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THE OXIDATION STATUS OF *ALDH3A1* IN HUMAN SALIVA AND ITS CORRELATION WITH ANTIOXIDANT CAPACITY MEASURED BY ORAC METHOD

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Abstract: Oxidation status of the salivary aldehyde dehydrogenase (ALDH) was measured in healthy human population using two-assay fluorimetric method and compared with antioxidant capacity (ORAC) in non-smoking and heavy smokers group. Influence of high or low antioxidant diet was also examined. Except for the group of smokers, the salivary ALDH oxidation degree in human saliva was not correlated with antioxidant capacity. Simultaneously direct administration of the antioxidant-containing drug, Fluimucil, resulted in short-term, but statistically significant increase of the reduced (active) form of the enzyme, presumably due to a radical-scavenging activity of the drug.

Keywords: aldehyde dehydrogenase, enzyme oxidation, saliva, antioxidant capacity, fluorimetric assay, radical scavengers

Oxidative stress is defined as an imbalance between production of reactive oxygen species (ROS) and a biological system's ability to neutralize them *via* antioxidant enzymatic and/or nonenzymatic activity. Large amounts of reactive intermediates lead to cell component damage and production of secondary toxic compounds, for instance reactive aldehydes and ketones (1, 2), in consequence causing an increased risk of many diseases including cancer.

Oxidative stress can be monitored in body fluids by various analytical methods including estimation of so-called total antioxidant capacity (ORAC, Oxygen Radical Absorbing Capacity). The ORAC-FL test uses fluorescein as a fluorophore (3), and follows time extinction of the fluorescence emission resulting from exposition to peroxyl radical generated with 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) as a radical source. This decay is substantially delayed in the presence of chemical and/or enzymatic antioxidants.

The saliva is probably the most easily available body fluid and exhibits variable antioxidant capacity (4). Moreover, the antioxidant enzymes present in saliva may play an important role in cancer prevention (2, 5). In the present work, we examine possible correlation between activity of the salivary aldehyde dehydrogenase [ALDH, E.C. 1.2.1.3, see ref. (6-9)], and particularly its degree of oxidation, and antioxidant capacity of the saliva samples.

The salivary ALDH3A1 is a homodimeric enzyme oxidizing mainly long- and medium-chain aliphatic and aromatic aldehydes (but not acetaldehyde), and utilizing both NAD^+ and $NADP^+$ as cofactors (6). It is involved in neutralization of a variety of aldehydes of plant origin, as well as those

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formed during food processing or applied as food additives, and therefore may be directly related to protection and prevention of chemical carcinogenesis (7-9). The same isozyme, expressed selectively in various tissues, is responsible, along with ALDH1A1, for cellular resistance to oxazaphosphorine drugs (10).

We have shown recently that salivary ALDH undergoes reversible oxidation in thiol-free media, and the degree of such oxidation can be measured using fluorimetric two-assay method, utilizing fluorogenic naphthaldehyde substrates (11). This method is profiting from the fact that dithiols like dithiothreitol (DTT) or dithioerythritol are able to regenerate the previously oxidized enzyme within a few minutes.

Preliminary data (11) indicated that salivary ALDH is in the most cases partially oxidized already in the moment of saliva collection. Moreover, both specific activity and the degree of inactivation vary with time and are highly variable in the healthy population (to be published elsewhere). It is therefore interesting to check, if the inactivation of salivary ALDH is dependent on antioxidant capacity of saliva, or in general, whether the chemical antioxidants, frequently found in food or applied as drugs, or food additives, can affect the general oxidation status of this enzyme in saliva.

EXPERIMENTAL

Chemicals

Syntheses of 6-methoxy-2-naphthaldehyde (MONAL-62) and the corresponding carboxylic acid were described previously (12), and both compounds are now available from Sigma. Concentration of the stock solutions was determined spectrophotometrically, by use of molar extinction coefficients 14400 for MONAL-62, and 7100 for the corresponding carboxylate. NAD^+, glutathione (GSH) and DTT were purchased from SIGMA (St. Louis, USA); all other chemicals were of analytical grade. Water was purified with a Mili-Q filter system (Milipore Corp., USA).

