

# Role of Nuclear Factor Erythroid 2-Like 2 in the Induction of Cytochrome P450 2a5 *in Vivo* of Nonalcoholic Fatty Liver Disease

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## Abstract

Mouse cytochrome P450 2A5 (Cyp2a5) is upregulated in various pathophysiological liver disease and induced by structurally variable hepatotoxic chemicals. A putative common feature for all of these conditions is altered cellular redox status. Nuclear factor erythroid 2-like 2 (Nrf2) is a transcription factor that is post-translationally regulated by oxidative stress and controls the transcription of numerous protective target genes. In order to determine if upregulation of Cyp2a5 by Nonalcoholic fatty liver disease (NAFLD) was mediated through Nrf2, we applied Nrf2 knockout mice (*Nrf2*<sup>-/-</sup>) to examine the Cyp2a5 mRNA and protein levels compared to wild type mice. Furthermore, the interaction of Nrf2 with Cyp2a5 was confirmed by co-immunoprecipitation (co-IP) *in vivo*. The NAFLD increased the Nrf2 mRNA and protein levels in wild type mice and also increased the Cyp2a5 mRNA and protein levels resulted from translocation of Nrf2 into the nucleus. However, the expression and activity of Cyp2a5 were no difference in the Nrf2 knockout mice. Nuclear immunohistochemical staining of Nrf2, an indicator of activation of the transcription factor, was evident in NAFLD. Co-IP experiments showed that Nrf2 binds to the Cyp2a5. Our current results unequivocally show that expression of Cyp2a5 is tightly controlled by Nrf2 in liver. Nrf2 is needed for constitutive expression of Cyp2a5, and Cyp2a5 is also sensitively upregulated by an increased level of Nrf2 protein. Therefore, Cyp2a5 upregulation could be a useful indicator for hepatic activation of the Nrf2 pathway.

**Keywords:** Coumarin 7-hydroxylase; Oxidative stress; High-fat diet; Knockout mice; Pyrazole

**Abbreviations:** ARE: Antioxidant Response Element; Nrf2: Nuclear Factor Erythroid 2-like 2; KO: Knockout; WT: Wild-Type; P450: Cytochrome P450; NASH: Non Alcoholic Steato Hepatitis; NAFLD: Non Alcoholic Fatty Liver Disease; co-IP: Co-Immuno Precipitation

## Introduction

Nonalcoholic fatty liver disease (NAFLD) composes a spectrum of etiologies ranging from simple fatty liver (steatosis) to the more severe Nonalcoholic Steatohepatitis (NASH). The proposed mechanism for progression of NAFLD involves a two-hit theory where lipid accumulation in hepatocytes (the “first hit”) is followed by a “second hit,” including insulin resistance, oxidative stress, and cytokine production [1]. Several of the factors regulating hepatic CYP2E1 and CYP4A expression have been linked to the pathogenesis of hepatic steatosis and NASH. In the context of hepatic steatosis, both CYP2E1 and CYP4A generate the “second hit” of cellular injury, particularly when antioxidant reserves are depleted [2]. In contrast, relatively little data has been reported with respect to the effect of NAFLD on hepatic Cyp2a5.

The mouse Cyp2a5 and its human ortholog CYP2A6 mediate the phase I metabolism of thousands of endogenous and xenobiotic substrates, including environmental pollutants, pharmaceuticals, steroids, prostaglandins, and fatty acids [3]. It is induced by classical inducers, such as pyrazole and carbon tetrachloride [4,5]. Elevated CYP2A5/CYP2A6 protein levels in hepatocytes are associated with the development of liver tumours in mice [6] and in humans [7] respectively. It has been proposed that Cyp2a5 may be one of the major xenobiotic-metabolizing CYP forms in a damaged liver [8]. However, till now, the mechanism(s) by which these xenochemical inducers and pathophysiological stressors, such as oxidant stress, regulate hepatic Cyp2a5 in mouse tissues are poorly understood, and little

data has been reported with respect to the effect of NAFLD on hepatic expression of Cyp2a5.

