EFFECT OF INTRAVENOUS INJECTION OF IRON ON INDUCED PULMONARY CYTOCHROME P450 1A1 (CYP1A1) AND CONSTITUTIONAL CYP2B1 ACTIVITIES

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Cytochrome P450s (CYPs) are membrane-bound heme-containing proteins (hemeproteins) that catalyze oxidation of xenobiotics producing less lipophilic substances thereby facilitating their excretion. The heme moiety of this protein is iron-protoporphyrin IX, which plays a major role in the catalytic activity of the enzyme. We hypothesized that the bioavailability of iron modifies the activity of inducible (CYP1A1) and constitutional (CYP2B1) CYP isozymes in rat lungs. Therefore, 39 male Sprague Dawley rats were allocated into three groups. The first group was injected IV with 50 mg /kg BW iron dextran. The second group was injected IV with 200 mg /kg BW iron dextran. The third group was injected with saline as a control. Eleven days later, all rats were injected intraperitoneally with the classic CYP1A1 inducer, beta-naphthoflavone (BNF; 50 mg/kg BW), to induce CYP1A1. Three days after BNF injection, rats were sacrificed and the lungs were perfused by phosphate buffer saline (PBS) through pulmonary artery to wash off the blood for measuring nonheme iron. Immediately after washing, the microsomes of the right lung lobes were freshly prepared to measure the CYP1A1-dependant enzymatic activity [ethoxyresorufin – O - deethylase (EROD)] and the CYP2B1-dependent enzymatic activity [pentoxyresorufin – O - deethylase (PROD)]. Before separation of microsomes by differential centrifugation, an equal portion of lung homogenate was freeze-dried for measurement of lung iron. The left lung lobes were collected in 10% neutral buffer formalin to prepare slides for staining with immunofluorescence for CYP1A1 and alveolar cell marker (cytokeratin 8) The results showed that iron content of the lung was increased in rats receiving 50 mg/kg (310 $\pm$ 31.45 µg/gm) and 200 mg/kg (796 $\pm$ 37.8 µg/gm) compared to control (180 $\pm$ 13.33 µg/gm). However, the activities of EROD and PROD were diminished with increasing iron content of the lung. Supporting that result was the reduction of the red fluorescence signal in animal receiving iron dextran compared to control. Taken together, these results suggest that increasing lung iron content modifies xenobiotic metabolism in lung by suppressing the activity of CYP2B1 and inducible CYP1A1. Further studies are therefore required to investigate the mechanism of iron-induced suppression of these metabolizing enzymes

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INTRODUCTION

Cytochrome p450 1A1 (CYP1A1) is a heme-containing metabolizing enzyme that is induced by polycyclic aromatic hydrocarbons (PAHs), such as the benza(a)pyrene in cigarette smoke. CYP1A1 activates pro-carcinogenic materials to produce potential carcinogenic intermediates through phase I enzymatic reaction. During phase II enzymatic reactions, these metabolites are further detoxified via conjugation in the presence of other metabolizing enzymes, such as glutathione S-transferases (GST) (**Ahmad et al., 2008)** to form less lipophilic materials, thus facilitating their excretion (**Mucci et al, 2001**). Therefore, the excessive expression of CYP1A1 enzyme may produce large amount of potential reactive carcinogenic intermediates. These reactive intermediates may covalently interact with DNA nucleotide bases, particularly guanine and adenine, producing DNA adducts with potential mutations of important genes. Cells with damaged DNA may be removed by apoptosis (programmed cell death) **(Venkatachalam et al, 1993**). Alternatively, if a permanent mutation occurs in a critical region, an oncogene may be activated, or a tumor suppressor gene, such as p53 may be inactivated (**Wang et al, 1995**). The excessive presence of such mutational events leads to the loss of normal growth control and, ultimately, to lung cancer (**Arif and Gupta, 1997; Liang et al, 2003**). Therefore, studying the modification of CYP1A1 induction is extremely important in terms of its relation to lung canrcinogenesis.