2,2-Azobis(2-amidinopropane) dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8--tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich, and uranin (fluorescein sodium salt) from POCh (Gliwice, Poland).

Stock fluorescein solution (80 nM) in PBS (0.8% NaCl; 0.02% KH_2PO_4; 8.1 mM Na_2HPO_4; 0.02% KCl; pH 7.4) was stored at 4°C for 2 months. Fluimucil® was a commercial product (Zambon Group S.p.A, Italy), approved in Poland for treatment of respiratory ways, as mucolytic drug.

Saliva collection

Saliva samples were collected directly to test tubes and used as collected for ORAC measurements. For ALDH activity determination, samples were diluted 1:1 with cooled 50 mM phosphate buffer pH 8, containing 0.5 mM EDTA and 1 mM GSH, then centrifuged and the precipitate was discarded. Samples were kept at 5°C. The activity was measured after 2-5 h from collection.

If not otherwise indicated, saliva collection was performed between 8 and 9 a.m., after carefully washing mouth with water, and before the first meal.

Diet description

A group of 16 volunteers was asked to strictly follow the described dietary rules for 3 days:

- DIET No. 1 – rich in high fat dairy products, pork meal, rye white bread with butter, wheat noodles, white rice. No fruit or vegetables were included. There were no energetic limits to this diet, but typically daily intake was 2000 kJ.
- DIET No. 2 – rich in boiled vegetables, salads, fruits and fruit juices, poultry, low-fat dairy products, boiled potatoes and whole-wheat bread, with total daily intake ~2000 kJ.

METHODS

ALDH assay

Fluorometric assays (12) were run on a thermostated Shimadzu 5001PC or Perkin-Elmer LS50B instruments, using time-drive mode, with excitation and emission wavelengths of 310 nm and 360 nm, respectively. Spectral bandwidths were 3-5 nm for excitation and 7-10 nm for emission beams. Assays utilize a highly fluorogenic naphthaldehyde substrate,6-methoxy-2-naphthaldehyde (MONAL-62), reacting with NAD^+ as co-substrate. The estimated apparent K_m values were 0.24 μM for MONAL-62 and 1.2 μM for NAD^+ (11).

Fluorimetric assays were run in 50 mM phosphate, pH 8.1, at 25°C, in the presence of 0.5 mM EDTA and either 0.5 mM DTT or 1 mM GSH. Typical substrate concentrations were 5 μM for MONAL-62, 100 μM for NAD^+. The saliva samples were diluted 20-fold with buffer, and an increase of fluorescence of the naphthoate was recorded for 6 – 10 min.

The purified reaction product (6-methoxy-2-naphthoate) at concentrations of 1-3 μM was used as an internal standard to obtain absolute reaction rates, which were calculated according to the formula:

$$V = \frac{dF}{dt} \cdot \frac{C_{st}}{F_{st}}$$

where dF/dt is the fluorescence slope, C_{st} is the concentration of standard, F_{st} is the fluorescence of the standard.

Specific activities were calculated as the ratio of the reaction rate to protein concentration, the latter determined by the Bradford method.

Oxidation status of salivary ALDH:

The degree of ALDH oxidation was determined by two consecutive assays (11):

1. in the buffer containing 1 mM GSH,
2. in the buffer containing 0.5 mM DTT

The ratio of the two measured activities was always less than 1, and represents the percent of the active (reduced) form of ALDH3A1 at the moment of saliva collection. Fraction of the inactivated (oxidized) form was calculated as:

$$1 - \frac{v(GSH)}{v(DTT)}$$

where v(GSH) and v(DTT) are reaction rates, in relative units, recorded in buffers 1. and 2., respectively. No activity in the presence of GSH (buffer 1.) means 100% inactivation of the enzyme.

