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The influence of single application of paracetamol and/or N-acetylcysteine on rats in subchronic exposition to trichloroethylene vapours. III. Effect on some isoforms of cytochrome P450 in liver

Wpływ pojedynczej dawki paracetamolu i/lub N-acetylocysteiny na szczury przewlekle eksponowane na trichloroetylen. III. Wpływ na wybrane izoformy cytochromu P450 w wątrobie

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ABSTRACT

Background: In case of overdose of paracetamol the ability of hepatic biotransformation is saturated and accumulation of toxic metabolite – NAPQI takes place. Main CYP isoforms considered to be responsible for bioactivation of APAP and promoting the same liver intoxication are CYP2E1, CYP1A2, CYP3A4 and in animals 2B1/2 isoforms additionally. Purpose of this work was examination of paracetamol influence and/or trichloroethylene on the composition of hepatic cytochrome P450 isoforms. **Materials and method**: Tests were carried out on rats which were treated with trichloroethylene, paracetamol and/or N-acetylcysteine. In the microsomal fraction content of three isoforms of cytochrome P450 i.e. CYP2E1, CYP2B1/2 and CYP1A2 were determined. **Results**: Paracetamol slightly stimulated CYP2B1/2 lowering simultaneously level of CYP1A2. Trichloroethylene stimulated CYP2B1/2. N-acetylcysteine stimulated all tested P450 isoforms. N-acetylcysteine given together with examinated xenobiotics induced studied P450 isoforms. **Conclusions**: N-acetylcysteine demonstrated a protective effect on studied CYP isoforms especially when was given upon termination of xenobiotics exposure.

Keywords: isoforms of cytochrome P450, liver, trichloroethylene, paracetamol, N-acetylcysteine

STRESZCZENIE

Wstęp: W przypadku przedawkowania paracetamolu, zdolność wątroby do detoksykacji zostaje wysycona i następuje akumulacja toksycznego metabolitu, jaki jest NAPQI. Główne izoformy CYP, uważane za odpowiedzialne za bioaktywację APAP-u i sprzyjające w ten sposób zatruciom wątrobowym, to CYP2E1, CYP1A2 oraz CYP3A4 a u zwierząt dodatkowo izoformy 2B1/2. Celem pracy było zbadanie wpływu paracetamolu i/lub trichloroetylenu na skład wątrobowych izoform cytochromu P450. **Materiał i metody**: Badania wykonano na szczurach, które traktowano trichloroetylenem, paracetamolem i/lub N-acetylocysteiną. We frakcji mikrosomalnej wątroby oznaczano zawartość trzech izoform cytochromu P450, tj., CYP2E1, CYP2B1/2 oraz CYP1A2. **Wyniki**: Paracetamol lekko stymulował CYP2E1 obniżając równocześnie poziom CYP1A2. Trichloroetylen stymulował CYP2B1/2. N-acetylocysteina miała stymulujący wpływ na wszystkie badane izoformy P450. N-acetylocysteina podawana łącznie z badanymi ksenobiotykami prowadziła do wyraźnych wzrostów CYP. **Wnioski**: N-acetylocysteina wykazywała ochronny wpływ na poziomy badanych izoform cytochromu P450, szczególnie, jeśli została podana zaraz po zaprzestaniu ekspozycji na ksenobiotyki.

Słowa kluczowe: izoformy cytochromu P450, wątroba, trichloroetylen, paracetamol, N-acetylocysteina

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INTRODUCTION

Acetaminophen (APAP) is one of the most commonly used analgesics and antipyretics in the world. It is a safe medication in therapeutic doses, however, is poisoning in certain risk group it can cause a severe necrosis located in the middle section of lobule [1].

In case of paracetamol overdose, detoxification ability of the liver becomes saturated and starts accumulation of a toxic metabolite, NAPQI. The main cytochrome P450 isoforms (CYP), which are thought to be responsible for APAP bioactivation, thus promoting liver poisoning (hepatotoxicity), are CYP2E1, CYP2C9, CYP1A2 and CYP3A4 [2, 3] and in animals additionally 2B1/2 isoforms [4].

