CHARLES UNIVERSITY IN PRAGUE
FACULTY OF SCIENCE
DEPARTMENT OF BIOCHEMISTRY

Study on metabolism of 3-aminobenzanthrone and induction of biotransformation enzymes

Summary of PhD Thesis

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INTRODUCTION

3-Nitrobenzanthron, precursor of 3-ABA

The nitroaromatic 3-nitrobenzanthrone (3-nitro-7*H*-benz[*de*]anthracen-7-one, 3-NBA) occurs in diesel exhaust and in airborne particulate matter[8, 9, 14]. 3-NBA is most likely formed during the atmospheric reaction of benzanthrone with nitrogen oxides, especially in the presence of ozone**,** or during imperfect burning of diesel. 3-NBA exhibits extremely high mutagenic activity[9, 14] and is also a genotoxic carcinogen causing lung tumors in rats[14]. 3-NBA is also evaluated to be a potential carcinogen for humans[14, 1, 9, 19]. The genotoxicity of 3-NBA was documented by the detection of specific 3-NBA-derived DNA adducts *in vitro*, in human cell lines and also *in vivo* in rats and mice[12, 13, 15, 2]. The predominant DNA adducts formed by 3-NBA after its metabolic activation by reduction of the nitro group are 2-(2'-deoxyguanosin-N^2-yl)-3-aminobenzanthrone and *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone[9, 14] and these are most probably responsible for the G to T transversion mutations induced by 3-NBA in Muta Mouse[14]. The uptake of 3-NBA in humans has been demonstrated by the detection of its metabolite 3-aminobenzanthrone (3-ABA) in urine samples of salt mine workers occupationally exposed to diesel emissions[21].

3-Aminobenzanthron

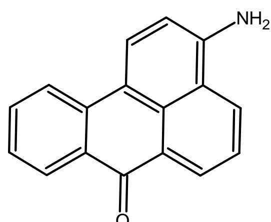


Fig. 1 Structure of 3-aminobenzanthrone

As mentioned above, 3-aminobenzanthrone (3-ABA) (Fig. 4) is the major reductive metabolite of 3-NBA, detected in urine samples of salt mine workers occupationally exposed to diesel emissions[21]. These miners use the diesel pneumatic drill, thereby being exposed to high concentrations of this diesel pollution.

Hence, 3-ABA might be a suitable biomarker of human exposure to 3-NBA.

In comparison with 3-NBA, 3-ABA is a less cytotoxic compound. Latest results show that 3-ABA might be involved in modulation of immune response, e.g. by increased induction of cytokines[16, 20].

Recently, a role of rat hepatic, pulmonary and renal NAD(P)H:quinone oxidoreductase (NQO1) in reducing 3-NBA to species that are further activated by *N*,*O*-acetyltransferases (NATs) and sulfotransferases (SULTs) to form DNA adducts was found[6, 9]. These enzymes are also the major activation enzymes for 3-NBA in human liver[6, 9]. Cytosolic xanthine oxidase, and microsomal NADPH:cytochrome P450 reductase are also capable of 3-NBA activation, but are less active than NQO1[6, 7, 9]. Cytochromes P450 (CYP) 1A1 and 1A2 are essential for the oxidative activation of 3-ABA in human and rat livers, leading to the same DNA adducts that are formed *in vivo* by 3-ABA or 3-NBA[5, 9]. CYP1A1 is also an efficient activator of 3-ABA in microsomal fractions from rat kidneys and lungs, while prostaglandin H synthase (cyclooxygenase, COX) plays a minor role in this subcellular fraction[3]. Previous results also indicate that besides microsomal CYP enzymes cytosolic peroxidases might play a role in the oxidative activation of 3-ABA, mainly in extrahepatic tissues such as kidneys and lungs. In *in-vitro* experiments, mammalian COX, lactoperoxidase (LPO) and myeloperoxidase (MPO) were found to be effective in activating 3-ABA[6, 9]. *N*-hydroxy-3-aminobenzanthrone (*N*-OH-ABA) is the intermediate generated from both 3-NBA and 3-ABA, which is responsible for DNA adduct formation[5, 9]. DNA adducts, qualitatively analogous to those formed by 3-NBA[7, 5, 6, 4], were observed *in vivo* in rats and mice treated with 3-ABA[7, 5, 15, 2, 9, 6, 4, 11, 12, 10].

