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Comparison of the effects of cold on cytochromes P450 1A in the rat lungs versus liver

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Abstract: During hypothermia, most cellular processes are inhibited, including expression of numerous genes. A small number of special genes are activated, which code for proteins participating in cold acclimation of the cell. We previously uncovered a paradoxical phenomenon: cytochrome P450 subfamily enzymes (CYP1A) in rat liver are not inhibited but activated by systemic cooling. In the present study, we have investigated the influence of cold exposure on the activity and expression level of the CYP1A proteins in a different organ, rat lungs, and did not find any changes either in the activity or quantity of these enzymes. We hypothesize that this phenomenon is due to the absence of an endogenous intermediary of CYP1A induction in the rat lung, in contrast to the liver.

Keywords: CYP1A Activation, Cold, Rat Liver, Rat Lung, Tocopherol, α-TBP

1. Introduction

Cytochromes of the P450 1A subfamily, such as proteins CYP1A1 and CYP1A2, are generally known as the enzymes that metabolize pro-carcinogenic xenobiotics, for example, polycyclic aromatic hydrocarbons (PAH) and arylamines, giving rise to carcinogenic and mutagenic metabolites. The only proven mechanism of induction of the *CYP1A1* gene is the arylhydrocarbon receptor (AhR) signaling pathway[1], although other possible mechanisms are being examined by researchers in this field. The *CYP1A2* induction, however, can be either AhR-dependent[2] or take place at the post-transcriptional level[3].

For a long time it has been thought that CYP1A substrates consist exclusively of anthropogenic xenobiotics such as PAH, but recent data suggest that this is not the case. CYP1A inducers are not only PAH-like xenobiotics, but also compounds from other classes of chemicals, including endogenous compounds, nutrients (vitamins, tryptophan)[4], hyperoxia[5], and mechanical factors, such as ultrasound[6], hydrodynamic shear[7], ultraviolet light[8], and cold[9].

A growing body of evidence points to CYP1A enzymes being able to perform some normal physiological function, for instance, it is known that they metabolize certain products of the arachidonic acid cycle[10, 11]. The physiological functions of the AhR transcription factor have been known for quite some time: it participates in the developmental and differentiation processes and in the immune response[12, 13].

We reported previously[9] that CYP1A1 and CYP1A2 in rat liver are induced by exposure to cold via the Ah-receptor-dependent signaling pathway. Apparently, this process involves some endogenous intermediary or intermediaries, which must either be AhR ligands or participate in metabolic pathways that synthesize a compound that is an AhR ligand. Several candidates failed to be confirmed as an intermediary during the initial testing, but we cannot rule them out completely either: the possible compounds are tocopherol, bilirubin, or corticosterone[14].

CYP1A are known to be expressed at different levels in different tissues of the same organism, and there are inter-species differences too. For instance, human CYP1A1 level of expression in the liver is very low[15], and also very low in extrahepatic tissues, but it is inducible by AhR ligands in lung, lymphocytes, mammary gland, and placenta[16]. In contrast, the expression of CYP1A2 is liver-specific because no CYP1A2 protein has ever been detected in any other human tissues[16]. In rats, CYP1A is mostly expressed and induced in the liver but also in lungs,

kidneys, brain and some other organs[17].

As mentioned above, CYP1A enzymes evidently perform some physiological role, in addition to the PAH-mediated activation and production of chemically-induced cancer and other pathologies. Therefore, some non-PAH inducers, including endogenous ones, can activate or modulate the expression of those genes and activity of the resulting enzymes via mechanisms that differ among different organs. The aim of this work was to investigate how systemic exposure to cold affects the CYP1A activity in the rat lung compared to liver tissue.