A common feature of all modern threats, both environmental and occupational, is a combined exposure, repeatedly leading to unpredicted biological answer of the body, resulting from interactions on cytochrome P450 system participating in biotransformation of both trichloroethylene and paracetamol. It is thought that CYP2E1, CYP2B1/2 and CYP1A1/2 [4] isoforms play a particular role in biotransformation of both of these xenobiotics in animals.

As it is known, NAPQI is a toxic metabolite of paracetamol, and is formed by oxidative metabolism with cytochrome P450 isoforms involved. At present, research is focused on the issue which isoenzymes and to what extent metabolize paracetamol. At least several of these isoenzymes were shown to be involved in this metabolism [5–7].

The aim of this study was to investigate the effect of paracetamol and/or trichloroethylene and N-acetylcysteine on the content of selected cytochrome P450 isoforms. It is commonly known that P450 2E1, 1A1/2 and 2B1/2 cytochromes participate in TRI metabolism [8]. If cytochrome P450 actively participates in the metabolism of the aforementioned xenobiotics, we wish to find out what is the effect of these xenobiotics on this cytochrome isoforms.

MATERIALS AND METHODS

Animals

The examinations were conducted on male Wistar rats with body mass 280–300 g. The animals were kept separately in plastic cages throughout the examination in controlled culture conditions with constant air humidity (60%), constant temperature (22 ± 2° C) and 12 hour cycle day/night. The animals were fed on Murigan type standard granulated fodder, with unlimited water access.

This research was approved by the Local Bioethics Committee of The Medical University in Poznań.

Experiment outline

The animals were divided into groups, 6 in each. They were administered xenobiotics separately and collectively according to the following regimen:

1. The control group
2. APAP – 250 mg/kg b.m.
3. TRI – 50 mg/m^3
4. NAC – 150 mg/kg b.m.
5. TRI 50 mg/m^3 + NAC (0 h) 150 mg/kg b.m.
6. TRI 50 mg/m^3 + NAC (2 h) 150 mg/kg b.m.
7. APAP – 250 mg/kg b.m.+ TRI 50 mg/m^3
8. APAP – 250 mg/kg b.m.+ NAC (0 h) 150 mg/kg b.m.
9. APAP – 250 mg/kg b.m.+ NAC (2 h) 150 mg/kg b.m.
10. APAP – 250 mg/kg b.m.+ TRI 50 mg/m^3 + NAC (0 h) 150 mg/kg b.m.
11. APAP – 250 mg/kg b.m.+ TRI 50 mg/m^3 + NAC (2 h) 150 mg/kg b.m.

The control group were the animals not exposed to the mentioned xenobiotics. Experimental group animals were exposed to TRI vapours through inhalation route in the dynamic toxicological chamber in concentration 50 mg/m^3 of air for the following 7 days, 6 hours daily. Exposure to TRI lasted between 9.00 and 15.00. On the last day of exposure, at 9.00 were administered APAP by stomach tube. NAC was administered along with examined xenobiotics right after the exposure (0 h) or 2 hours following their application (2 h).

The level of the cytochrome P450 isoforms (CYP) were measured at the following time points: 4-, 12-, 24-, 48- and 120 hours after treatment.

Isolation of liver microsomes

Rats were sacrificed between 8.30 and 9.30 a.m. to avoid circadian fluctuations in the activity of the cytochrome P450-dependent monooxygenase system [9, 10]. Liver samples were placed in ice-cold physiological saline. The microsomal fraction was isolated by the method of Dallner [11].