AIM OF THE STUDY

The aim of this study was to extend our knowledge on metabolic activation of 3-nitrobenzanthrone and its reductive metabolite, 3-aminobenzanthrone. Another aim of the study was to evaluate the potential of both compounds to induce biotransformation enzymes involved in their own metabolism.

The aims of the present work are as follows:

- To study metabolism of 3-aminobenzanthrone by cytochromes P450 present in rat hepatic microsomes. To identify the major cytochromes P450 responsible for 3-ABA metabolism in this subcellular fraction**.**
- To evaluate metabolism of 3-aminobenzanthrone by rat and human recombinant cytochromes P450. To identify the major rat and human cytochromes P450 metabolizing 3-ABA.
- To study metabolism of 3-aminobenzanthrone by peroxidases.
- To investigate potential of 3-nitrobenzanthrone to induce the expression of biotransformation enzymes in kidney and lung after its intraperitoneal application to rats.
- To examine potential of 3-aminobenzanthrone to induce the expression of biotransformation enzymes in kidney and lung its intraperitoneal application to rats.
- To investigate potential of 3-nitrobenzanthrone to induce the expression of biotransformation enzymes in liver, kidney and lung after its intratracheal instillation to rats.

RESULTS AND DISCUSSION

The results shown in this study extend our knowledge on oxidation of 3-aminobenzanthrone, the main metabolite of carcinogenic 3-nitrobenzanthrone. They also contribute to identify the major rat and human cytochromes P450 involved in oxidation of this compound. In addition, the results expand our knowledge on potency of 3-nitrobenzanthrone and 3-aminobenzanthrone to induce biotransformation enzymes participating in their own metabolism. The results obtained in the study also increase our knowledge on biochemistry of these processes.

The main results found in this work are summarized as follows:

3-Aminobenzanthorne is oxidized by rat hepatic microsomes of rat

The metabolism of 3-aminobenzanthrone was studied *in vitro*, using hepatic microsomes of rats treated with several inducers of individual cytochromes P450 (CYP). We have found that 3-aminobenzanthrone is oxidized by miccrosomal CYP enzymes to three metabolites, *N*-hydroxy-3-aminobenzanthrone (*N*-OH-ABA), a parental compound, 3-NBA, and another metabolite with still unknown structure (metabolite M18). The hepatic microsomal system of rats treated with phenobarbital (rich in CYP2B) was the most effective in 3-ABA oxidation (*Fig. 2, pg. 5*). Hepatic microsomes of rats treated with PCN (rich in CYP3A) were also efficient to catalyze 3-ABA oxidation, followed by hepatic microsomes of rats treated with ß-naphthoflavone (rich in CYP1A1/2). These results suggest the participation of CYP1A, 2B and 3A in metabolism of 3-ABA in rat hepatic microsomes (*Fig. 2, pg. 5*). To confirm these results, the inhibition studies utilizing selective inhibitors of individual cytochromes P450, were performed (*Table 1, pg. 5*). Such a study confirmed previous results, showing participation of cytochromes P450 1A, 2B and 3A in 3-ABA oxidation in rat hepatic microsomes.

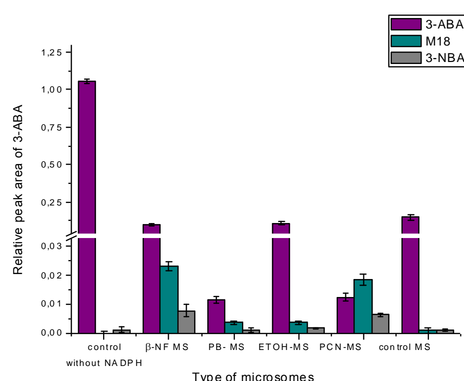


Fig. 2 Oxidation of 3-ABA by rat hepatic microsomes treated with CYP inducers. The values are averages and standard deviations of triplicate incubations.