2. Methods

2.1. Animals

This study used male Wistar rats weighting 150-200 g from a stock maintained in the Animal Facility of the Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences (Novosibirsk, Russia). The animals were housed in plastic cages under standard conditions (12:12 h light/dark regimen; food and water available *ad libitum*). All experimental procedures were approved by the Animal Care Committee of the Institute of Molecular Biology and Biophysics, Siberian Branch of the Russian Academy of Medical Sciences (Novosibirsk, Russia). Rats were subsequently sacrificed by decapitation under ethyl ester anesthesia.

2.2. Cold Exposure

Experimental rats lived at air temperature of 4ºC for 1, 5, or 10 days. The control group was kept at the room temperature.

2.3. Preparation of Rat Liver Microsomes

Rat liver microsomes were prepared by differential ultracentrifugation[18]. Livers were perfused by cold 1.15% KCl, 20 mM Tris-HCl buffer, pH 7.4, removed and homogenized in the same buffer. Liver homogenates were spun at 10,000 *g* for 20 min, and the resulting supernatants were ultracentrifuged at 105,000 *g* for 60 min. The pellets were resuspended in 0.1 M KH_2PO_4 buffer pH 7.4, containing 20% glycerol. Protein concentrations were measured using the Lowry method[19] with bovine serum albumin as a standard.

2.4. Enzyme Assays

The selective activities of CYP1A isoforms 7-ethoxyresorufin-O-deethylase (CYP1A1) and 7-methoxyresorufin-O-demethylase (CYP1A2) were measured by the spectrofluorometric method described by Burke[18].

2.5. SDS-PAGE Electrophoresis and Immunoblot Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the Laemmli method[20]. For protein identification, 100 μg of microsomal proteins were loaded onto each lane of a 10% acrylamide gel, separated at 20 mA for 2 h, and transferred to a nitrocellulose membrane using Fastblot B34 (Biometra, Germany).

The proteins were then visualized by staining with Ponceau Red. The membrane was blocked with 5% non-fat dry milk in 1× TBS buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl) containing 0.05% Tween 20 overnight at +4°C and incubated for 1 h at 37°C on a shaker. The membrane was washed with 0.05% Tween/TBS and then incubated with an antibody that reacts with both rat CYP1A1 and CYP1A2[21] for 1.5 h at room temperature.

After that, the membrane was washed and incubated with a secondary antibody for 1 h. The proteins under study were visualized using SIGMA FAST™ 5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium tablets (Sigma-Aldrich, USA).

The membrane was then washed and incubated with a mouse monoclonal antibody against rat β-actin, washed, incubated with a goat anti-mouse IgG conjugated with horseradish peroxidase as a secondary antibody. The proteins under study were visualized using 3, 3′-diaminobenzidine tetrahydrochloride tablets.

2.6. RNA Isolation, Reverse Transcription and PCR

Total RNA was isolated using VektoRNK-ekstraktsiya RNA isolation kit (Vector-Best, Russia) as per the manufacturer's protocol.

The RNA extracts were treated with RNase-free DNase to remove contaminating DNA: the reaction mixture contained 5 μg of total RNA, 1 unit of RQ1 RNase-Free DNase, 1x reaction buffer and 20 units of RNasin® in a 20 μL reaction volume. The mixture contained 400-800 ng of total RNA, reaction buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and 10 mM DTT), 1 mM dNTPs, 200 units of M-MuLV reverse transcriptase, 4 μg of random hexamer primers, and 25 units of RNasin® in a 25 μL reaction volume. The samples were incubated for 120 minutes at 37°C.

We used the following PCR primers: for *α-TPB,* forward 5'-ATGGAGTTCTGAGGTCC-3' and reverse 5'-CTCATTGGATGGTCTCAGAAAT-3', amplicon size 537 bp; for *rpl29,* forward 5'-GCTTACCGTGCAGACATGGCCA-3' and reverse 5'-TTGGCATCTTGGGCTTGACAGC-3', amplicon size 274 bp. The *rpl29 gene* was used as an internal control. PCR was performed in a total volume of 20 μl in 1x PCR buffer, 0.25 mM dNTP, 1 μM of the primers (0.25 μM each of the target gene primers and 0.25 μM each of the housekeeping gene primers), 2 units of Taq polymerase, 2 μL of cDNA, and 3.5 mM $MgCl_2$. The PCR program started with an initial denaturation at 95°C for 3 min followed by 30 cycles of

amplification (94°C for 15 s, 60°C for 15 s, and 72°C for 15 s) and a final extension at 72°C for 4 min.