CYPs are hemeproteins that consist of a protein (the apoprotein or apoenzyme) and a heme moiety, called iron-protoporphyrin IX (**Testa, 1995**). The significance of the liganded heme in CYPs structure is extremely important. It can exist in different electronic states (either ferric or ferrous oxidation state) which are responsible for many of the properties of CYP, most significantly for the binding of ligands and the activation of molecular oxygen (**Testa, 1995**). Therefore, the heme portion is crucial for the metabolic activity of CYP enzymes.

Data concerning the effect of iron on CYP1A1 and other forms of cytochromes are contradictory and not clear. Some studies showed that

intratracheal instillation of benza(a)pyrene, which is a polycyclic aromatic hydrocarbon inducer for CYP1A1 similar to beta-naphthoflavone (BNF), coated with iron hematite significantly increased CYP1A1 protein concentration and EROD activity in rats (**Garcon et al., 2004**). Moreover, **Madra et al. (2004)** attributed the mechanism of the iron-enhanced toxicity to the oxidative damage associated with chronic induction of CYP1A1 isoforms. However, other investigators reported that CYP1A1-depenedant metabolic activity (EROD) induced by the polycyclic aromatic hydrocarbon hexachlorobenzene was significantly depressed in rats with high iron load produced by intraperitoneal injection of iron dextran (600mg/kg Fe) (**Smith et al., 1993**).

Therefore, this work was designed to establish a relationship between the iron content of the lung and the induced activity and alveolar localization of CYP1A1 and the activity of other non-inducible CYPs, more specifically CYP2B1.

MATERIALS AND METHODS:

Animals and Experimental Design

Thirty nine male Sprague-Dawley rats (~220-270g) were purchased from Hilltop Labs (Scottsdale, PA). Food and water were supplied ad libitum. Rats were housed in Shoebox cages on autoclaved hardwood and cellulose (Alpha-Dri) bedding in HEPA filtered laminar-flow, ventilated cage racks (Thoren). Rats were allowed to acclimatize in their cages for at least 7 days before the experiment. They were randomized and assorted into 6 groups by using a research randomizer program (http://www.randomizer.org). 13 rats were assigned to each group (3 groups) based upon the power analysis using the means and standard deviation of the previous experiments. Summary of experimental design is shown in Table 1.

Table 1: Experimental design showing the different groups and treatments.

Group	Number of animals	IV injections	IP injections
1 (control)	13	Saline	BNF
2 (low iron)	13	Iron dextran (50 mg/kg BW)	BNF
3 (high iron)	13	Iron Dextran (200 mg/kg BW)	BNF

Iron Dextran

Iron dextran (Dexferrum, American Regent Laboratories, INC, Shirley, NY 11967) was used for intravenous injection (IV). Two doses were used; low dose (50 mg/kg) and high hose (200 mg/kg). Since the iron dextran used in this study contains sodium chloride for tonicity, we injected the normal saline (0.09 % sodium chloride, Abbot Laboratories, North Chicago, IL) at a dose of 0.0004 ml/gm IV) as a control vehicle. For the higher dose of iron, 0.004 ml/gm BW was administered IV. For the lower dose of iron, the solution was diluted 4 times with normal saline and 0.004ml/gm BW was administered. Acepromazine maleate (Phoenix Pharmaceutical INC., St. Joseph, MD) was injected with a dose of 2mg/kg intramuscular (IM) as a tranquilizer. Acepromazine has the advantage of

producing peripheral vasodilatation that could be helpful in the IV injection of iron dextran and saline.

Beta-naphthoflavone Preparation

Beta-naphthflavone (BNF) (Sigma) was injected intraperitoneally (IP) as an inducer for CYP1A1 while the control group received corn oil as a control vehicle. Solutions of 5% of BNF in filtered corn oil were prepared as previously described (**Ghanem et al., 2004)**

Necropsy and Collection of Tissues

Rats were necropsied after euthanasia using 0.5 ml 26% sodium pentobarbital (Sleepaway®, Fort Dodge Animal Health, Fort Dodge, IA, USA) then the aorta was transected as previously described (**Ghanem et al., 2006).** The lungs and attached organs including tracheal bronchial lymph nodes, thymus, heart, aorta, and esophagus were then removed. The lungs were perfused by phosphate buffer saline as describes later then the right lung lobes were collected and weighed for microsomal preparation. On the other hand, the left lung lobes were inflated with 3 cc neutral buffer formalin (NBF) 10% to prepare formalin-fixed tissues for immunofluorescence staining. Fixed tissues were trimmed the same day, routinely processed in a tissue processor and embedded in paraffin the following morning. Five-micrometer lung tissue sections were used for immunofluorescence staining of CYP1A1 and cytokeratin 8.