ORAC assay

The ORAC-FL (Oxygen Radical Absorbance Capacity-fluorescein) assay was performed according to Ou et al. (3). All solutions used (fluorescein, AAPH) were prepared daily in PBS buffer, pH 7.4. For measurements, 300 µL of diluted saliva (100-250 fold with PBS, pH 7.4) and 1800 µL of 80 nM fluorescein solution were mixed and thermostated for 10 min at 37°C. Then, 900 µL of 40 nM AAPH solution was added. AAPH is widely used as a free radical generator since its thermal decomposition produces molecular nitrogen and two carbon radicals. A fraction of free radicals generated by AAPH is inactivated by salivary antioxidants while the rest oxidizes fluorescein, causing a decay of fluorescence. Fluorescence of the remaining fluorescein was measured every minute for 90 min after AAPH addition.

The ORAC value was determined as a difference between the area under the curve (AUC) values of the fluorescence decay curve for the saliva sample and the blank (PBS buffer). The AUC was calculated according to the equation:

Table 1. Basic statistics for ALDH activity and ORAC measurements in saliva of healthy volunteers.

	Control groups			Smokers groups	
	Warsaw group (a) (N = 42)	Olsztyn group (b) (N = 37)	Warsaw group (c) (N = 34)	Heavy-smokers group (d) (N =14)	Smokers group (e) (N = 15)
age [yr] females	17-60 24	19-62 22	18-70 20	24-56 11	20-65 5
average ALDH activity [U/g]*	0.60	2.00	1.37	0.49	1.25
C.V.	163	106	168	232	114
average ALDH inactivation [%]	64	60	54	74	68
C.V.	108	42	104	81	72
average ORAC [µM Trol]	2246	nd**	nd**	4170	nd**
C.V.%	96	-	-	114	-

*Activity measured in the presence of 1 mM GSH; ** not determined

Table 2. Effect of fat-rich and fat-low diets, applied for 3 days, on salivary ALDH activity and ORAC, determined in a group of 16 non-smoking, healthy volunteers.

	After diet #1		After diet #2	
	average	C.V. [%]	Average	C.V. [%]
Average ALDH activity [U/g]	0.24	204	0.23	83
Average ALDH inactivation [%]	95	304	85	108
Average ORAC value [µM Trol]	2668	65	3155	102

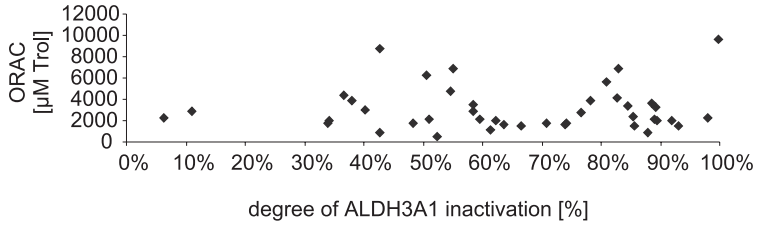

Figure 1. Saliva antioxidant capacity and degree of ALDH inactivation in saliva collected throughout the day of healthy non-smoking volunteers (group a, n = 42). The correlation parameters: r = 0.048, p = 0.76 (insignificant).

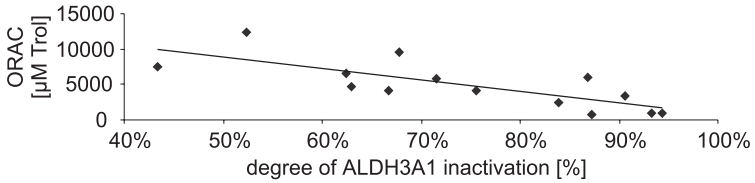

Figure 2. Correlation between antioxidant capacity (ORAC) and ALDH3A1 inactivation degree in saliva of 14 heavy smokers. Correlation parameters: r = 0.81; p = 0.0013.