Each sample was amplified twice. PCR products were separated by electrophoresis in a 2% agarose gel and stained with ethidium bromide. PCR bands were visualized using UV light, photographed using DNA Analyzer Video System (Lytech, Russia) and semiquantitated. The final results were calculated by normalizing the *CYP1A1 and CYP1A2* levels to the *β-actin* level.

2.7. Statistical Analysis

Statistical analysis was performed using the STATISTICA software package (StatSoft, Inc., USA). All data were analyzed using Student's t test and the results confirmed using the Mann-Whitney rank sum test.

3. Results

To study the effect of cold on CYP1A activity *in vivo* in the rat liver and lungs, we measured the CYP1A1 and CYP1A2 activities in both tissues. The results are shown in Fig. 1.

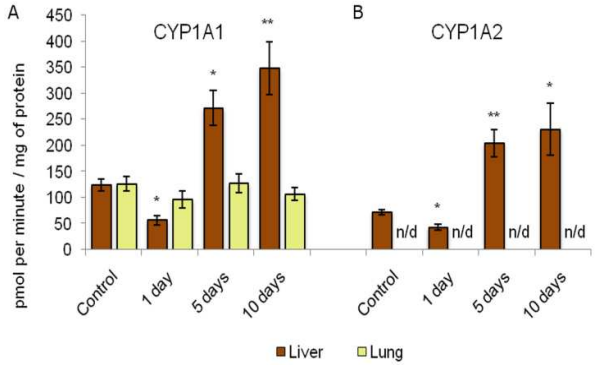

Figure 1. *Effects of the 1-, 5-, and 10-day cold exposure (+4ºC) on liver and lung CYP1A1 activity (A) and on liver and lung CYP1A2 activity (B). The data are shown as mean±S.E.M. (n= 6-21 in each group). Significantly different from the control animal group: * indicates p<0.005 and ** means p<0.001.*

Previously we demonstrated that cold is a weak inducer of CYP1A in rat liver when rats were exposed to 4ºC for 5 or 10 days[9]. In the present study, we exposed rats to 4ºC for 1, 5 or 10 days. In the liver and lung of animals, we measured CYP1A1 and CYP1A2 activity. In the liver, there was a decrease in both CYP1A1 and CYP1A2 activity on day 1 and an increase on days 5 and 10. In contrast, in the lungs, there was no change in CYP1A1 activity, whereas CYP1A2 activity was below the detection limit at all time points during exposure to cold.

Immunoblot analysis with a monoclonal antibody against rat CYP1A1 and CYP1A2 showed that CYP1A1 protein, which is usually undetectable in control rat liver microsomes and after 1 day of cold exposure, is detectable in the hepatic microsomes of rats after 5 and 10 days of cold exposure (Fig. 2A). CYP1A1 protein level increased in the hepatic microsomes of rats on day 10 of cold exposure. As shown in Fig. 2B, in the rat lung, both in control animals and in those who underwent exposure to cold for 1, 5, and 10 days, the CYP1A proteins are present in negligible amounts (almost undetectable).