Recent studies have indicated that induction of Cyp2a5 is not directly related to the nature of the inducing agents, but instead may be an indirect consequence of a specific cellular event associated with the pathogenesis of liver injury. For example, certain agents may disturb cellular redox status, a common denominator that may consequently induce the expression of CYP2A5 through activation of stress-related transcription factors, such as the transcription factor nuclear factor E2-related factor 2 (Nrf2) [9-11]. Nrf2 is primarily a cytosolic protein due to proteasomal degradation regulated by Keap1 [12,13]. When a cell is undergoing oxidative stress, Nrf2 escapes Keap1-mediated degradation, translocates to the nucleus, binds to ARE sequences within the promoters of target genes, and induces expression of phase I and II metabolizing enzymes and transporters pivotal to the maintenance of oxidative stress-inducing molecules [14]. Until present, it is unclear to whether induction of Nrf2 can induce Cyp2a5 and the Nrf2 interaction with Cyp2a5 in NAFLD.

The aim of the present study was to elucidate the potential role of Nrf2 in NAFLD-mediated Cyp2a5 regulation. The results show existence of Nrf2 binding Cyp2a5 and that NAFLD activates binding

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of Nrf2 to Cyp2a5, which in turn up-regulates the Cyp2a5. The present evidence represent a novel mechanism in the regulation of phase I xenobiotic-metabolizing gene.

## Materials and Methods

### Animal studies

All animals received proper care in agreement with animal protocols approved by the Institutional Animal Use and Care Committee of the University of Northeast Agricultural. Six to eight-week-old, male ICR wild-type (*Nrf2*<sup>+/+</sup>) and Nrf2 knockout mice (*Nrf2*<sup>-/-</sup>) mice were purchased from the Animal Experiment Center of General Hospital of Nanjing Military Area Command [Certification No. SCXK (JUN) 2007-012]. The mice were treated for eight consecutive weeks, the control group (Control) was fed with the standard diet, high-fat group (NAFLD) was fed with a high-fat feed (68.5% standard diet, 15% lard, 1% cholesterol, 0.5% bile and 15% dextrin) [15], and the pyrazole group was used as positive control (PRY+) and fed with the standard diet but injected intraperitoneally with pyrazole (150 mg/kg body wt) once per day in the last three days before sacrificed [16]. Twenty-four h after the last pyrazole injection, the mice in all groups were euthanized with CO<sub>2</sub> gas. The livers were removed, rinsed in ice-cold saline, and divided for various assays, as outlined below.

### Immunofluorescence assay

Hepatocytes were isolated by adaptation of the calcium two step collagenase perfusion techniques as previously described [17,18]. Hepatocytes were plated on collagen coated six well plates at 250 000 cells/well. The cells were grown on glass coverslips in 24-well plates, then were washed with PBS, fixed with 4% paraformaldehyde for 10 min at RT, permeabilized for 5 min with 0.2% Triton X-100 and blocked for 45 min with 5% bovine serum albumin in PBS. The cells were next incubated with primary antibodies Nrf2 (1:100) for 1 h at RT, and followed by incubation with FITC conjugated goat anti-mouse IgG (1:50 dilution) for 1 h at room temperature. After washing with PBS, cells were mounted with an anti-fade reagent containing DAPI (4, 6-diamidino-2-phenylindole; Invitrogen) and observed under a fluorescence microscope equipped with the Nikon Metamorph digital imaging system.

### Immunohistochemistry

Livers were fixed in formalin and embedded in paraffin. Deparaffinized sections (5 μm) were used for immunohistochemistry. For antigen retrieval, sections were heated in 100 mM sodium citrate, 90°C, for 10 min followed by gradual cooling to room temperature. Endogenous peroxidases were inactivated with 0.3% H<sub>2</sub>O<sub>2</sub> for 10 min at room temperature and sections were blocked with 5% BSA in 1×PBST for one hour. Incubation with affinity purified anti-Nrf2 antibody was performed overnight at room temperature followed by standard washes and one-hour incubation with goat HRP-conjugated anti-rabbit IgG (Bio-Rad). Following washes, sections were developed using diaminobenzamidine at pH 7.0 and counter-stained with hematoxylin.

### Isolation of nuclear and cytoplasmic proteins from mouse liver

Nuclear and cytoplasmic extracts were prepared with Nuclear and Cytoplasmic Protein Extraction Kit according to their protocol (Beyotime Institute of Biotechnology, Beijing, China). Protein concentrations were determined with the BCA Assay Kit from Beyotime Biotechnology. Briefly, approximately 0.2 g of mouse liver was homogenated with 2 ml of ice cold hypotonic buffer A and

buffer B (20:1). The homogenate was incubated at 4°C for 15 min and centrifuged at 12,000 g for 10 min at 4°C. The supernatant was obtained as cytoplasmic extract and the residue was added with 0.2 ml of hypertonic buffer C. The pellet suspension was shaken gently for 30 min at 4°C and then centrifuged at 12,000 g 10 min at 4°C. The supernatant was obtained as nuclei extract. PMSF and leupeptin were added into buffer A and buffer C before use.