Table 1 Inhibition of 3-ABA metabolism by specific inhibitors of CYPs

Inhibitors of CYP 450	Rat hepatic microsomes	IC_{50} [μM]
α-naphthoflavone (CYP1A1/2)	β-NF- MS (CYP1A1/2)	10.9
furafylline (CYP1A2)	β-NF- MS (CYP1A1/2)	16.6
diamantane (CYP2B)	PB-MS (CYP2B)	0.90
ketoconazole (CYP 3A1/2)	PCN-MS (CYP 3A1/2)	20.0
DDTC (CYP2E1)	EtOH-MS (CYP2E1)	80.6
DDTC (CYP2E1)	control MS	96.8
sulfaphenazole (CYP2C)	control MS	10.8
ketoconazole (CYP 3A1/2)	control MS	3.5

Rat and human recombinant cytochromes P450 oxidize 3-aminobenzanthrone

Among rat recombinant cytochromes P450, the enzymes of 3A and 1A subfamilies were the most effective to oxidize 3-ABA. Likewise, among human recombinant cytochromes P450, the orthologous enzymes, CYP3A and 1A, exhibited the highest efficacy to oxidize 3-ABA. In addition, rat CYP2C11 and human CYP2D6 also oxidize 3-ABA (*Fig. 3, pg. 6*). Cytochrome b_5 stimulated 3-ABA oxidation catalyzed by CYP3A and 1A (*Fig. 3, pg. 6*). Kinetics of 3-ABA oxidation catalyzed by the most effective cytochromes P450 was examined (*Table 2, pg. 6*).

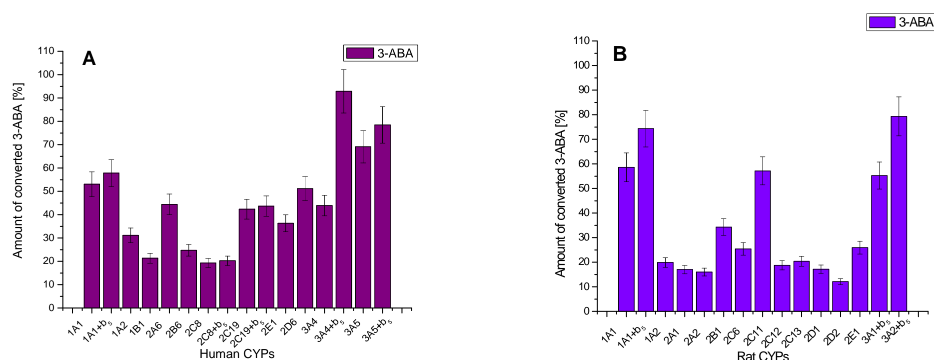


Fig. 3 Oxidation of 3-ABA by human (A) and rat (B) recombinant CYPs. The values are averages and standard deviations of triplicate incubations.

Table 2 Kinetics parameters K_m a V_{max} for oxidation of 3-ABA by rat and human recombinant CYPs

Cytochromes P450	K_m [μM]	V_{max} [min^{-1}]	K_m/V_{max} [μM . min]
human CYP1A1	75.5	21.0	3.60
human CYP1A1+b_5	122.8	24.0	5.12
human CYP3A4	68.27	10.0	6.83
human CYP3A4+b_5	38.41	28.0	1.37
rat CYP1A1	27.7	7.0	3.96
rat CYP1A1+b_5	42.11	8.0	5.26
rat CYP3A1+b_5	49.99	13.0	3.85
rat CYP3A2+b_5	62.51	20.0	3.13

3-Aminobenzanthrone is oxidized by peroxidases

Among peroxidases tested in this work to be active in 3-ABA oxidation, horseradish peroxidase was found to be the most active, followed by myeloperoxidase and lactoperoxidase (*Fig. 4*).

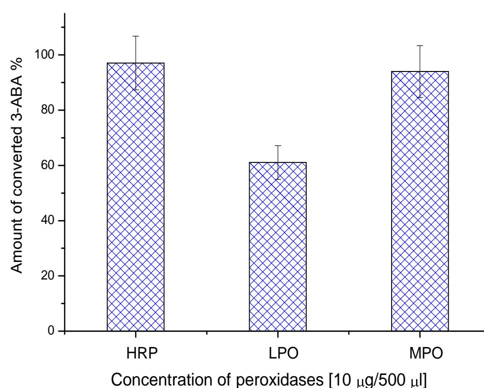


Fig. 4 Oxidation of 3-ABA by peroxidases. The values are averages and standard deviations of duplicate incubations.

3-Nitrobenzanthrone induces expression of biotransformation enzymes in kidney and lung after intraperitoneal application to rats

3-Nitrobenzanthrone was found to induce expression of CYP1A1 and NQO1 enzyme proteins in kidney and lung of rats treated intraperitoneally (i.p.) with this compound. Such an induction corresponded to an increase in enzymatic activities of these enzymes. In addition, by inducing lung and kidney CYP1A1 and NQO1, 3-NBA increases its own enzymatic activation as well as that of its metabolite, 3-ABA, thereby enhancing the genotoxic and carcinogenic potential of both compounds (*Fig. 5 and 6*).