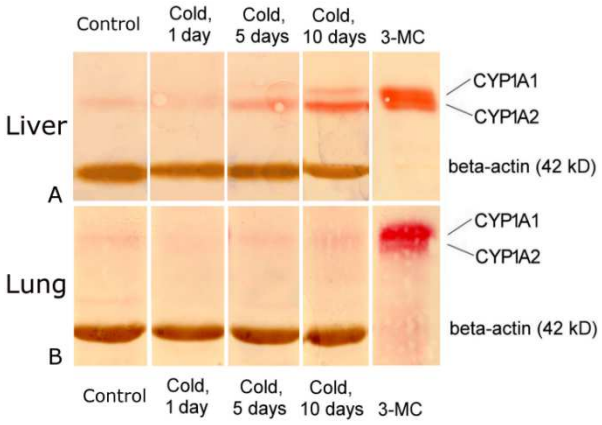

Figure 2 *Western blot analysis of the effects of cold exposure on CYP1A1 and CYP1A2 proteins in the in rat liver (A) and lung (B) microsomes. The microsomal protein samples (100 µg/lane) were applied to 10% SDS-PAGE, transblotted onto nitrocellulose membrane and incubated with a mouse anti-rat CYP1A1/2 monoclonal antibody [21]. Each treatment was done in triplicate. Lanes (left to right): control animal group, 1-day cold exposure, 5-day cold exposure, 10-day cold exposure, and positive control (shown as 3-MC; 10 µg/lane): animals treated with 3-methylcholanthrene at the dose of 5 mg/kg of body weight per day in vegetable oil during 4 days.*

We also measured the level of tocopherols and alpha-tocopherol-binding protein (α-TBP) mRNA in the liver of cold-exposed rats. Starting on day 5 of cold exposure, the liver content of tocopherols increases. At the same time (day 5), there is a pronounced increase of the mRNA level of alpha-tocopherol-binding protein.

Thus, in the rat liver as a result of exposure to cold, we see an increase in the activity of CYP1A1 and CYP1A2, the protein content of CYP1A1/2, and the amount of tocopherols and α-TBP mRNA. On the other hand, in the lungs of cold-exposed rats, we don't see any changes in either the level of activity or protein content of CYP1A1/2.

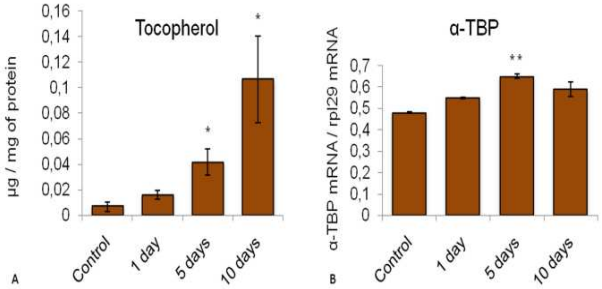

Figure 3. *Effects of the 1, 5, and 10 days of cold exposure (+4ºC) on the liver content of tocopherol (A) and alpha-tocopherol-binding protein mRNA (B). The data are shown as mean±S.E.M. (n= 4-5 in each group). Significantly different from the control animal group: * indicates p<0.05 and ** means p<0.0001.*

4. Discussion

According to literature, CYP1A is inducible not only in the liver but also in other organs, including lungs. Some reports show that one of the classic inducers of CYP1A, benzo[a]pyrene, increases the activity and mRNA level of CYP1A in the lungs of Wistar rats approximately 3-fold[22]. Benzo[a]pyrene and related compounds also enhance the activity of CYP1A1 according to Pushparajah and coauthors[23]. Non-classical CYP1A inducers are also able to enhance CYP1A1 activity in the rat lung. For example, Oltipraz® increases CYP1A1 activity in the lungs and kidneys of Wistar rats by about 3.5-fold[17].

Literature shows that mechanical factors can also induce CYP1A1 activity in the rat lung. Hyperoxia (95% oxygenation) increases activity, mRNA and protein level of CYP1A1 in the lungs and liver of Sprague-Dawley rats[5]. This is not the case in AhR-null mice[5], suggesting that the process is AhR-dependent. It is possible that in response to hyperoxia, the cellular level of hydrogen peroxide increases, which can enhance CYP1A1 activity[5]. Increased oxidative stress is one of the hypothetical mechanisms underlying the cold-induced CYP1A activation in the rat liver, but the lack of changes in the malonic dialdehyde content (unpublished data, Perepechaeva M.L.) in the rat liver contradicts that hypothesis.