Electrophoresis and Western Blot analysis of cytochrome P450 isoforms

The working medium was polyacrylamide gel (1 mm in thickness and 15 cm in length). The stacking gel was composed of 4% polyacrylamide dissolved in 0.125 mM Tris-HCl (pH 6.8) containing 0.1% SDS. The running gel was composed of 10% polyacrylamide dissolved in 0.375 mM Tris-HCl (pH 8.8)

containing 0.1% SDS. Microsome samples (10 µg of protein) were separated (as described above) according to the method of Laemmli [12].

The levels of cytochrome P450 isoforms in rat liver microsomes were determined by Western blot analysis. Separated proteins were transferred into a PVDF membrane (Milipore) and were stained immunochemically. Polyclonal anti-CYP rabbit antibodies were purchased from Chemicon Int. Inc. The antibody-CYP binding was visualized by using secondary antibodies coupled with alkaline phosphatase and by using the BCIP/NBT substrate (Sigma) as suggested by the manufacturer. Blots were analyzed using a densitometer with software One D-scan (Scanalytics company). In this paper only changes in studied groups were evaluated in relation to control which was adopted as 100%.

Statistical analysis

Characteristics of the examined parameters was presented in a form of arithmetic mean as a measure of central tendency and standard deviation as a measure of variability. Distribution normality of the examined parameters was verified with Kolmogorov-Smirnov accordance tests and Shapiro-Wilk test and visually evaluated by histograms. Distributions close to normal were considered those for which significance level as a result of testing was greater than 0.05 in both tests, and whose histogram shape was symmetrical. Distributions of all examined parameters were considered normal.

To estimate the effect of the examined xenobiotics on the level of cytochrome 450-dependent hepatic monooxygenases system (in subsequent time periods of 12-, 24-, and 120 hours) ANOVA variance analysis was used. Assumptions concerning variance homogeneity in this analysis were verified by means of Levene's test. As post-hoc test (to compare individual means with controls) in this ANOVA analysis, Dunnett's multiple comparison test was performed. The results were considered statistically significant with $p < 0.05$ and were marked in tables with the symbol "*". Statistical analyses were conducted using a professional set of STATISTICA PL statistical procedures, version 8,0.

Results

APAP had a stimulating effect on CYP2E1 (Table I). Since approximately 165% of control group value, reached in 4 hour since experiment completion, its level tended to rise and reached maximum after 24 hours. After 5 days of screening, it was still higher than in control group. A similar course of alterations was observed in case of CYP2B1/2 isoform, although, the stimulation of this protein was weaker. Paracetamol decreased the level of CYP1A2 isoform. Throughout the investigated period the level of this isoform was lower than in control. A particularly potent decrease was shown in 24 hour of screening.

Observed effect of trichloroethylene on CYP2E1 was different from the image obtained after APAP administration (Table I). A subtle stimulation was revealed, only limited to the first hours since experiment completion. CYP2B1/2 isoform immediately after experiment completion exceeded control level, which was still rapidly increasing. After 24 hours it exceeded control by approximately 150% and remained on this level till day 5 of screening. The

Tab. I. Effect studied of xenobiotics on some isoforms of cytochrome P450 in rat liver
Tab. I. Skutki badanych ksenobiotyków na skład wątrobowych izoform cytochromu P450 u szczurów

Xenobiotic	Isoforms	Time after exposition [in hours]				
		4	12	24	48	120
		The control group = 100%				
APAP	CYP 2E1	167*	142*	242*	133	124
	CYP 2B1/2	144*	98	172*	163*	126
	CYP 1A2	88	77*	65*	88	90
TRI	CYP 2E1	117	129*	104	111	94
	CYP 2B1/2	133*	129	145*	152*	146*
	CYP 1A2	116	102	89	88	105
NAC	CYP 2E1	135*	133*	158*	173*	141*
	CYP 2B1/2	107	160*	190*	153*	145*
	CYP 1A2	110	121	181*	175*	148*
APAP + TRI	CYP 2E1	109	120	131*	120	110
	CYP 2B1/2	147*	196*	148*	111	96
	CYP 1A2	110	103	143*	177*	123*

level of CYP1A2 immediately after the experiment was slightly increased, but after several hours it returned to control level.