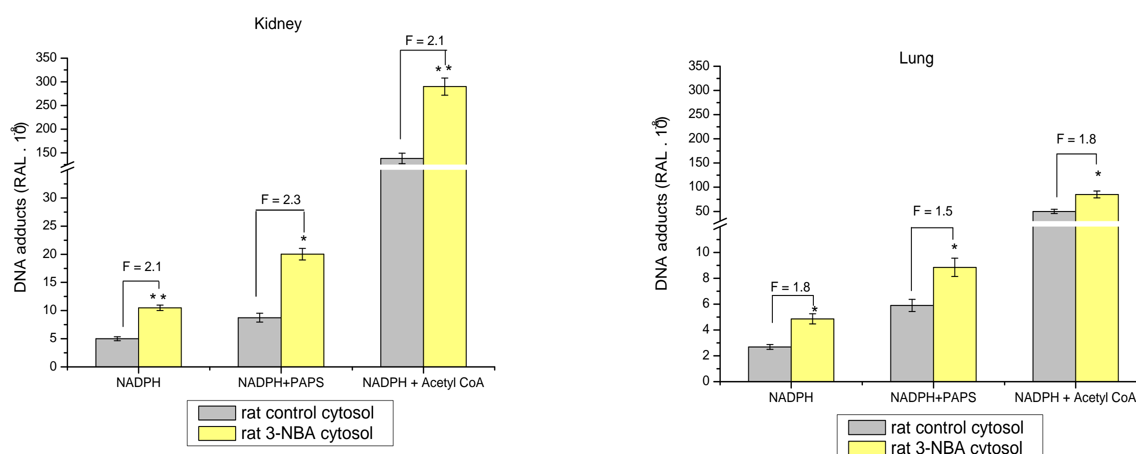


Fig. 5 DNA adduct formation by 3-NBA activated with cytosols isolated from lungs and kidneys of rats control and treated with 40 mg/kg bw of 3-ABA *p<0.05, **p<0.01 The values are averages and standard deviations of triplicate incubations.

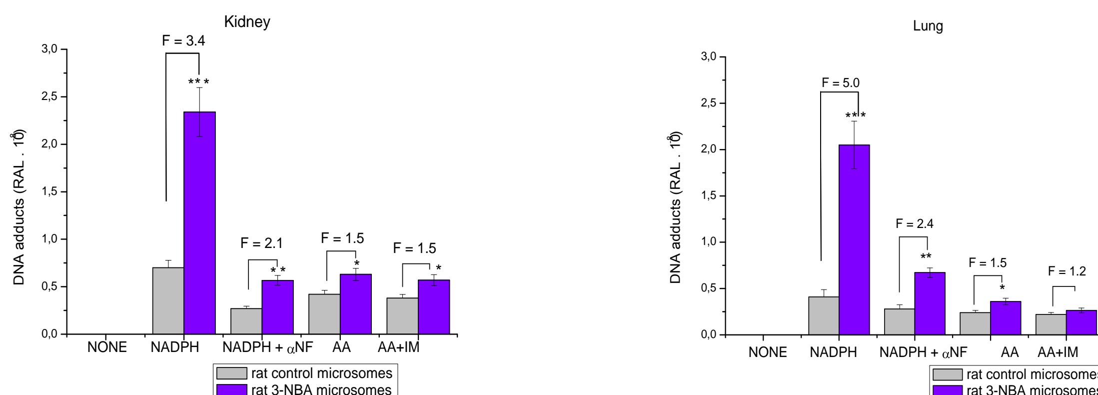


Fig. 6 DNA adduct formation by 3-ABA activated with microsomes isolated from lungs and kidneys of rats control and treated with 40 mg/kg bw of 3-NBA **p<0.01, **p<0.001 The values are averages and standard deviations of triplicate incubations.