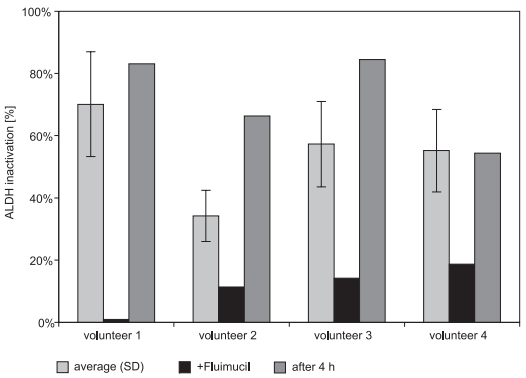

Figure 3. Effect of Fluimucil® (600 mg tablet) on salivary ALDH inactivation degree: first bar, average inactivation for each volunteer prior to Fluimucil® intake; second (black), inactivation after intake, third, inactivation 4 hours later.

$$AUC = 1 + f_1/f_0 + f_2/f_0 + ...+ f_{90}/f_0$$

where f_i and f_0 are fluorescence intensities at *i-th* minute and at the starting point, respectively.

The ORAC value in Trolox equivalents (TE) was determined using the standard curve for Trolox, a water soluble vitamin E derivative, which read:

$$AUC(Trolox) = 2.415c + 3.13$$

where c is Trolox concentration in µM.

Therefore, taking into account saliva dilution (D), we calculate ORAC in Trolox Equivalents (TE), expressed in µM, as:

$$ORAC = D \cdot \frac{AUC - 3.13}{2.415}$$

Fluorimetric assay was run on thermostated Shimadzu 5001PC or thermostated Perkin-Elmer LS55 spectrometers. LS55 spectrometer was equipped with a 4-position automatic cell changer. Instrumental settings were as follows: excitation wavelength 485 nm, emission wavelength 520 nm; for the Shimadzu 5001PC spectrometer spectral bandwidths was 5 nm for excitation and 3 nm for emission, whereas for the Perkin-Elmer LS55 instrument, respectively, 5.5 nm and 3 nm.

RESULTS AND DISCUSSION

Oxidation status of salivary ALDH3A1 in non-smoking volunteer's group

We have shown previously, that salivary ALDH3A1 is in majority of samples markedly, but reversibly inactivated, presumably *via* air oxidation, already at the time of saliva collection (11). Both absolute activity and the degree of oxidation of the salivary ALDH3A1 is intra- and interpersonally highly variable, and additionally high variability was recorded throughout the day time, with the highest activities observed between 8 and 10 a.m. (B. Wojno, J. Giebułtowicz et al., to be published elsewhere). In order to study ALDH sensitivity to oxidative stress, drugs and diet in healthy popula-

tion, a simple two assay method (11) to evaluate degree of salivary ALDH3A1 inactivation was used. Additionally, the ALDH3A1 oxidation status was correlated with anti-oxidant capacity (ORAC) of the volunteers' saliva estimated using fluorescence method, and the results have been presented in Table 1 and Figure 1.

Table 1 summarizes the basic statistics for three different control groups of volunteers: (a) forty two non-smoking volunteers from Warsaw, with non-specified time of saliva collection; (b) thirty seven volunteers, (7 of them moderately smoking), from less polluted area of Olsztyn, with saliva collected at 9-10 a.m.; (c) group of thirty four volunteers from Warsaw, saliva collected at ~ 9 a.m. Additionally, a separate group (d) of fourteen heavy smokers was selected on the basis of taking at least one pack of cigarettes a day for more than five years and their saliva was examined with non-specified time of collection. Similar group of fifteen smokers (e) was examined with saliva collected at ~ 9 a.m. Group (e) included 6 individuals from group (d).

Relation between antioxidant capacity of saliva samples and the degree of salivary ALDH3A1 inactivation, determined for the control group (a), is presented on Figure 1. In general, no statistically significant correlation between these parameters was found, with one exception for the group of heavy smokers, explained in the next section.

