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Interaction of Volatile Anesthetics with Rat Hepatic Microsomal Cytochrome P-450

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The addition of halothane, methoxyflurane, or enflurane to hepatic microsomes caused the appearance of a trough at 420 mμ and simultaneous appearance of an absorption band at 385 mμ (type I), indicative of an interaction between cytochrome P-450 and the anesthetics. Diethyl ether had no effect. Halothane, methoxyflurane and enflurane acted as competitive inhibitors of the spectral change produced by addition of hexobarbital (type I substrate), while the spectral change caused by addition of aniline (type II substrate) was increased in amplitude by these volatile anesthetics. N-demethylase activity of aminopyrine (type I substrate) was inhibited and hydroxylase activity of aniline was enhanced by the anesthetics studied. It is concluded that biotransformation of volatile anesthetics (halothane, methoxyflurane, and enflurane) is preceded by interaction with cytochrome P-450. (Key words: Anesthetics, volatile: halothane; Anesthetics, volatile: methoxyflurane; Anesthetics, volatile: enflurane; Biotransformation, anesthetics.)

IT IS NOW GENERALLY RECOGNIZED that several inhalation anesthetics are metabolized *in vivo*, and that this may be related to their toxicity.[1,2] The enzymes responsible for metabolism of inhalation anesthetics are mixed-function oxidases found within microsomes of hepatic endoplasmic reticulum. The most important component of these enzymes is cytochrome P-450. Cytochrome P-450 has been shown to be the oxygen-activating terminal oxidase for many mixed-function oxidations.[1-6]

The purpose of the present study was to define the nature of the interaction of volatile anesthetics with cytochrome P-450.

Methods

Adult male Wistar rats weighing 200–250 g were pretreated with phenobarbital (80 mg/kg, ip, daily for four days) to increase the amount of hepatic microsomal enzymes by enzyme induction. The animals were sacrificed by decapitation 24 hours after the last dose and the livers removed as quickly as possible. Following irrigation with physiologic saline solution through the portal vein to remove the blood, the livers were minced with scissors and then homogenized with 10 volumes of 0.1 M phosphate buffer, *p*H 7.4, in a motor-driven RKI homogenizer with a Teflon pestle.

Hepatic microsomes were prepared from the resulting homogenate by differential centrifugation, as described below. Cell debris, nuclei, and mitochondria were removed by 15-minute centrifugation at 8,500 rpm and 11,000 rpm, using a Hitachi refrigerated centrifuge. The supernatant fraction was removed and centrifuged for 60 minutes at 105,000 × *g* in a Hitachi ultracentrifuge, following which the microsomal pellet was removed and suspended in 0.15 M KCl prior to being recentrifuged to insure removal of adventitious hemoglobin. The washed microsomal pellet was resuspended in 5 mM $MgCl_2$–50 mM Tris buffer, *p*H 7.4, to give a protein concentration of about 30 mg/ml as measured by biuret methods.

Measurement of microsomal cytochrome P-450 was made by the methods of Omura and Sato.[7] A Hitachi split-beam/dual-wavelength spectrophotometer was used to measure the absorption spectra. It was quantified from the CO-difference spectra of dithionate-reduced microsomes between the peak wavelength of 450 mμ and 500 mμ. An extinction coefficient of 91 cm^{-1} mM^{-1} for cytochrome P-450 was used.

The microsomal suspension, diluted with Tris buffer, was divided equally between two cuvettes and the spectral baseline was recorded with a Hitachi double-beam wavelength-scanning spectrophotometer. Halothane, methoxyflurane, enflurane, or diethyl ether was added as a liquid (about 6 mM) to the sample cuvette and the resultant

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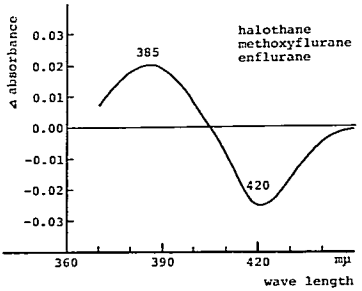

FIG. 1. Spectral change observed on addition of volatile anesthetics to rat hepatic microsomes.

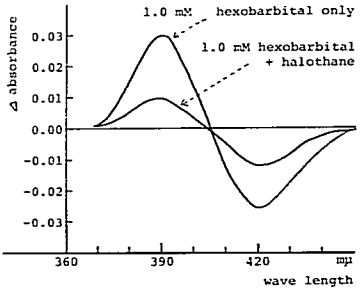

FIG. 2. Effect of halothane on hexobarbital-induced type I spectral change.

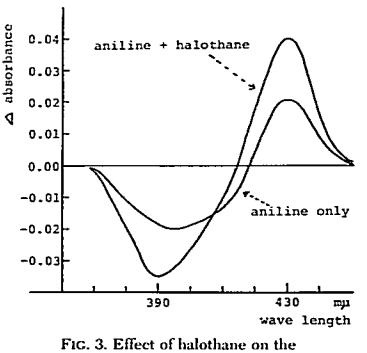

FIG. 3. Effect of halothane on the aniline-induced spectral change.

change in spectrum recorded. (The sum of the absorbance changes at the wavelength of the peak and trough of the difference spectrum was determined in each case.)