3-Aminobenzanthrone induces expression of biotransformation enzymes in kidney and lung after intraperitoneal application in rats

3-Aminobenzanthrone was also investigated for its ability to induce CYP1A1 and NQO1 in kidney and lung of rats, and for the influence of such induction on DNA adduct formation by 3-ABA and 3-NBA. 3-ABA is capable of inducing CYP1A in rat lung and kidney and NQO1 in lung. In contrast, no induction of NQO1 expression by 3-ABA has been found in kidneys. Even though 3-ABA is also effective to induce these enzymes, its inducing potential is, however, lower than that of 3-NBA. The results found in this study show that by inducing lung and kidney CYP1A and NQO1, 3-ABA increases its own enzymatic activation as well as that of the environmental pollutant, 3-NBA, thereby enhancing the genotoxic and carcinogenic potential of both compounds (*Fig. 7 and 8*).

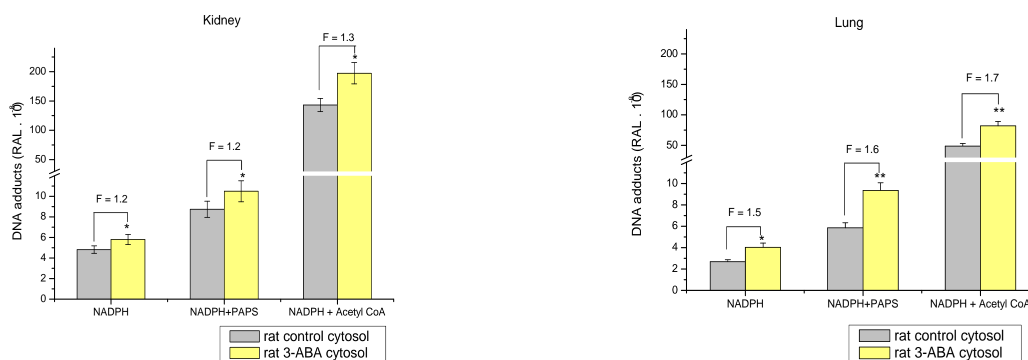


Fig. 7 DNA adduct formation by 3-NBA activated with cytosols isolated from lungs and kidneys of rats control and treated with 40 mg/kg bw of 3-ABA *p<0.05, **p<0.01 The values are averages and standard deviations of triplicate incubations.

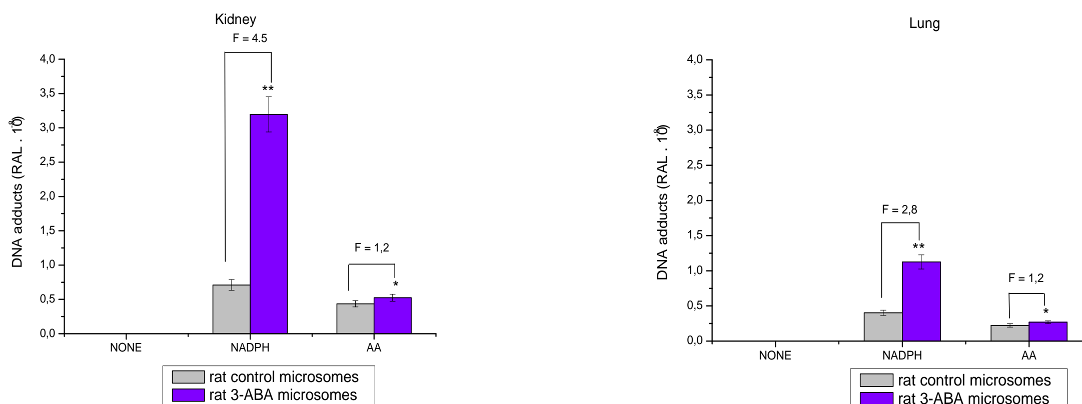
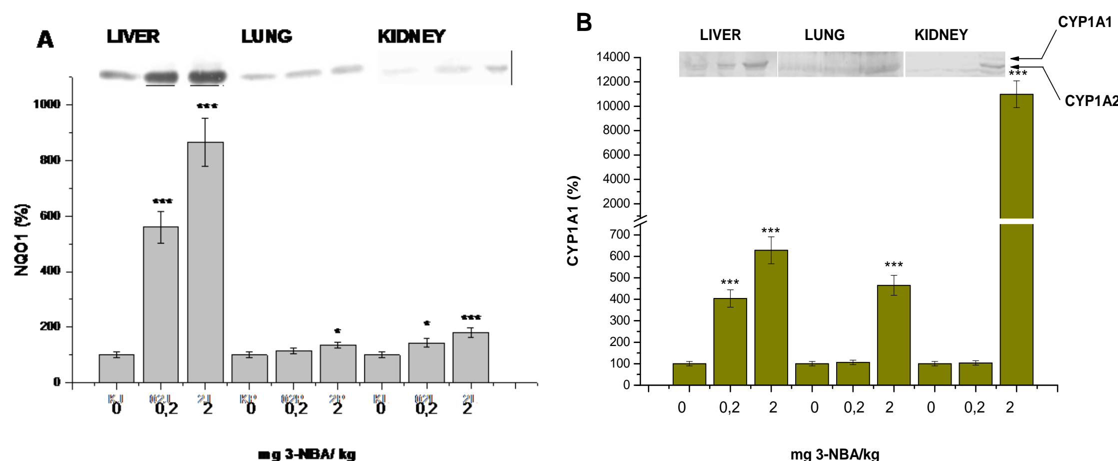