Lung Perfusion Technique:

Immediately after necropsy, the left atrium was transected and the right ventricle was injected with a 10 cc Phosphate buffer saline (PBS) free from magnesium and calcium (BioWhittaker A Cambrex company) to allow the fluid to pass into the lungs through the pulmonary arteries. The fluid (Mg and Ca free) was observed coming out of the transected left atrium allowing perfusion of the lungs and removing the intravascular blood. The process of infusion may be repeated until the lung color turns white, indicating no blood within lung capillaries. The lung perfusion was conducted to clear lung from blood before measuring the iron content (nonheme iron).

Preparation of Freeze-Dried Lung Tissue

Approximately 5 % of the right lung tissue was chopped and homogenized and then lyophilization occurs by freezing the sample at -80ºC and then placing in the vacuum freeze dryer (Edwards- model Pirani 1001) as previously described (**Nettelbladt et al., 1989**). The dried lung tissue was kept at -20 ºC until measuring of iron content.

Measurement of Iron Content of the Lung

Iron concentration was measured with a standard colorimetric assay (Sigma, St. Louis, MO) as previously described (**Ghio et al., 2008**). The lung concentration was expressed as µg/mg fried lung tissue.

Microsomal Preparation:

Microsomal preparations were prepared as previously described (**Ma et al, 2002**). Briefly, at the day of necropsy, the right lung lobes were weighed and shopped for 4 times. The chopped tissue was homogenized with phosphate buffer solution at a ratio of 1gm lung/4 ml phosphate buffer. The homogenized solution was then centrifuged and the supernatant was ultracentrifugated to obtain the microsomal pellet. The pellets were then resuspended in buffer at a ratio of 1gm lung / 1 ml buffer and kept at -80 ºC until assayed.

The Bicinchoninic Acid (BCA) Method for Determination of Total Lung Protein

The protein content of lung microsomes was measured by the bicinchoninic acid (BCA) method as previously described (**Smith et al, 1985, Ma et al, 2002**) using the BCA protein assay kit (Pierce, Rockford, IL) in a spectra Max 250 Spectrophotometer (Molecular Devices Corporation, Sunnyvale, California). Bovine serum albumin was used as the standard.

Dual Immunofluorescence

The formalin-fixed left lung tissues were used for immunofluorescence. The immunofluorescence was performed for the CYP1A1 and cytokeratin 8, a cytoskeletal protein used as a marker of alveolar type II cells. Immunofluorescence was a 2-day procedure as previously described (**Ghanem et al., 2006**). On day one, the slides were heated in oven at 60 ºC for 20 minutes. Then deparafinization in 3 baths of xylene 6 minutes each, 3 minutes in 100 %

alcohol, 3 minutes in 90 % alcohol, 3 minutes in 80 % alcohol, and 5 minutes in distilled water. Antigen retrieval was maximized by using EDTA retrieval method. Slides immersed in EDTA (0.01M, pH 8) were heated for 1.45 minutes on high then 6 minutes on defrost in the microwave. Defrosting was repeated after addition of more EDTA. The slides were removed from microwave and allowed to stand for 20 minutes for coaling. To avoid the non-specific binding, the tissue samples were blocked with 5 % BSA/PBS (IgG free) (Sigma) for 10 minutes at room temp. (RT). Then the slides were rinsed with distilled water and blocked with 5% pig serum (Biomeda) for 10 minutes at RT (pig serum was diluted with PBS) . The slides were then rinsed with distilled water and primary antibodies were applied. For cytokeratin 8 staining, sheep anti-human antibody was used diluted 1: 10 with PBS. For CYP 1A1 staining, Rabbit anti-rat antibody (Xenotech) was used and diluted 1: 5 with the diluted Cytokeratin 8 antibody. Both primary antibodies were applied by utilizing the capillary action between 2 slides that help withdraw the antibodies. Then the slides were incubated overnight at RT through which the primary antibody is allowed to bind to the antigen (CYP1A1 and Cytokeratin 8). On the second day, the slides were placed in the oven at 37 °C for 2 hours. Then the slides were rinsed with distilled water and the secondary antibodies were applied. For cytokeratin 8 staining, donkey anti-sheep antibody (Alexa 488) was diluted 1:20 with PBS and centrifuged for 5 minutes and only the supernatant was applied to the slides. For CYP1A1 staining, goat antirabbit antibody (Alexa 594) was diluted 1:20 with PBS and spun for 5 minutes and only the supernatant was applied. The secondary antibodies were allowed to bind the primary antibodies for 2 hours in the dark. Anti-fade gel and cover slip were applied and slides allowed to dry for 2 hours after which the slides were ready for examination under the fluorescent microscope.