N-acetylcysteine through the first several hours since experiment completion had a delicate stimulating effect on CYP2E1. However, after 24 hours the level of this protein was nearly 1.5-fold higher than in control (Table I). Such elevated level remained through a certain period, and still after 5 days did not approach control level. NAC also distinctly stimulated CYP2B1/2. Throughout screening period, revealed levels of this isoform were higher than in control (especially since 12 hour since experiment completion). This relation had no significant effect on CYP1A2 immediately after experiment completion, however, since 24 hour a potent, 180% stimulation was found, fixed in time.

Combined administration of APAP and TRI resulted in a very mild stimulation of CYP 2E1 level, which virtually remained through 5 days since experiment completion (Table I). Combination of these two xenobiotics had also a stimulating effect on CYP2B1/2. This time, more distinct increases were observed, reaching 200% of control value (in 12 hour of screening), while this stimulation rapidly faded after 24 hours. CYP1A2 isoform did not change its concentration in this treatment through the first hours since screening completion. Beginning with 24 hour, the level of this isoform tended to increase up to 175% of control value and then decreased, but even after 5 days the level exceeded control values.

Paracetamol combined with NAC within hours since experiment completion, modified CYP2E1. Since hour 4 its level clearly increased, reaching nearly 180% of control (Table II), retaining high levels even after 5 days. APAP and NAC noticeably stimulated CYP 2B1/2. In more than ten hours since the experiment completion the levels of this protein were revealed, exceeding 16% of control, which slightly decreased only after 48 hours. In these conditions CYP1A2 did not change its concentration, except for a delicate stimulation in a period 12–24 hours.

Delayed administration of NAC following exposure to APAP in initial stage after experiment completion, stimulated CYP2E1 a little more subtly than in the previous experiment (Table II). After 24 hours the observed levels of this isoform were similar to the group in which the animals were simultaneously exposed to these xenobiotics. This manner of exposure of rats had no significant effect on CYP2B1/2

in comparison to the previous experimental group. Similar observations also concern CYP1A2 isoform.

Combined exposure to TRI and NAC led to approximately 150% increase of CYP2E1 already after 4 hours since the experiment completion. It rose delicately in successive hours and remained on that level virtually throughout the screening period (Table II). In these conditions of experiment CYP2B1/2 isoform behaved in a similar manner, reaching levels slightly higher than 2E1. CYP1A2 through the period since experiment completion showed the levels in a range 110–130% of control value, then decreased to control group level after 5 days.

If after TRI inhalation, NAC was administered with 2 hour delay, it resulted in a potent 150% stimulation of CYP2E1 just after 4 hours since the exper-

Tab. II. Effects of APAP or TRI with exposition of N-acetylcysteine on some isoforms of cytochrome P450 in rat liver
Tab. II. Wpływ APAP lub TRI po potraktowaniu N- acetylocysteiną na skład wątrobowych izoform cytochromu P450 u szczurów

Xenobiotic	Isoforms	Time after exposition [in hours]				
		4	12	24	48	120
		The control group = 100%				
APAP + NAC [0 h]	CYP 2E1	132*	183*	176*	144*	160*
	CYP 2B1/2	134*	116	162*	169*	134
	CYP 1A2	100	120	128*	119	87
APAP + NAC [2 h]	CYP 2E1	122	155*	161*	161*	148*
	CYP 2B1/2	157*	140*	171*	190*	148*
	CYP 1A2	106	94	122	119	92
TRI + NAC [0 h]	CYP 2E1	152*	160*	199*	187*	159*
	CYP 2B1/2	107	154*	218*	197*	145*
	CYP 1A2	111	123	111	130*	108
TRI + NAC [2 h]	CYP 2E1	158*	144*	172*	180*	148*
	CYP 2B1/2	133*	130*	121	139*	93
	CYP 1A2	91	106	98	117	99

iment completion (Table II). It increased to 180% in 48 hour, then delicately decreased to approximately 150% after 5 days. CYP2B1/2 level for 2 days ranged within 130%, then decreased to control group value. In these conditions of experiment the level of CYP1A2 did not change.