It is known that the AhR signaling pathway has many intersections with other transcription factor-related signaling pathways. Let's examine some of the pathways that may be influenced by cold temperatures, in the lung tissue in particular, which is in close contact with the cold air.

We can hypothesize that exposure to cold activates alveolar macrophages, which release inflammation factors, which suppress AhR function possibly via activation of the nuclear factor kB (NF-κB)[24]. But Lim and coauthors[25] have reported that hypothermia inhibits cytokine release of alveolar macrophages and activation of NF-κB; similar results were obtained by another laboratory using cultured rat microglia[26]. In other words, if NF-κB were activated, this would explain our results. But literature shows the opposite, namely that NF-κB should be less active during hypothermia.

Another regulator, HIF (*hypoxia-inducible factor), which* belongs to the PER-*ARNT*-SIM subfamily, can affect the AhR pathway by competing with the messenger protein Arnt. Nonetheless, the expression of HIF-dependent genes *in vitro* during cold exposure and hypothermia (28-32 degrees C) doesn't change if oxygenation is normal[27].

The increase of activity and CYP1A apoprotein content in the liver of rats on days 5 and 10 of cooling are discussed at length in our previous report[9]. In that work we made some conclusions regarding the mechanisms of CYP1A induction in the rat liver as a result of exposure to cold: CYP1A1 is induced transcriptionally via the AhR-dependent signal transduction pathway, whereas the activation of CYP1A2 by cooling is a posttranscriptional phenomenon.

The present work reports the data on the activity and protein content of CYP1A in the rat liver after one day of cold exposure: the CYP1A1 and CYP1A2 activity decreases (Fig. 1), and the protein level remains barely detectable, similar to the control group (Fig. 2). Indeed, cooling leads to slowing of almost all activities in the cell: transcription, translation, and enzymatic reactions[28]. The small group of genes that is activated are genes that play the role of modulators of gene expression during hypothermia, mostly genes belonging to the group of cold-inducible RNA-binding proteins[29, 30]. In this context, it is rather unusual that CYP1A is activated and not inhibited by exposure to cold.

One plausible explanation is that during exposure to cold some endogenous compound either appears or increases in quantity, and this compound participates in the physiological response to cold and concomitantly activates the Ah-receptor pathway. We theorized previously that such a compound could be tocopherol, corticosterone, or bilirubin [14]. It should be noted that the level of bilirubin in the liver microsomes of cold-exposed rats increases only on day10, and the tocopherol level doesn't change on day 1 of cold exposure (Fig. 3A) but increases on days 5 and 10[14]. The increase of the tocopherol level in the rat liver (Fig. 3A) takes place without nutritional supplementation with tocopherol, that is, it is redistributed into the liver from the common pool.

Redistribution of tocopherol within the system and its accumulation in the liver can be mediated by α-TBP, a protein that selectively binds to α-tocopherol and promotes its inclusion into lipoproteins[31]. Our data showing an increase of the relative content of α-TBP mRNA in the liver on day 5 of the exposure to cold (Fig. 3B) are indicative of enhancement of tocopherol redistribution within the system at this time point. The lowering of α-TBP expression by day 10 of cooling suggests that the accumulation of tocopherol within the cell has reached a sufficient level, so that the negative feedback loop can trigger mechanisms suppressing α-TBP expression. It is noteworthy that tocopherol itself can control α-TBP expression via negative feedback[32].

7. Conclusion

The results show that, unlike the liver, the rat lung doesn't undergo changes in the activity and level of CYP1A and CYP1A2 proteins during exposure to cold. If the intermediary of the induction of CYP1A in the liver is tocopherol (accumulates in the liver via redistribution from other organs as a result of cooling), then this can explain the lack of CYP1A activation in the lung. In the absence of a specific "cold-related" intermediary, CYP1A logically is not activated—the normal state of affairs during hypothermia.

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