### Immunoprecipitation

Liver fractions were extracted as described above. Sufficient amount of Nrf2 antibody or Cyp2a5 antibody or mouse IgG negative control was added into 200 μg proteins and gently rotated at 4°C overnight. The immunocomplex was captured by adding 25 μl protein A+G agarose beads (Beyotime, Beijing, China) and gently rotating at 4°C for 3 h. Then the mixture was centrifuged at 2500 g for 5 min at 4°C and the supernatant was discarded. The precipitate was washed for three times with ice-cold RIPA buffer, resuspended in 3×sample buffer and boiled for 5 min to dissociate the immunocomplex from the beads. The supernatant was collected by centrifugation and subjected to SDS-PAGE.

### Western blot analysis

Approximately 0.2 g of mouse liver was homogenated with 1 ml of ice cold RIPA Lysis Buffer (Beyotime, Beijing, China). The homogenate was centrifuged at 12,000 g for 5 min at 4°C. Supernatants were denatured; 30 μg of protein was separated by polyacrylamide gel electrophoresis, and then transferred to nitrocellulose membranes (Invitrogen). Membrane blotting was performed using rabbit polyclonal antibody to Nrf2, β-actin and histone H1 polyclonal antibody (Biosynthesis Biotechnology Co., LTD, Beijing, China) or chicken polyclonal antibody to Cyp2a5 (University of Kuopio, Kuopio, Finland).

### Real-time qPCR

Total RNA was extracted using Trizol reagent™ (Invitrogen, CA), followed by column purification (RNeasy™ mini kit, Qiagen, MD) and treated with DNase (Roche, CT). A total of 0.5 μg of RNA was reverse transcribed per reaction using Sprint™ RT Complete (Clontech Laboratories, CA). RT-qPCR was performed on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, CA). Primers sequence are as follows:

#### Cyp2a5

Forward 5'-GGACAAAGAGTTCCTGTCACCTGCTTC-3'

Reverse 5'-GTGTTCCACTTCTTGTTATGAAGTCC-3'

#### Nrf2

Forward 5'-CCA GCA CAT CCA GAC AGA CAC-3'

Reverse 5'-GAT ATC CAG GGC AAG CGA CTC-3'

#### GADPH

Forward 5'-ACAGTCCATGCCATCACTGCC-3'

Reverse 5'-GCCTGCTTCACCACCTTCTTG-3'

### Coumarin 7-hydroxylase assay

Cyp2a5 activity was determined using the protocol described previously (18), modified for 96-well plates. In brief, coumarin 7-hydroxylase activity was assessed using 0.1 mg of microsomal

protein incubated with coumarin (100 mM) and NADPH for 15 min. The amount of hydroxylated coumarin was determined by fluorescence with an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a Thermo Fluoroskan Ascent spectrophotometer.

## Results

### Effect of high-fat diet and pyrazole on cellular distribution of Nrf2

Cellular localization of Nrf2 protein was determined by immunofluorescence, immunostaining and Western blotting. As shown Figure 1, in untreated cells, Nrf2 fluorescence was found to be distributed throughout the cells, in both the cytoplasm and nucleus. After treatment with high-fat, most of the Nrf2 fluorescence was localized to the nuclei, as was also observed for pyrazole treatment, and immunostaining for Nrf2 showed increased nuclear staining in many hepatocytes in NAFLD as compared to control liver sections. As shown in Figure 2, a significant decrease in Nrf2 protein levels in the cytoplasm was observed after treatment with high-fat diet and pyrazole. However, Nrf2 protein levels in the nucleus were higher in high-fat diet group mice livers than that in control mice livers. These results suggested that the high-fat diet and pyrazole induced a translocation of Nrf2 from the cytoplasm to the nucleus.

