabolism of 7,8-benzoflavone by caffeine has been examined in a number of species. Huang et al. (12) demonstrated that the mechanism of activation of 7,8-benzoflavone on benzo(a)pyrene oxidation in rabbit and hamster liver microsomes was in part a result of increased affinity between cytochrome P450 and its reductase. They also conducted incubations in guinea pig and female rat liver microsomes. 7,8-Benzo-flavone inhibited benzo(a)pyrene oxidation in the former and had essentially no effect in the latter. In the presence of 7,8-benzoflavone, the affinity of CYP3A6 for substrate is increased (13). As discussed above, studies by Shou et al. suggest that 7,8-benzoflavone activates CYP3A4 by simultaneously occupying the active site with the substrate, forcing an allosteric change that enhances electron flow. The mechanism by which 7,8-benzoflavone activates the formation of NAPQI by CYP3A2 is different from that of caffeine and remains to be established.

References