To investigate the effects of volatile anesthetics on binding of hexobarbital and aniline to cytochrome P-450, equal amounts of halothane, methoxyflurane, or enflurane were added to the microsomal suspension; each microsomal suspension was divided equally between two cuvettes, and then hexobarbital or aniline was added to the sample cuvettes and the difference spectrum recorded. The spectral dissociation constant (K_s) was obtained by means of a double reciprocal plot of spectral changes associated with the addition of increasing concentrations of aniline to the microsomal suspension with or without volatile anesthetic.[8]

Enzymic activities of the mixed-function oxidase system were determined by aminopyrine and aniline assays in a medium containing 50 mM Tris buffer, pH 7.4, 5 mM $MgCl_2$, and NADPH (25 C). N-demethylase activity of aminopyrine was determined by measuring formaldehyde produced using the Nash reagent. The demethylase reaction was terminated by the addition of 10 per cent trichloroacetic acid to a reaction medium. After removal of the protein by centrifugation at 15,000 rpm for 10 minutes, the supernatant fluid was mixed with Nash reagent and incubated at 58 C for 8 min in a water bath. The samples were then cooled to room temperature and the intensity of the yellow color was determined at 412 mμ.

Ring hydroxylase activity of aniline was determined by measuring P-aminophenol formation. The hydroxylation reaction was terminated by the addition of 20 per cent trichloroacetic acid, and the protein removed by centrifugation at 15,000 rpm for 10 minutes. One ml of the supernatant fluid was added to 1 ml of 0.5 M NaOH containing 1 per cent phenol, and after mixing, 1 ml of Na_2CO_3 was added. After 20 minutes of incubation at room temperature, the intensity of the blue color was determined at 630 mμ.[8,9]

Results

The concentration of cytochrome P-450 in induced rat hepatic microsomes was 2.50 nM/mg protein.

The spectral changes illustrated in figure 1 were obtained by addition of halothane, methoxyflurane, or enflurane to the hepatic microsomal suspension. These spectral changes were characterized by the appearance of a trough at 420 mμ and an absorption peak at 385 mμ (type I). The addition of diethyl ether to the hepatic microsomal suspension produced no spectral change.

As shown in figure 2, the spectral changes caused by addition of hexobarbital (type I substrate) were inhibited by halothane. Methoxyflurane or enflurane produced similar inhibition.

The addition of aniline to the hepatic microsomal suspensions produced a trough at 393 mμ and the simultaneous appearance of an absorption band at 430 mμ (type II), the resulting broad trough giving an asymmetrical appearance to the spectrum. Halothane enhanced the intensity of these spectral changes, and the asymmetry of the trough was removed, as shown in figures 3 and 4, but the K_s value of aniline was not changed (K_s = 0.35 mM). Methoxyflurane and enflurane had similar effects.

N-demethylase activity of aminopyrine (type I substrate) was inhibited by halothane, methoxyflurane and enflurane (fig. 5).

Ring hydroxylase activity of aniline was enhanced by methoxyflurane (fig. 6). Similar responses were observed with halothane and enflurane.

Discussion

Twenty years ago, Brodie and Axelrod showed that the hepatic endoplasmic reticulum or its fragments, microsomes, contain an enzyme system which requires NADPH and molecular oxygen and which is capable of converting various drugs and aromatic substances to more polar, water-soluble compounds.[10,11,12] In 1958, Klingenberg[13] and Garfinkel[14] reported the presence of a carbon monoxide-binding pigment in hepatic microsomes. This pigment was shown by Omura and Sato[15] to be a cytochrome, which was named "cytochrome P-450" from the spectral characteristics of the absorption peak at 450

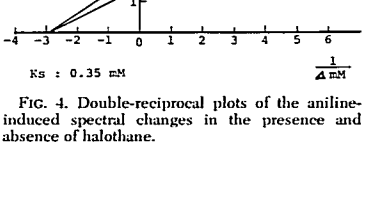

FIG. 4. Double-reciprocal plots of the aniline-induced spectral changes in the presence and absence of halothane.

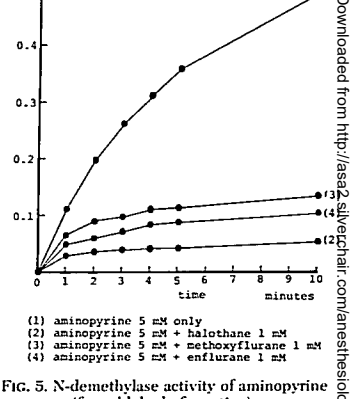

FIG. 5. N-demethylase activity of aminopyrine (formaldehyde formation).