### Effects of high-fat diet and pyrazole on Nrf2 and Cyp2a5 expression in Nrf2<sup>+/+</sup> mice

As shown in Figure 3, levels of Nrf2 were increased 24% and 49 % by high-fat diet and pyrazole compared with the mice in the control

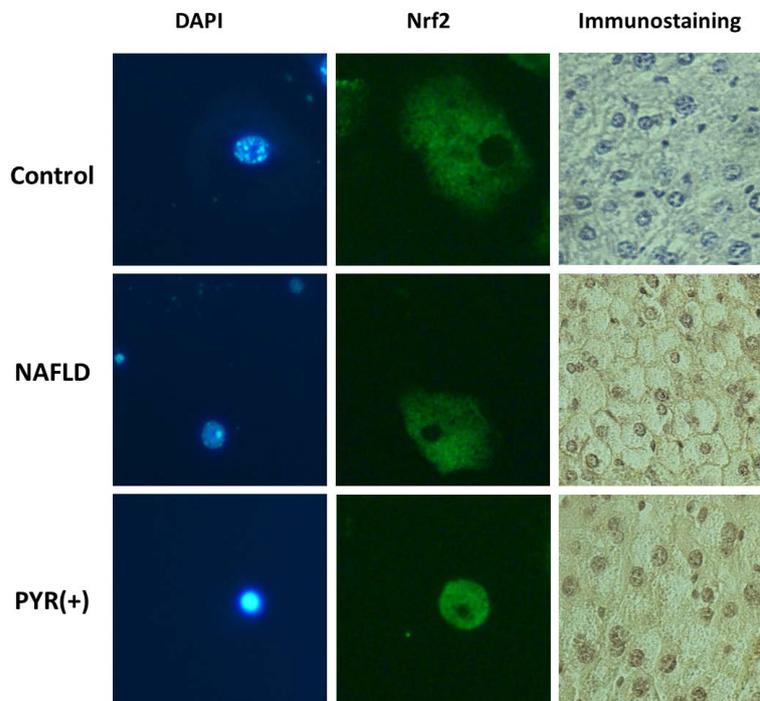
group (Figure 3A and 3B), respectively. Levels of Cyp2a5 were also were higher by high-fat diet and pyrazole (63% and 130 %). Coumarin 7-hydroxylase activity was increased approximately 71% by high-fat diet and 177% by pyrazole compared with the mice in the control group (Figure 3C). The increase in Cyp2a5 mRNA was highest treatment with pyrazole, analogous to the high protein levels and activity of Cyp2a5 in these mice. The high-fat also elevate levels of Nrf2 mRNA in the WT mice (113%) confirming that NAFLD induction of Nrf2 and Cyp2a5.

### Effects of high-fat diet and pyrazole on Cyp2a5 expression in Nrf2<sup>-/-</sup> mice

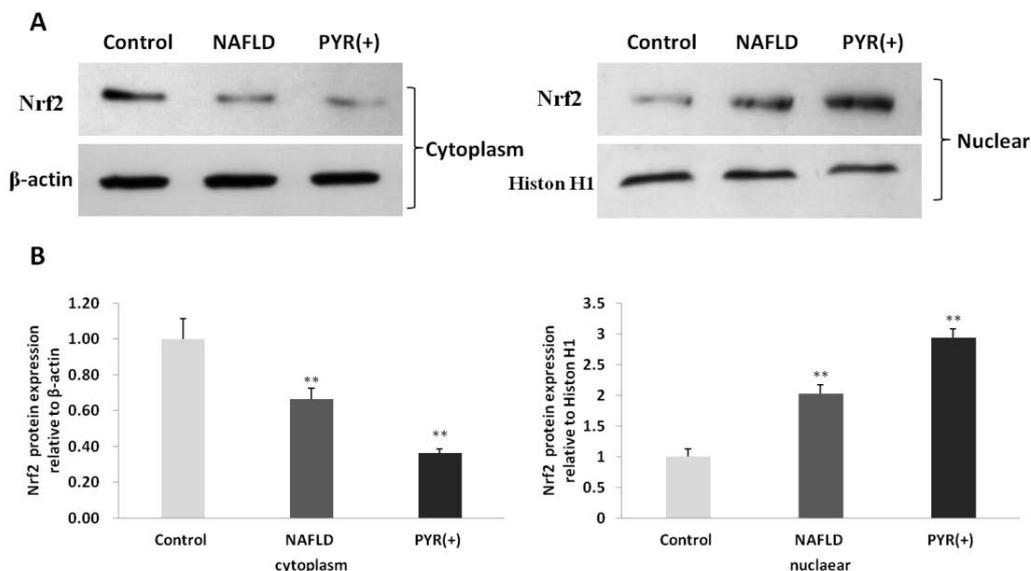
To evaluate the role of Nrf2 in high-fat diet induced Cyp2a5, levels and activities of Cyp2a5 in livers from KO mice were assayed (Figure 4). However, high-fat diet and pyrazole did not significantly elevate Cyp2a5 in the Nrf2 KO mice. The above studies show that NAFLD elevates Cyp2a5 protein levels and activity in WT mice but not in Nrf2 KO mice.

