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Effect of green tea on NADPH-cytochrome P-450 reductase activity in selected human organs

NADPH-cytochrome P-450 (CPR) catalysis with one electron reduction is involved in the toxicity of various drugs like doxorubicin and bipyridylium herbicides, e.g., paraquat. Doxorubicin – an anthracycline antibiotic – has been used in cancer therapy for over 30 years. Its CPR dependent cardiotoxicity complicates therapeutic processes and limits the effective dose (14). Unlike doxorubicin, target organs in paraquat intoxication are lungs, kidneys and liver (3).

Independently of the substrate used, CPR transfers one electron from NADPH to doxorubicin or paraquat which in turn transfers electron to O2 forming superoxide radical. That is the first crucial step in the sequence of events leading toward cell death (1). As a result, changes in the redox status and subsequent apoptosis in cardiac muscle cells were observed in case of doxorubicin intoxication (9). In paraquat toxicity, redox cycling leads not only to the generation of superoxide anion but also to a potent depletion of intracellular NADPH (12). However, the same CPR molecular mechanism is desirable in some kinds of therapy. It plays a crucial role in the reductive activation of some drugs, e.g., tirapazamine. The enzyme is able to bioactivate tirapazamine to its radical form, which reoxidizes rapidly in the presence of O2 forming a parent compound (11). Under hypoxic conditions the toxic radical species live longer and interact with DNA leading to chromosomal damage. Such reaction is suggested as a cytotoxic mechanism against malignant neoplasmatic changes (4).

Taking into account the dual effect of the enzyme activation, it seems to be useful to search for new compounds, which might modulate CPR activity. Such substances would improve the therapeutic efficiency of tirapazamine and/or diminish doxorubicin and paraquat toxicity. Recently, great interest is given for natural medicine, including mixed medicinal herbs.

One of the most common studied natural products is green tea obtained from *Camellia sinensis* plant. It contains mainly polyphenols, flavonols, flavandiols, phenolic acids and flavanols (catechins) such as (-)-epigallocatechin-3-gallate (EGCG), (-)-epicatechin, (+)-gallocatechin and catechin (10).

The extract has a potent antioxidant activity (2). Therefore, the aim of this study was to evaluate the effect of green tea extract on the CPR activity in human heart, liver and lungs *in vitro*.

MATERIAL AND METHODS

The samples were collected during the medical autopsy according to the national law and followed guidelines of the Declaration of Helsinki and Tokyo for humans. All chemicals obtained from commercial sources, were of the highest quality and were used without further purification. NADPH and cytochrome c (oxidised form) and Bradford Reagent were purchased from Sigma Chemical Co. (St. Louis, USA) and Fluka Chemica (Switzerland), respectively.

Tissue preparation. Seven samples of human male livers, hearts and lungs were collected 24 h after death, and stored at –70°C until used. Immediately after defrosting samples of human tissues were rinsed with buffer (150 mM KCl, 50 mM Tris, pH 7.4), minced with scissors, and homogenized on ice in a motor-driven Potter-Elvehjem homogenizer with three volumes of buffer. The crude homogenate was centrifuged at 8,750 g for 15 min at 4°C. Microsomes were sediment from the 8,750 g supernatant by centrifugation at 165,000 g for 38 min at 4°C. After two washings microsomes were dispersed in homogenizing buffer to provide a protein concentration of approximately 30 mg/ml.

Solution preparation. Green tea (2.5 g) was added to 50 ml of boiling water and was steeped for 15 min. The infusion was cooled to room temperature and then filtered. The tea leaves were extracted subsequently and used twice and every time 25 ml of boiled water and filtrates were added to obtain a 2.5% tea water extract – 2.5 g tea leaf/100 ml water.