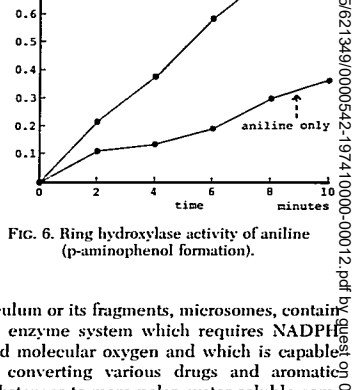

FIG. 6. Ring hydroxylase activity of aniline (p-aminophenol formation).

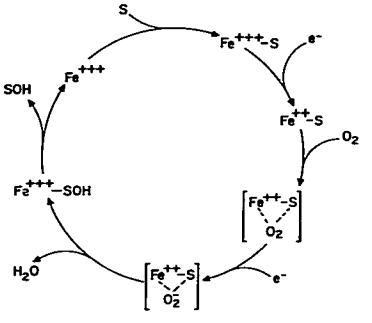

FIG. 7. A schematic representation of the cytochrome P-450 reduction and oxidation cycle during substrate hydroxylation. (The valence state of cytochrome P-450 is indicated by the charge associated with the Fe. Substrate is designated by "S" and the site of interaction of electrons as "e⁻.")

mμ of the CO derivatives of the reduced pigment. It is now recognized that cytochrome P-450 acts as the oxygen-activating enzyme, as well as acting as the site of substrate interaction for oxidative transformation of various lipophilic foreign compounds and possibly also steroids.[16,17,18,19]

A schematic representation of the cytochrome P-450 reduction and oxidation cycle during substrate hydroxylation is illustrated in figure 7. The ferric form of cytochrome P-450 (Fe^{+++}) initially forms a complex with the substrate (S), and is reduced by a suitable electron donor (first electron from NADPH). The ferrous-substrate complex ($Fe^{++} - S$) can then undergo a reaction with molecular oxygen. The interaction with oxygen suggests the formation of an oxygenated intermediate $\left(\begin{smallmatrix} Fe^{++}\text{–}S \\ \diagdown\ \diagup \\ O_2 \end{smallmatrix}\right)$ during the course of the reaction. This oxygen is activated by a second electron $\left(\begin{smallmatrix} Fe^{++}\text{–}S \\ \diagdown\ \diagup \\ O_2^- \end{smallmatrix}\right)$. Finally, one atom of oxygen molecule is incorporated into water, another into substrate, and cytochrome P-450 is reoxidized.[20]

As shown in figure 8, two types of spectral changes result from substrate interaction with cytochrome P-450. One class of spectral change (type I) is characterized by appearance of a trough at 420 mμ and an absorption peak at 385–390 mμ. The second class of spectral change (type II) is characterized by appearance of an absorption peak at about 430 mμ and a trough at about 390 mμ.

In the present experiments, addition of the volatile anesthetics halothane, methoxyflurane and enflurane to rat hepatic microsomal suspensions caused a type I spectral change characterized by the appearance of a trough at 420 mμ with the simultaneous appearance of an absorption band at 385 mμ. Van Dyke and others reported that halothane and methoxyflurane bind to cytochrome P-450, producing a type I spectral change.[2] The present results confirm that the three halogenated anesthetics tested interact with cytochrome P-450 and that volatile anesthetic–cytochrome P-450 complexes are formed, but that metabolism of diethyl ether does not involve an oxidative reaction, as diethyl ether fails to produce this spectral change.

Halothane, methoxyflurane, and enflurane acted as competitive inhibitors of the spectral change produced by addition of hexobarbital (type I substrate), while the spectral change caused by addition of aniline (type II substrate) was increased in amplitude when these volatile anesthetics were added. These

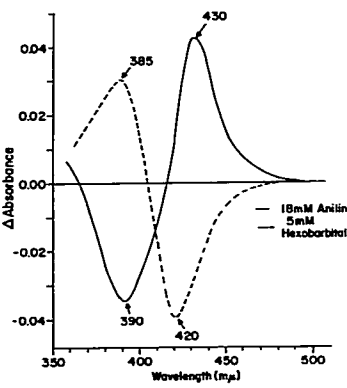

FIG. 8. Spectral changes caused by addition of aniline and hexobarbital to suspensions of rat hepatic microsomes.

findings agree with those in the previous studies of Schenkman and Leibman, in that type I substrates enhance type II spectral changes, while type I substrates competitively inhibit type I spectral changes.[22]

In the present study, N-demethylase activity of aminopyrine (type I substrate) was also inhibited and hydroxylase activity of aniline (type II substrate) was enhanced by addition of halothane, methyoxyflurane, or enflurane (type I substrate).

These phenomena could be explained on the basis that type I substrates act as competitive inhibitors to type I substrates, and that type I substrates change the hemoprotein to a form in which it could readily react with a type II substrate.

The present findings indicate that biotransformation of halothane, methoxyflurane, and enflurane is dependent upon cytochrome P-450 (oxygen-activating terminal oxidase of mixed-function oxidase system), that induction of microsomal mixed-function oxidases in individuals changes the rates of metabolism of halothane, methoxyflurane, and enflurane, and that halothane, methoxyflurane, and enflurane competetively inhibit cytochrome P-450 metabolism of other substrates.

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