### The interaction between Nrf2 and Cyp2a5

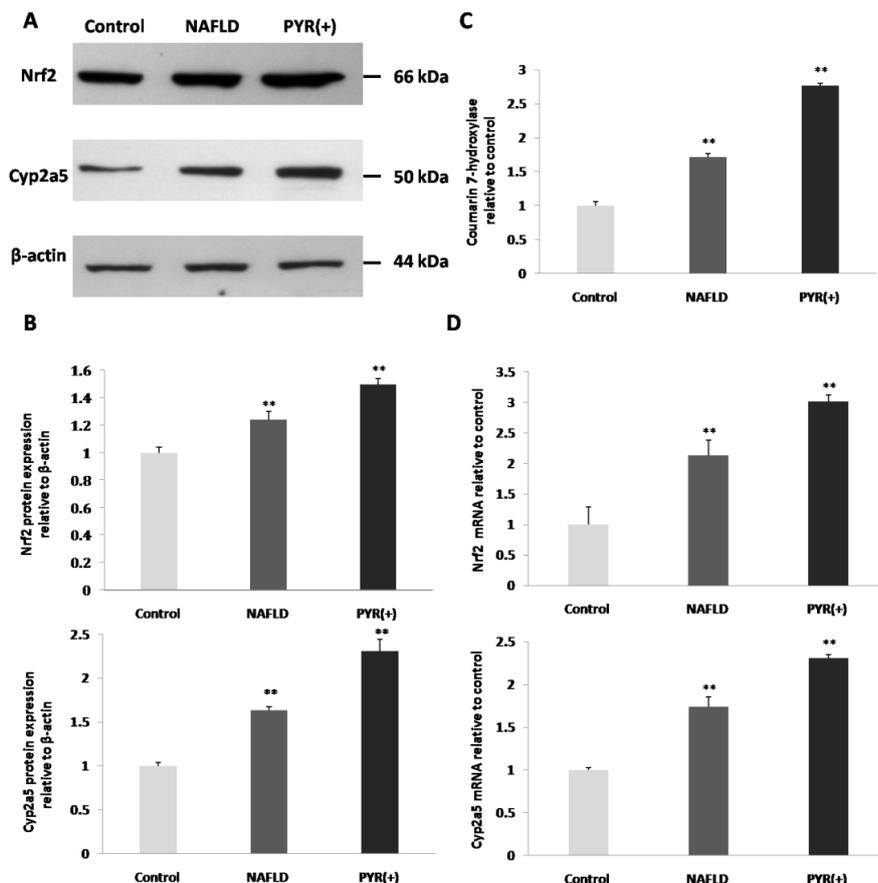
As shown in Figure 5, Cyp2a5 protein was detected in the protein complex from the immunoprecipitation of cell lysates using an Nrf2 antibody (Figure 5A), and Nrf2 protein also was detected in the immunoprecipitations of Cyp2a5 (Figure 5B). Furthermore, in the high-fat diet group and pyrazole group both Nrf2 and Cyp2a5 protein expression were higher significantly ( $P < 0.05$ ) than in control group mice. There was no co-immunoprecipitation of Nrf2 with Cyp2a5 in the absence of antibody.