Enzyme assay. The activity of NADPH-cytochrome P-450 reductase (EC 1.6.2.4.), was evaluated by measuring the rate of cytochrome c reduction at 550 nm. Microsomes were incubated in 33 mM phosphate buffer pH 7.7 in the presence of 1 mM KCN. Cytochrome c was used (0.025 mM) as an electron acceptor. The green tea infusion was added (1, 2, 5 and 10 µl) separately to the reaction mixture (total volume of 1000 µl). After 5 min incubation at 25°C, reaction was started by addition of NADPH (0.1 mM). Activity of NADPH-cytochrome P-450 reductase was monitored for 2 min (at 30 s intervals) after NADPH addition, using Shimadzu UV-160A spectrophotometer. In control samples the tested compounds were replaced with buffer. Milimolar extinction coefficient (ε =0.021 mM-1cm-1) was used for the calculation and the enzyme activity was expressed in nmoles of reduced cytochrome c/min/mg microsomal protein. The content of microsomal protein was measured using Bradford Reagent. Bovine serum albumin served as a standard.

Statistical analysis. Results are given as minimum (Min) and maximum (Max) value, arithmetic mean (M), standard deviation (SD) and median (Me). The differences between experimental and control group were evaluated using the Mann-Whitney's U test. To calculate differences between CPR activities with all used concentrations of tested preparations in particular organ the Friedman ANOVA and Wilcoxon tests were used. To compare the CPR activity in different organs at the same concentration of tested compound the Kruskal-Wallis ANOVA test were used. An (α=0.05, $p<0.05$) was considered significant. The data were analyzed using STATISTICA 5.0 (StatSoft Inc., USA).

RESULTS

The effect of green tea on human P-450 reductase activity at the amount of 1, 2, 5 and 10 µl in total volume of 1,000 µl reaction mixture was tested. The findings were presented in Table 1.

Table 1. The statistical characteristics of NADPH cytochrome P-450 activity [nM cyt.c/min/mg microsomes protein] in the presence of green tea infusion

Organ/ Concentration		Min	Max	M	SD	Me	ΔM	Δ% M	p'	ANOVA	p
Liver	C	121.00	192.00	161.29	24.55	167.00					
	1 µl	135.00	242.00	177.57	32.65	174.00	16.28	10.09	ns	21.0000	0.0001
	2 µl	173.00	248.00	211.43	27.23	214.00	50.14	31.09	< 0.01		
	5 µl	192.00	281.00	247.00	32.27	253.00	85.71	53.14	< 0.01		
	10 µl	189.00	257.00	227.86	24.90	234.00	66.57	41.27	< 0.01		
Heart	C	8.00	22.00	13.71	4.64	14.00					
	1 µl	12.00	24.00	16.71	3.73	17.00	3.00	21.91	ns	6.0455	0.1094
	2 µl	15.00	22.00	18.43	2.37	19.00	4.72	34.42	< 0.05		
	5 µl	16.00	24.00	19.14	2.79	19.00	5.43	39.63	< 0.05		
	10 µl	15.00	24.00	18.71	3.09	17.00	5.00	36.50	< 0.05		
Lung	C	138.00	213.00	168.86	27.34	157.00					
	1 µl	181.00	211.00	193.71	9.38	193.00	24.85	14.72	ns	15.2647	0.0016
	2 µl	186.00	214.00	202.14	8.78	201.00	33.28	19.71	< 0.05		
	5 µl	211.00	243.00	226.57	10.74	227.00	57.71	34.18	< 0.01		
	10 µl	198.00	234.00	214.29	11.93	211.00	45.43	26.90	< 0.01		

p' vs. control; C – control; ns – not significant

and Figure 1. The activity of CPR *in vitro* was significantly increased in the liver, heart and lung microsomes in the presence of 2, 5 and 10 µl of added aqueous extract from green tea. The most potent effect after addition of 1 and 2 µl of the extract was observed in microsomes obtained from the heart (21.91 and 34.42%). However, the rise in the Δ% of CPR activity caused by 5 and 10 µl was in the following order: liver > heart > lung. When the green tea was added in the amounts of 2, 5 and 10 µl to the reaction mixture containing liver microsomes the mean values of CPR activity increased to 31.09, 53.14 and 41.27%, respectively. The same amounts of green tea added to the lung microsomes caused the rise in the mean value of CPR activity about 19.71, 34.18 and 26.19%, respectively.