Statistical Analysis

The means of different groups were compared by using one-way analysis of variance (One-way ANOVA) followed by Holm-Sidak post hoc test. The statistical analysis was performed by using Sigma Stat software (version 3.1,

SPSS Inc., Chicago, IL). The data were expressed as means ± standard error. The differences in means were considered significant if $P < 0.05$.

RESULTS

1- Effect of Iron Dextran Administration on CYP1A1-Dependent EROD Activity and CYP2B1-Dependent PROD Activity

EROD activity was significantly reduced by IV administration of iron dextran in induced lung microsomes of rats receiving 50 mg/kg BW and 200 mg/kg BW compared to control rats injected with BNF and saline (**Figure** 1A). PROD activity was reduced by the IV administration of iron dextran in induced lung microsomes received 50 mg/kg BW and was significantly reduced in induced rats injected with 200 mg /kg iron dextran compared to rats injected with BNF and saline (**Figure 1B**).

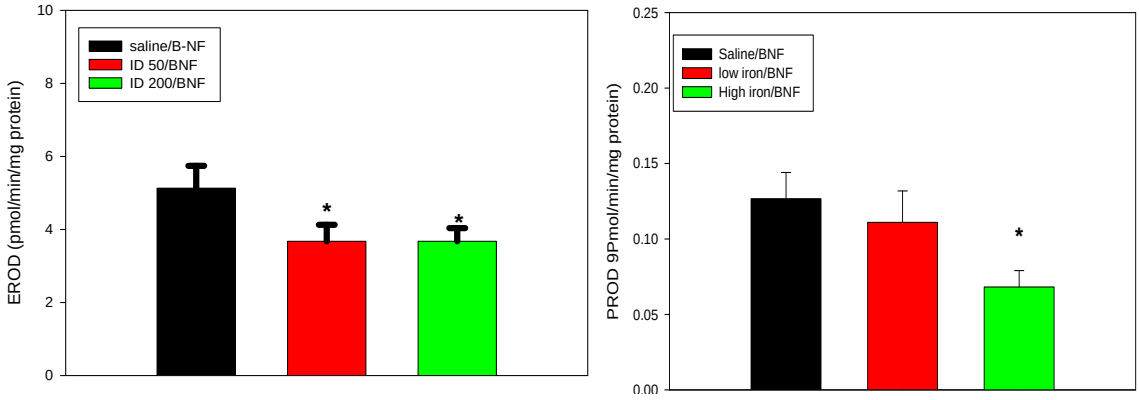

Figure 1. The enzymatic activity of induced CYP1A1 (EROD) (A) and the constitutional CYP2B1 (PROD) (B) are significantly reduced by IV administration of 50 and 200mg/kg iron dextran. The bars represent means and standard errors. Asterisk indicate significant difference from control when $P < 0.05$.

2- Effect of IV Administration of Iron Dextran on Iron Content of lungs

The iron content of the lung was significantly increased by intravenous injection of 50 and 200 mg/kg compared to control injected with saline (Figure 2)

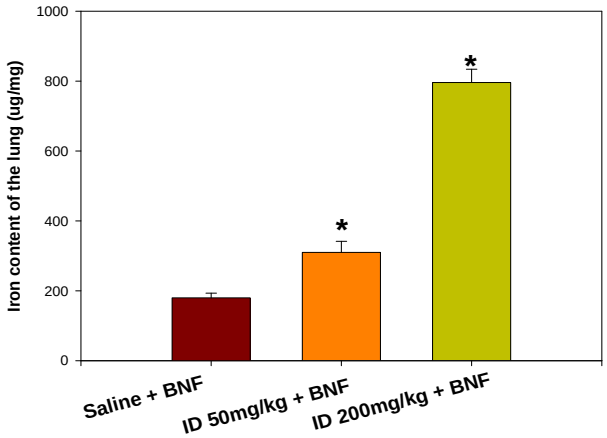

Figure 2. The iron content of the lung is significantly increased by intravenous injection of 50 and 200 mg/kg compared to control injected with saline. $P < 0.05$.