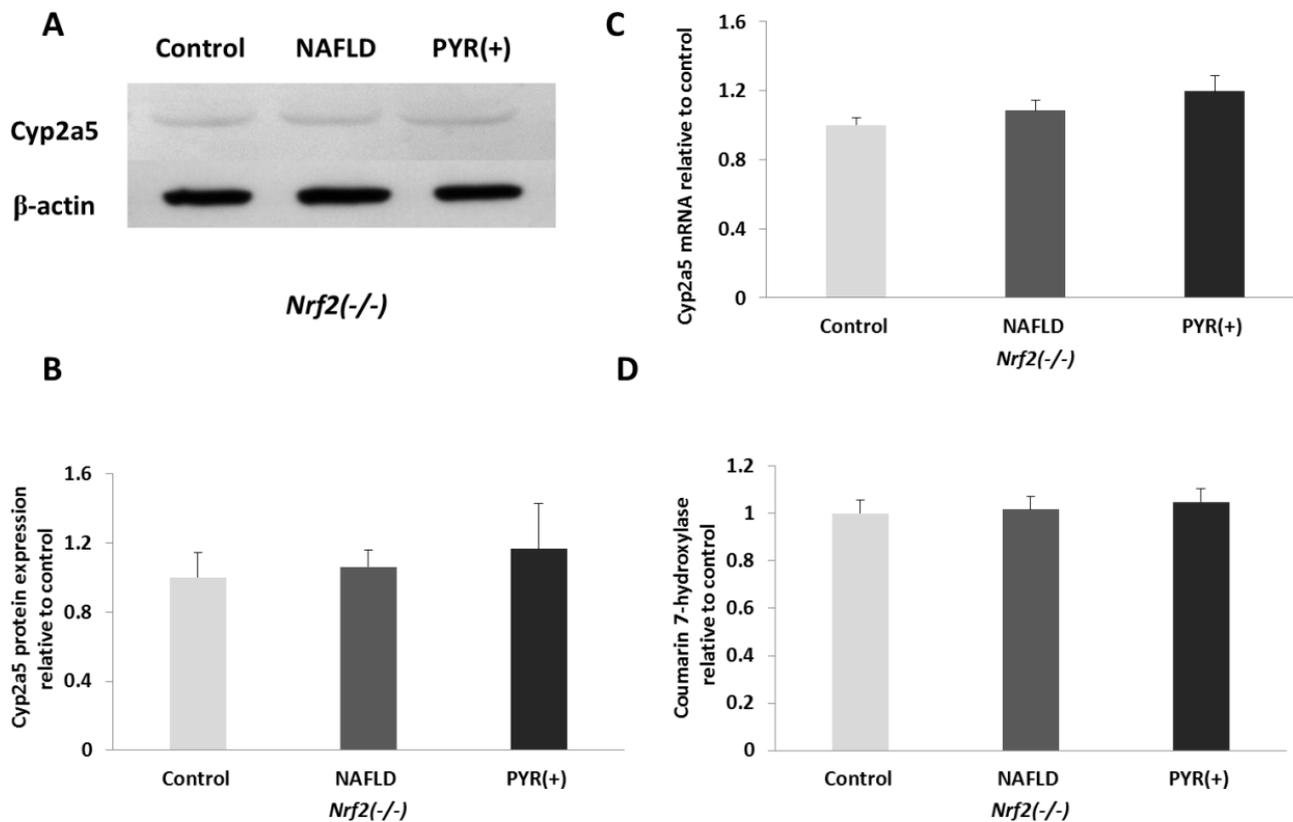
**Figure 1: Nrf2 nuclear accumulation induced by NAFLD** (Cellular distribution of Nrf2 obtained from the liver of mice treated with control, high-fat diet and pyrazole. An immunofluorescence assay was performed followed by detection with confocal microscopy. The cells were fixed, permeabilized and stained with antibodies against Nrf2. DAPI was used as a nuclear stain. Nrf2 specific nuclear staining by immunohistochemistry. Paraffin sections of livers of control, high-fat diet and pyrazole were stained using antibodies against Nrf2 followed by anti-rabbit horseradish peroxidase conjugate, as indicated. Chromagen development yields a brown color; sections were counter-stained with hematoxylin (blue). The increased brown staining of hepatocytic nuclei in upper panel as opposed to the more blue color of these nuclei in lower control panels indicated that Nrf2 was more abundant in high-fat diet and pyrazole nuclei. The image shows the results of a representative experiment from three independent experiments performed. Scale bars 20 mm).