Combined exposure to three evaluated xenobiotics induced CYP2E1. From approximately 170% right after the examination completion, to almost 190% after 48 hours (Table III). After that period, the concentration of this isoform tended slightly to decrease. A similar behavior exhibited CYP2B1/2 isoform, but in this case, just after experiment completion the absence of induction was observed. CYP1A2 responded to a combined administration of three xenobiotics in an increase of that protein level to over 140%, which then decreased and faded after 5 days of screening.

If exposure to TRI and APAP was supplemented with a delayed exposure to NAC, over 175% increase of CYP2E1 was found just after 4 hours of screening (Table III). In a later period, it tended to increase slightly and remained on a high level till day 5. Also, CYP2B1/2 isoform underwent stimulation, but not so distinctly and it reached 160% at its highest. CYP1A2 in the first hours since experiment completion had a tendency to a slight stimulation, but after 24 hours a continuous decrease of this isoform concentration was observed, up to 65% of control value after 5 days.

Tab. III. Effects of APAP and TRI with exposition of N-acetylcysteine on some isoforms of cytochrome P450 in rat liver
Tab. III. Wpływ APAP lub TRI po potraktowaniu N- acetylocysteiną na skład wątrobowych izoform cytochromu P450 u szczurów

Xenobiotic	Isoforms	Time after exposition [in hours]				
		120	120	120	120	120
		The control group = 100%				
APAP + TRI + NAC [0 h]	CYP 2E1	171*	180*	172*	188*	157*
	CYP 2B1/2	107	147*	178*	166*	135*
	CYP 1A2	144*	143*	118	126*	108
APAP + TRI + NAC [2 h]	CYP 2E1	176*	186*	186*	206*	182*
	CYP 2B1/2	131*	158*	138*	161*	128
	CYP 1A2	106	115	98	88	66*

DISCUSSION

As it was presented earlier in this study, NAPQI is a toxic metabolite of paracetamol. Firstly, it is formed by oxidative metabolism with CYP isoforms involved, which was shown in animal studies in the last century [2, 3, 13, 14]. At present, the studies are focused on the question which isoenzymes metabolize paracetamol, both in people and in various animals. Therefore, such isoenzymes are examined like CYP1A2, CYP2A6, CYP2D6, CYP2E1 or CYP3A4. Secondly, initial treatment of mice with CYP inhibitors before exposure to acetaminophen can significantly decrease the extent of liver poisoning (hepatotoxicity). Thirdly, it was shown recently, that "knockout" mice with cyp2e1 and double cyp2e1 and cyp1a2, are less susceptible to liver poisoning with acetaminophen [6, 15, 16] than their wild type counterparts.

Hepatotoxicity caused by paracetamol is defined as an increase of aspartate aminotransferase level and/or alanine aminotransferase to the level above 1000 IU/L. In patients with no elevated level of these aminotransferases, there is no liver damage, thus they are not threatened by a severe liver failure or death [17, 18]. Exceeding the level of 1000IU/L by aspartate or alanine aminotransferase is generally concerned with hepatic histological alterations, with necrosis of varied intensity, located in the middle section of acinus, the so-called zone III of hepatic lobule. It is significant to note that for most patients these alterations are completely reversible, not resulting in a long term organ damage. The data concerning patients with paracetamol poisoning who did not undergo a therapy with an antidote, showed that approximately 25% of patients died with a diagnosed liver failure. That means that even without N-acetylcysteine, over 75% of patients with acute poisoning will completely recover.

