Chinese Chemical Letters Vol. 12, No. 5, pp. 449 – 452, 2001

In Vitro Interaction of Nicotine and Hemoglobin under Liver Cell Metabolizing Condition

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Abstract: The *in vitro* interaction of nicotine and hemoglobin (Hb) in a metabolizing system was studied by spectroscopy assays. Visible spectra showed two isobestics, and fluorescence spectra showed static quenching with increasing of nicotine dose. Meanwhile, the CD spectra intensity reduced, showing the conformation of Hb varied markedly through the interaction. All these results suggested that the interaction of nicotine or its metabolites and Hb might do harm to physicological function of Hb.

Keywords: Nicotine, hemoglobin, visible absorption, fluorescence, CD.

Nicotine, 3-(1-methyl-2-pyrrolidinyl)pyridine, the main alkaloid in tobacco products, has been well known for its addiction effect. Habitual smokers are usually maintaining nicotine concentration constant in their blood at about 10-15 ng/mL[1]. Therefore the interaction of nicotine and hemoglobin (Hb) is essential to be studied. Our recent study by accelerator mass spectrometry (AMS) has shown that nicotine could form adducts with Hb after *in vivo* administration, and there is a positive dose response correlation[2]. Since Hb is the main blood protein that carries oxygen for man, the adduction of nicotine with Hb may change the structure of Hb, doing some harm to the function of Hb. The present study focuses on the chemical interaction of nicotine and Hb by employing liver cell culture to imitate *in vivo* metabolism. Visible, fluorescence and circular dichroism (CD) spectrometries were used in the study.

Experimental

Liver cells were isolated from fresh Kun Ming mice (~20 g) liver according to reference[3]. The fresh liver tissue was picked up and washed it with D-Hanks buffer pH 7.2 (prepared following reference[4]) for 3 times to remove fat, blood and connective tissues. Then sheared it into 1 mm^3 mince and washed again by D-Hanks for 3 times. Later, ~50 mL 0.25% trypsin solution was added and it was digested at 37℃ for 20-40 min. Then it was filtered through two layers of gauze to remove undigested tissue and centrifuged at 1000 rpm for 10 min. The harvested cells were washed and centrifuged again, and it was suspended in 3 mL DMEM culture media (prepared following reference[4]) for use. All the process was operated under asepsis. Trypan blue dyeing assay showed the cell

survival ratio >90%.

The *in vitro* interaction experiments were performed as following: 0.5 mL liver cell suspension was mixed with a certain amount of nicotine (0, 0.7, 2.8 and 5.6 mg) in the culture solution at 37°C for 4 h, then 46 mg Hb was added to the mixture and the total volume was adjusted to 2 mL. After incubating at 37°C for another 2 h, the mixture was centrifuged at 1000 rpm and the supernatant was collected. The supernatant was dialyzed against 0.1 mol/L phosphate buffered saline (PBS) for 24 h, with 4 times renewal. Finally, the obtained Hb solution was diluted to proper concentration and assayed by visible spectrometry, fluorescence spectrometry and circular dichroism (CD) spectrometry.

Hemoglobin (human, oxide) was purchased from HuaMei Bioengineering Co., Beijing, China. Nicotine (>98%) was purchased from Aldrich, USA. Visible absorption spectra were recorded in a Shimadzu UV 240 spectrophotometer. Fluorescence measurements were performed in a Shimadzu RF540 spectrophotometer. Circular dichroism (CD) spectra were obtained by a JASCO-500 CD spectrophotometer.