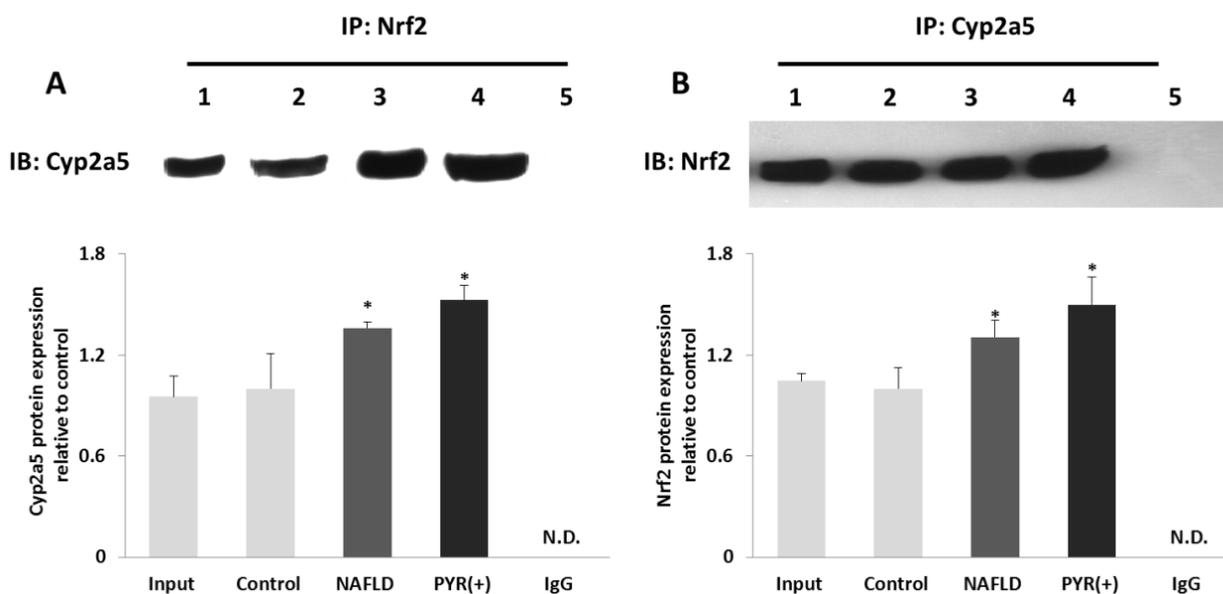
**Figure 2:** NAFLD induces a translocation of Nrf2 from cytoplasm to nuclear in WT mice liver (Western blot (A) and densitometric analysis (B) of Cyp2a5 and Nrf2 protein expression in the cytosolic and nuclear fractions obtained from mice treated with control, high-fat diet and pyrazole. Histon H1 was used as the nuclear marker. Values represent the mean  $\pm$  SD (n=3). Values are significantly different from controls (\*\* P<0.01).



**Figure 3:** NAFLD and pyrazole increase Nrf2 and Cyp2a5 protein expression *in vivo* (Western blot (A) and densitometric analysis (B) of Cyp2a5 and Nrf2 protein expression in liver obtained from mice treated with control, high-fat diet and pyrazole. Values represent the mean  $\pm$  SD (n=3) expressed as a percent relative intensity of control. Values represent the mean  $\pm$  SD (n=3). Values are significantly different from controls (\*\* P<0.01). (C) COH activity was assessed in liver obtained from mice treated with control, high-fat diet and pyrazole. (D) Cyp2a5 and Nrf2 mRNA levels were quantified by RT-qPCR and normalized to GAPDH: average expression obtained from mice treated with control, high-fat diet and pyrazole. Values represent the mean  $\pm$  SD (n=3). Values are significantly different from controls (\*\* P<0.01).



**Figure 4:** Cyp2a5 protein expression, mRNA levels and coumarin 7-hydroxylase activity in the *Nrf2*<sup>-/-</sup> mice (Western blot (A) and densitometric analysis (B) of Cyp2a5 and *Nrf2* protein expression in liver obtained from mice treated with Control, high-fat diet and pyrazole. (C) Cyp2a5 mRNA levels were quantified by RT-qPCR and normalized to GAPDH. (D) COH activity was assessed in liver obtained from mice treated with control, high-fat diet and pyrazole. Values represent the mean  $\pm$  SD (n=3) expressed as a percent relative intensity of control).



**Figure 5:** *Nrf2* protein expresses along with *Cyp2a5* protein interactively in WT mice and NAFLD induces the two protein expression ((A) Co-immunoprecipitated with anti-*Nrf2* and Western blot with anti-*Cyp2a5* showing expression of *Cyp2a5*. (B) Co-immunoprecipitated with anti-*Cyp2a5* and Western blot with anti-*Nrf2* showing expression of *Nrf2*. Relative amount of the co-immunoprecipitation complex *Nrf2* and *Cyp2a5* valuated by the ratio of band optical density. All values represent the mean  $\pm$  SD (n = 3) against the control levels. Mean differences are significant from control group at  $P < 0.05$  (\*). (1) liver extracts (input) were used as positive control; (2) immunoprecipitates from wild-type control group mice livers; (3) immunoprecipitates from high-fat diet group mice livers; (4) immunoprecipitates from pyrazole group mice livers; (5) immunoprecipitates with IgG used as negative control; IP, co-immunoprecipitation; IB: immunoblot).