4- Effect of IV administration of iron cellular expression of CYP1A1

The cellular expression of CYP1A1 produced red color with indirect immunofluorescent staining. On the other hand, green color indicated staining of alveolar cells, more specifically the alveolar type II cells. IV administration of iron reduced the red signal in rats given 50 and 200 mg/kg iron dextran (Figure 3)

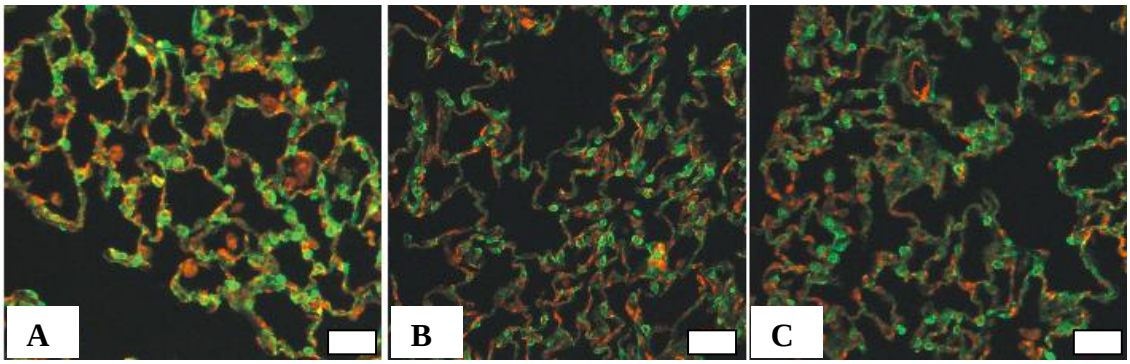

Figure3. Double labeled immunofluorescence for CYP1A1 (red) and alveolar cell marker (green, cytokeratin 8). The red color of CYP1A1 is reduced in animals injected with 50 mg/kg (B) and 200 mg/kg (C) iron dextran saline (A) compared to control animals injected with saline (A). Magnification bar is 20 microns.

DISCUSSION

CYP1A1 has recently acquired much attention worldwide because of its critical role in the metabolic activation of procarcinogens, such as the benzo(a)pyrene in cigarette smoke (**Endo et al., 2008**). Because the metabolic activity of this enzyme is mainly dependent upon its heme moiety (**Testa, 1995**), it is important to study the effect of increasing iron bioavalability in alveolar tissue on its activity and alveolar expression.

We have selected the iron dextran solutions for increasing iron content of the lung because this compound has been previously used as an effective and safe method for treatment of iron deficiency anemia in animals such as lambs (Green et al., 1997) and calves (Heidarpour Bami et al., 2008) as well as pregnant women (Ayub et al., 2008). To avoid contamination of lung tissue with blood heme, we perfused lungs to remove the blood from capillaries before freeze-drying of lung tissues. As expected, the intravenous injection of iron dextran significantly increased the iron content of lung compared to lungs in animals injected with saline (control). Surprisingly, the enzymatic activity of induced CYP1A1 (EROD) and constitutional CYP2B1 were significantly reduced in animals injected with iron dextran. This result indicated that the loading of lung tissue with iron had a suppressive effect on pulmonary CYP enzymes. This result is consistent with that obtained by Smith et al. (1993). Our results were confirmed by immunofluorescet staining of CYP1A1 in lung tissue sections. The red color of CYP1A1 was clearly reduced in rats administered with iron dextran, suggesting a reduction in cellular CYP1A1 protein. On the other hand, Garcon et al., (2004) demonstrated that intratracheal instillation of benza(a)pyrene, which is a PAH inducer for CYP1A1 similar to beta-naphthoflavone (BNF), coated with iron hematite significantly increased CYP1A1 protein concentration and EROD activity in rats. However, our result could not be compared to this study because the CYP1A1-inducer was directly instilled into the lung through the tracheal route and the iron was coated to the benzo(a)pyrene.