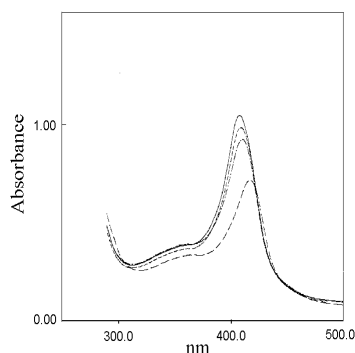
Results and Discussion

The visible spectra of Hb after reacting with nicotine are shown in **Figure 1**. Two isobestic points appear at 310 nm and 425 nm, as nicotine concentration increases. Meanwhile, the maximal absorption peak of Hb shifts to longer wavelength and lowers. That is similar to what have been reported in other studies[5], when biomacromolecule reacts with small molecule and forming a mutualconvertible complex. Since the absorption peak around 400 nm is owing to the Soret band of Hb[6], resulting from the π-π^* transition between porphyrin and surrounding aromatic amino acids, the changes around this region may be assigned to the interaction of nicotine or its metabolites with Hb porphyrin and/or its surrounding amino acids (Trp, Tyr, Phe, *etc*.).

After interacting with nicotine in the above system, the fluorescence spectra of Hb also varies. A static quenching phenomenon is observed, as nicotine concentration increases from top to bottom in **Figure 2**. The maximal fluorescence peak shifts blue. This result together with the above visible absorption result indicates that nicotine or its metabolites might interact with Hb forming adducts or complexes. Since these adducts or complexes can not be removed from Hb by dialysis, the interaction between them is presumably occurred through covalent binding. This kind of interaction may influence the conformation of Hb. The following results of CD study could support this point.

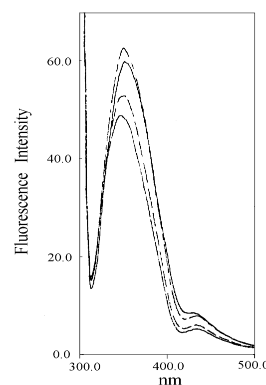
Figure 3 presents the CD spectra of Hb after interacting with nicotine *in vitro*. The bottom line is a typical CD spectrum of Hb in absence of nicotine. The peaks between 210-220 nm indicates the α-helix structure of normal Hb, with $[\theta]_{220\ nm}$=-22500 $deg \cdot cm^{-2} \cdot mol^{-1}$, similar to that reported in reference[7]. With increasing of nicotine dose, their intensities reduce gradually. When nicotine dose is 16.8 mmol/L, $[\theta]_{220\ nm}$ reduces to -13400 $deg \cdot cm^{-2} \cdot mol^{-1}$. According to reference[8], it can be calculated that the α-helix structure reduces about 40%. The conformation of Hb is changed by interaction of nicotine with Hb.

Figure 1 Visible absorption spectra of Hb after reacting with nicotine *in vitro*



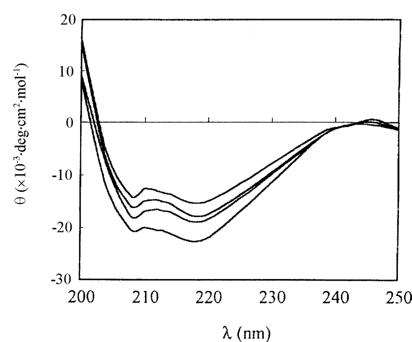
Concentrations of nicotine are 0, 2.1, 8.4, 16.8 mmol/L from top to bottom. Hb concentration in measurement is 0.94 mg/mL.

Figure 2 Fluorescence spectra of Hb after reacting with nicotine *in vitro*



Concentrations of nicotine are 0, 2.1, 8.4, 16.8 mmol/L from top to bottom. Hb concentration in measurement is 0.94 mg/mL. The excitation wavelength is 285 nm.

Figure 3 CD spectra of Hb after reacting with nicotine *in vitro*



Concentrations of nicotine are 0, 2.1, 8.4, 16.8 mmol/L from bottom to top.
Hb concentration in measurement is 0.94 mg/mL.

To sum up, the results of the three assays clearly demonstrate that the interaction of Hb and nicotine in this metabolizing system *in vitro* does form some kinds of adducts or complexes, which demonstrate different spectra characteristics(*e.g.* molar extinction coefficients) from normal Hb. Such interaction causes the molecular conformation of Hb varied, thus does some harm to the physicological function of Hb.

Acknowledgments

We thank the financial support of the National Natural Science Foundation of China (Grant. No. 19935020) and Research Fund for Doctoral Program of Higher Education.

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Received 17 October, 2000