It seems that TRI has a delicate stimulating effect on CYP2B1/2, although in relation to other isoforms it has rather inhibitory character [19]. However, we demonstrated in our studies that CYP2E1 virtually did not change its level after inhalation with TRI. What is more, we also found that CYP1A2 did not decrease its level. These two facts correspond with

the lack changes in general concentration of P450 cytochrome, which makes the results of our study unique [19–21]. Among other factors, it is due to the fact that we showed 2B1/2 isoform induction and, as literature data indicate, another constitutive isoform, CYP2C11 also undergoes induction in these conditions [19]. Based on extensive literature data, it is known that other P450 isoforms are not engaged in TRI metabolism, or their participation is negligible [22, 23].

Summing it up, it should be emphasized that TRI shows its effect on several P450 cytochrome isoforms, including CYP2E1, CYP1A2 and CYP2B1/2. Now these observation must be confirmed on men. Changes in relative proportions of individual P450 isoenzymes at high concentrations of the solvents, which take place due to their improper use and accidental occupational exposure, may result in changes of metabolism routes of chemicals different from normal ones.

In general, we observed spectacular alterations in CYP2E1, CYP2B1/2 and CYP1A2 levels in groups with APAP and NAC, which is confirmed in literature [24].

We may think that NAC can relieve liver damage caused by paracetamol. This effect does not include modification of significant CYP-s, that is CYP2E1, CYP2B1/2 or CYP1A1/2. A complex mechanism based on cellular GSH protection, is likely to play a role here [25, 26]. We demonstrated that APAP, TRI and NAC are CYP2E1 and CYP2B1/2 stimulators, but rather decrease CYP1A2 level.

Studies *in vitro* of CYP1A2 and CYP2E1 inhibition suggest that CYP1A2 contributes to 30–50% metabolism of paracetamol, and 30–80% of this alteration involves CYP2E1. Studies in mice showed that in contrast to CYP2E1, CYP1A2 activity increased after higher doses of paracetamol [27]. The importance of these findings in animal studies in unclear, since the studies on human volunteers suggest that CYP1A2-inducing factors do not increase NAPQI production following therapeutic doses of paracetamol [28]. CYP2E1 is commonly known as a major isoenzyme responsible for NAPQI formation [29]. Inhibition of CYP2E1 *in vitro* significantly reduces the amount of produced NAPQI. CYP2E1 is thought to be the most efficient isoenzyme in metabolism of toxic paracetamol doses [27].

The evidence for extent of CYP3A1 involvement in paracetamol metabolism in animals is still insufficient. Studies *in vitro* suggest that human CYP3A4 (counterpart of animal CYP3A1) contributes to 1%-20% in total metabolism of this compound, especially at lower doses. Although paracetamol is a substrate of this isoform, it also appears to inhibit its activity [30]. Recent studies *in vitro* suggest that paracetamol bioactivation, both at therapeutic and toxic concentrations, to a great degree takes place with CYP3A4 participation [7, 31]. It seems that contribution of CYP3A4 in paracetamol metabolism and NAPQI formation in humans is probably limited.

Literature data indicate that CYP2E1 is significant in APAP bioactivation at low doses of this medication, but this isoenzyme may have only a marginal effect when there is a high level of paracetamol. CYP1A2 appears to play a greater role in APAP bioactivation and toxicity in its high doses [27, 32]. It was shown that in the liver, CYP2E1 induction, makes the liver susceptible to damage by acetaminophen. Therefore, earlier findings which emphasized a major role of CYP2E1 in APAP bioactivation, might underestimate the role of isoenzymes from CYP3A subfamily.

It strengthens the belief that CYP3A subfamily is an important CYP isoform which catalyses APAP alteration into NAPQI. Therefore, we may assume that in humans the role of CYP3A4 was earlier underestimated, after hepatotoxic APAP overdose, though it is the most abundant CYP in the liver and that it is the most efficient APAP bioactivator, according to demonstrated studies [7, 31].

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