The mechanism by which iron load of lung suppresses CYP1A1 protein and activity and CYP2B1 activity is not fully clear. It has been found that in rodent liver, oxidative stress may be caused by iron overload through enhancement of glutathione synthesis (**Brown et al., 2003**). Recent findings suggest that phase I xenobiotic metabolism enzymes are sensitive to oxidative stress (**Bassi et al., 2000**). Moreover, down-regulation of P450 1A1 and 1A2 gene expression was reported to occur in cultured cells exposed to H2O2 (**Barker et al., 1994; Morel and Batouki, 1998**). Therefore, reduction of CYP1A1 activity and protein could be attributed to the oxidative stress caused by overload of lung tissue with iron.

In conclusion, this work demonstrated for the first time a relationship between increased lung iron concentration and CYP1A1 protein and activity and CYP2B1 activity. However, the mechanism by which pulmonary iron load suppresses cytochrome isoforms requires further investigations.

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الملخص العربى

تتكون انزيمات السيتوكروم 450 من بروتينات محتوية على عنصر الحديد وتقوم بتحويل الملوثات الخارجية الى مواد اقل دهنية لتسهيل اخراجها من الجسم ويعتمد نشاط هذة الانزيمات على وجود عنصر الحديد فى تركيب هذة الانزيمات. ولذلك تم اختبار النظرية الافتراضية بان وجود عنصر الحديد ممكن ان يغير نشاط هذة الانزيمات وخاصة CYP1A1 وCYP2B1 . ولذلك استخدم 39 فار قسمت الى ثلاث مجموعات. المجموعة الأولى تم حقنها فى الوريد بمركب ديكستران الحديد بجرعة 50 مج/كج من وزن الجسم . المجموعة الثانية تم حقنها فى الوريد بمركب ديكستران الحديد بجرعة 200 مج/كج من وزن الجسم. المجموعة الثالثة تم حقنها فى الوريد محلول الفسيولوجى واتخذت كمجموعة ضابطة. بعد 11 يوما تم حقن كل الفئران بمادة البيتانافثوفلافون (50مج/كج) فى البريتونى لزيادة انتاج انزيم CYP1A1 . بعد 3 ايام تم قتل الئران ثم غسلت الرئة بمحلول الفوسفات المتعادل من خلال الشريان الرئوى لازالة الدم من الرئة حتى يمكن قياس محتوى الحديد الغير دموى فى الرئة. بعد ذلك تم تحضير ميكروسومات الرئة من الرئة اليمنى لقياس نشاط انزيمى (EROD) CYP1A1 و (PROD) CYP2B1 . قبل فصل اليكروسومات تم اخذ جزء مماثل من خليط النسيج الرئوى وذلك لتجفيفه عن طريق التجميد لقياس المحتوى الرئوى للحديد. كما تم اخذ الرئة اليسرى فى فورمالين متعادل (10%) لتحضير شرائح تصبغ بالفلروسينت المناعى لكل من CYP1A1 و محدد الخلايا الرئوية الحويصلية (السيتوكيراتين 8). أظهرت النتائج ان حقن محلول ديكستران الحديد ادى الى زيادة نسبة الحديد الغير دموى فى الرئة ومع ذلك نقصت انشطة انزيمات ال CYP1A1 و CYP2B1 فى الميكروسومات. ومما دعم هذه النتائج نقص اللون الاحمر الفلوريسنتى الدال على وجود انزيم CYP1A1 فى الخلايا الرئوية للحيوانات المحقونة بمركب ديكستران الحديد. خلصت النتائج الى ان زيادة محتوى الرئة من الحديد يؤثر على التمثيل الايضى للملوثات الخارجية التى تدخل الرئة عن طريق تثبيط نشاط وبروتينات انزيمات السيتوكروم الرئوية وخاصة CYP1A1 و CYP2B1 ولكن كيفية هذا التثبيط غير محددة حتى الان وتحتاج الى دراسات مستقبلية.