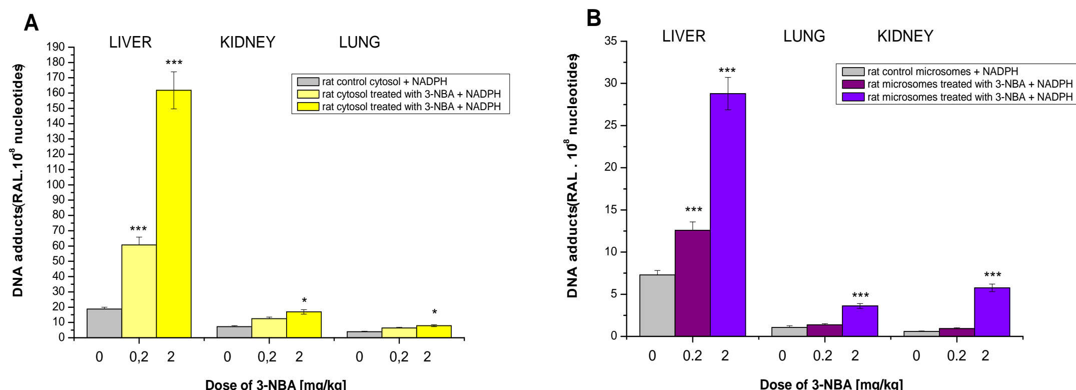
Fig. 8 DNA adduct formation by 3-ABA activated with microsomes isolated from lungs and kidneys of rats control and treated with 40 mg/kg bw of 3-ABA **p<0.01, **p<0.001 The values are averages and standard deviations of triplicate incubations.

3-Nitrobenzanthrone induces expression of biotransformation enzymes in liver, kidney and lung after intratracheal instillation in rats

Inhalation is the major route by which airborne material gain access to the human body. Therefore, we selected intratracheal instillation, which naturally simulate exposition of human to 3-NBA, to examine whether 3-NBA induces CYP1A1/2 and NQO1 also by such a type of exposition. 3-Nitrobenzanthrone is capable of inducing expression of proteins and enzyme activities of CYP1A1/2 and NQO1 in hepatic, renal and pulmonary tissues of rats also after intratracheal instillation of this carcinogenic compound (0.2 a 2 mg 3-NBA/kg) (Fig. 9). By inducing liver, lung and kidney CYP1A and NQO1 after intratracheal instillation, 3-NBA also increases its own enzymatic activation as well as that of its metabolite, 3-ABA, thereby enhancing the genotoxic and carcinogenic potential of both compounds (*Fig. 10, page 10*).



Obr. 9 Induction of NQO1 (A) and CYP1A1/2 (B) in livers, kidneys and lungs of rats after intratracheal instillation. The values are averages and standard deviations of triplicate incubations. For NQO1: **p*<0.05, ****p*<0.001, for CYP1A1/2: **p*<0.05, ****p*<0.01



Obr. 10 DNA adduct formation by 3-NBA activated with cytosols isolated from rats after intratracheal instillation (0.2 a 2 mg/kg 3-NBA) (**A**), $*p<0.05$, $***p<0.01$. DNA adduct formation by 3-ABA activated with microsomes isolated from rats after intratracheal instillation (0.2 a 2 mg/kg 3-NBA) (**B**), ***p<0.001. The values are averages and standard deviations of triplicate incubations

The major part of results of this PhD. thesis has already been published in scientific journals (see papers 1-5 in List of publications). Another paper, containing the results found in this work, is in preparation (see paper 6 in List of publications).

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