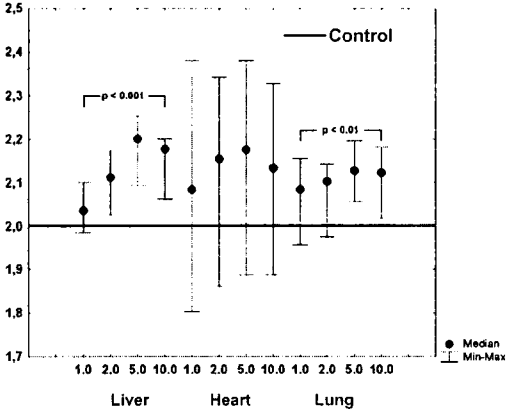

Fig. 1. The activity of NADPH-cytochrome P-450 reductase expressed as l g% of control value in human tissue microsomes incubated with green tea (control = 2)

There were no significant changes in CPR activity in the heart microsomes treated with different concentrations of green tea. In liver microsomes, statistical significance between subsequent concentrations of green tea infusion was observed. In lung microsomes statistical significance was found between 2 and 5 µl after addition to every tested mixture. Additionally, the differences in CPR activity between samples containing 5 and 10 µl of green tea infusion were statistically significant.

DISCUSSION

Some of catechins from green tea can decrease the production of reactive oxygen species (ROS) that are generated e.g. in the presence of CPR. The major polyphenolic catechin in green tea – EGCG is an antioxidant which is able to prevent the formation of tumour by protecting cellular components against oxidative damage via free radical scavenging (2, 13). It has to be stressed that while low concentrations of EGCG inhibited DNA damage induced by reactive oxygen and nitrogen species, high concentrations of phenolic phytochemicals by themselves caused the oxidative damage to cellular DNA (8). Unno et al. (15) reported that consuming four cups (800 ml) of green tea daily results in ingestion of 17.7 µmol EGCG/kg-bw/day. However, Jankun et al. (7) found that a single cup of green tea contains about 150 mg EGCG. On the basis of these data it was calculated that volumes of green tea extract, in our study ranging from 1 to 10 µl represent the concentration which is the equivalent to 0.001–0.01 µM or 0.004–0.04 µM of EGCG.

The present study showed that green tea increased CPR activity in all studied organs. The most potent and significant effect for low doses of the tea extract (1 and 2 µl) was observed in heart, while 5 and 10 µl rise the mean enzyme activity in the following order: liver>heart>lung. Hasaniya (6) reported that EGCG, one of the most potent compound of green tea, blocked the production of reactive oxygen species derived from CPR-mediated oxidation of 2-amino-3 methylimidazol (4, 5-f) quinoline, a carcinogen found in cooked meat. In the same study it was found that EGCG acted as competitive inhibitor of CPR reductase with Ki = 9.7 µM. It was also reported that EGCG inhibited human CPR with Ki value of 2.5 µM. Therefore, it may be concluded that a low concentration of green tea (in which EGCG amount is below 0.04 µM), enhances human CPR activity, but a higher concentration ranging from 2–10 µM inhibits the enzyme.

Addition of green tea to the reaction mixture caused the increase in CPR activity, which was statistically significant in lung between 2–5 µl and 5–10 µl. In liver microsomes, the statistical significance between subsequent concentrations of green tea infusion was found. However, there were no significant changes in CPR activity between tested amounts of green tea in heart microsomes.

There a question arises why the same concentrations of compound tested result in different activation (Δ) of CPR in microsomes obtained from different tissues. It seems that the expression of this enzyme is different in tested tissues. Observation of physiological activity (controls – a buffer instead of tested compound) in the liver, heart and lung might confirm this suggestion. Moreover, the studies conducted by Hall (5) revealed specific cellular localization of CPR in some human tissues.