## Discussion

In this study, we found that feeding mice a high-fat diet results in an increase in Cyp2a5 levels and catalytic activity compared with those in controls. In addition, the observation of no change in the expression of Cyp2a5 mRNA in the liver of treated knock-out (*Nrf2*<sup>-/-</sup>) mice, in contrast to the changes seen in the wild-type (*Nrf2*<sup>+/+</sup>) mice suggests that NAFLD induction of Cyp2a5 requires the transcription factor Nrf2. This is consistent with the observations that pyrazole and heavy metals were ineffective in inducing Cyp2a5 in *Nrf2*<sup>-/-</sup> mice. These results suggest that NAFLD-induced the Cyp2a5 expression in mice is due to the Nrf2.

Nrf2 regulates many important antioxidant genes and phase II detoxification genes. Oxidative stress plays an important role in NAFLD [19,20]. However, little is known about changes of Nrf2 in nonalcoholic fatty liver disease. The study shows that in chronic high-fat-fed mice, liver Nrf2 protein and mRNA expression is increased. Induction of Nrf2 by injecting pyrazole into mice also increased Cyp2a5 protein expression. Nrf2 levels were elevated 1.5 fold in mice after pyrazole treatment. These increases were associated with similar increases in Cyp2a5 protein levels. These results demonstrate that Cyp2a5 is activated by increased expression of Nrf2 in pyrazole treated group. Levels of Nrf2 were also elevated in the livers of mice high-fat diet, and it is interesting to speculate that this increase may play a role in the elevated mRNA levels of Cyp2a5 caused by high-fat treatment.

Recently it was found that Nrf2 can regulate Cyp2a5 induction, which contains an ARE in its promoter [21]. Pyrazole-induced Cyp2a5 was lower in *Nrf2*<sup>-/-</sup> mice, compared with that in *Nrf2*<sup>+/+</sup> mice, suggesting that Cyp2a5 induction by pyrazole was mediated by Nrf2. Cyp2a5 induction by heavy metals including CdCl<sub>2</sub> is also dependent on Nrf2 [22,23]. Ethanol induces Cyp2a5, at least in part, at the level of transcription because ethanol increased Cyp2a5 mRNA levels [24]. We found that the expression and activity of Cyp2a5 were no difference in the *Nrf2*<sup>-/-</sup> mice (Figure 4). In addition to Nrf2, several other transcription factors have been implicated in the basal and inducible expression of the Cyp2a5 gene [25-27]. It is possible that one or some of those transcription factors may take part in regulating NAFLD induction of Cyp2a5, especially because Cyp2a5 was still induced by NAFLD in *Nrf2*<sup>-/-</sup> mice although the extent of induction was lower compared with that in *Nrf2*<sup>+/+</sup> mice.

We believe the most novel information in this study is the close association between levels of Nrf2 and Cyp2a5 and the fact that the induction of Cyp2a5 by high-fat diet seems to be potentiated by the induction of Nrf2. Increased expression of Nrf2 has been reported in livers of mice fed a high-fat diet for 12 weeks [28]. In the current study, the Nrf2 signaling pathway was activated by the high-fat diet as indicated by increased nuclear accumulation of Nrf2 proteins (Figure 2). Immunohistochemical staining showed that the distribution of the elevated Nrf2 in the nucleus. As shown in Figure 1, we observed that Nrf2 was translocated from the cytoplasm into the nucleus in the high-fat diet and pyrazole treated group. However, the control group neither decreased the Nrf2 levels in the cytoplasmic fraction or increased it as great as in the livers treated with high-fat diet or pyrazole observed in the nuclear fraction. Many of these can respond to the Nrf2 pathway and Nrf2 protein preferentially accumulated in the high-fat diet and pyrazole treated group as compared to control liver nuclei.

In summary, we have investigated the molecular mechanism of NAFLD elevates Cyp2a5 protein expression and mRNA levels. We first validated a direct interaction of Nrf2 with Cyp2a5. Co-IP experiments

showed that Nrf2 interacts directly with Cyp2a5 supplies a novel insight of a potential role of Nrf2 and suggests a possible mechanism of Nrf2 participation in the regulation of Cyp2a5 in NAFLD.

## Authors' Contributions

YC and Pro. XZ designed the experiments and contributed to the description and writing. QW contributed to feed mice and did animal treatments, and contributed to the tissue collected. CW contributed to co-immunoprecipitation. GL contributed to the data analysis. XY and YQ contributed to the immunohistochemical staining. YC contributed to the RT-PCR assay. All authors read and approved the final manuscript.

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