Despite different expression of CPR protein in particular tissue it may be expected that a relative increment (Δ%) of CPR activity in the liver, heart and lung should be more or less the same. Nevertheless, 1 µl of green tea results in the elevation of Δ% value in the liver, lung and heart by about 10.09, 14.72 and 21.91%, respectively. Taking into consideration that these finding are relative (Δ%), different amounts of protein in microsomes from particular tissues do not explain this finding. As reactions were conducted under the same conditions (temp., pH, time of incubation, and the same amount of green tea infusion) it may be suggested that the increase in the relative activity of CPR at the same concentration but in different tissue depends on the ratio of CPR protein to

the concentration of the compound tested. Such effect could be also explained by the presence of different CPR isoforms in particular tissues but up to now no information confirming their existence were found. Even thought such explanation seems to be pure theory, there is no other way to prove this hypothesis now.

In conclusion, green tea infusion increases CPR activity in heart, lung and liver. The present results are promising for future studies to obtain the more beneficial therapeutic effect of bioreductive agents like tirapazamine. However, to prove the potential toxicity enhancement, as well as beneficial effect of green tea, further studies including doxorubicin and tirapazamine are desirable.

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SUMMARY

The purpose of the study was to evaluate the effect of green tea infusion on NADPH-cytochrome P-450 reductase (CPR) activity. The activity of the enzyme in human heart, liver and lung homogenates was determined spectrophotometrically by the measurement of the rate of cytochrome c reduction at 550 nm *in vitro*. After addition of green tea infusion to the reaction mixture, generally, the CPR activity raised in all tested organs in the following sequence: liver > heart > lung. There were no significant changes in CPR activity in the heart microsomes between different concentrations of green tea. In liver microsomes, statistical significance between subsequent concentrations of green tea infusion was observed. In lung microsomes statistical significance was found between 2 and 5 µl after addition to every tested mixture. Additionally, the differences in CPR activity between samples containing 5 and 10 µl of green tea infusion were statistically significant. The present results are promising for future studies to obtain the more beneficial therapeutic effect of bioreductive agents like tirapazamine. However, to prove the potential toxicity enhancement, as well as beneficial effect of green tea, further studies including doxorubicin and tirapazamine are desirable.

Wpływ zielonej herbaty na aktywność reduktazy NADPH cytochromu P450 w wybranych narządach ludzkich

Celem pracy była ocena aktywności reduktazy NADPH cytochromu P450 (CPR) w obecności naparu z zielonej herbaty. Aktywność enzymu była mierzona w mikrosomach otrzymanych z ludzkich skrawków tkanek, pobranych z serca, wątroby i płuca. Metodą spektrofotometryczną oznaczano stopień redukcji cytochromu c przy długości fali 550 nm. Dodanie naparu z zielonej herbaty w objętości 1, 2, 5 i 10 µl do środowiska reakcji prowadziło do wzrostu aktywności CPR we wszystkich przebadanych narządach. Największy wzrost aktywności badanego enzymu obserwowano w wątrobie i sercu, a najmniejszy w płucach. Nie stwierdzono różnic istotnych statystycznie między aktywnością CPR w mikrosomach serc w obecności różnych objętości naparu zielonej herbaty. W mikrosomach wątroby zaobserwowano statystycznie istotne różnice aktywności enzymu w obecności wzrastających ilości naparu. Natomiast w mikrosomach otrzymanych z płuc różnice statystyczne stwierdzono między oznaczeniami wykonanymi dla 2 i 5 µl oraz 5 i 10 µl. Przedstawione wyniki mogą sugerować pożądane działanie zielonej herbaty w przypadku leków, których skuteczność terapeutyczna jest uzależniona od bioaktywacji. Jednak zarówno możliwość nasilenia toksyczności takich leków, jak doksorubicyna, czy uzyskanie większej skuteczności terapeutycznej (np. tirapazaminy) w obecności zielonej herbaty wymaga dalszego poszerzenia badań.