Smokers *vs*. non-smoker group

It has been shown that an average degree of aldehyde dehydrogenase inactivation in saliva of the control groups, collected throughout the day (Warsaw group #1), was about 64%, but when saliva was collected in the morning, this value was 54% (Table 1). Corresponding values for smokers were slightly higher, 74% and 68% (Table 1). These differences are, however, not statistically significant at a confidence level $\alpha = 0.05$.

Statistical test revealed a significant correlation between antioxidant capacity (ORAC) and the degree of ALDH3A1 inactivation for heavy smokers group. We found that for 14 patients who had been selected on the basis of smoking at least one pack of cigarettes a day for more than 5 years, the coefficient of correlation reached 0.81 (cf. Figure 2). Moreover, the Mann-Whitney test revealed a statistical difference between ORAC value in the smoker and non- smoker group ($p = 0.003$). Antioxidant capacity for smokers had a greater range and was significantly higher comparing with non-smoking subject (cf. Table 1). Taken together, these facts indicate that (1) exposure to free radicals during heavy smoking tends to generate increased antioxidant level in saliva, presumably as a defence mechanism; (2) negative correlation between ORAC and ALDH inactivation (Fig. 2) can be explained assuming that inadequate increase in antioxidant level, leading likely to higher activity of reactive oxygen species, causes elevated salivary ALDH inactivation.

High and low antioxidant diet group

In a separate group of 16 volunteers, consisting of no smoking females, age 20 ± 1, obeying for 3 days DIET no. 1, and, after ~2 weeks break, diet no. 2, the saliva was collected in the morning before their first meal and examined as described previously. The average ALDH activity, measured in the presence of GSH, in this group was lower than in the control group (Table 2), and the average ALDH3A1 inactivation reached 85% (Table 2). Moreover, application of different dietary rules for 3 days apparently did not affect average salivary ALDH activity nor its inactivation degree (Table 2).

We conclude that short-term diet does not directly affect the oxidation status of the salivary ALDH3A1. There was also no significant influence of the changed dietary rules on the antioxidant capacity of saliva within this group (see Table 2).

The above data, although somewhat surprising, must be treated as preliminary, and more experiments are necessary to draw final conclusions.

Effect of Fluimucil®

The antioxidant-containing drug Fluimucil® (active substance: acetylcysteine, 600 mg, acting as a cysteine prodrug (13, 14)) was used to check if the salivary ALDH oxidation degree may be affected by high doses of antioxidants. The influence of this drug was discovered somewhat accidentally, when saliva sample of a person suffering from minor throat infection, and treated with Fluimucil®, was examined and showed nearly zero % ALDH inactivation. This experiment was then repeated on 4 healthy volunteers (members of the "Olsztyn" group, see above), chewing 600 mg Fluimucil® tablet in the morning (approx. at 8.30 a.m.), and degree of oxidation of their saliva was examined after 30 min, and repeated after 4 h. The ALDH activity of the same 4 persons was monitored for 4 months preceding the experiment, and average values of ALDH inactivation degree for each person were compared to those obtained directly after Fluimucil® treatment. The results are shown in Figure 3.

From Figure 3 it is evident that after treatment by 600 mg of Fluimucil®, in all 4 persons the degree of salivary ALDH inactivation was markedly reduced, relative to the average value recorded for each person for previous 4 months. Since the average ALDH inactivation degree for each individual, examined in the morning hours (8-10 a.m.) for several months, is relatively stable (C.V. ~20% (B. Wojno, master thesis, Olsztyn, 2008)), this result must be treated as statistically significant, and demonstrates protective effect of Fluimucil® towards salivary ALDH. However, this effect has virtually disappeared after 4 hours, as shown in Figure 3 (third column bars).

This experiment indicates that antioxidant-containing drug significantly reduces oxidation of the salivary ALDH. It has been shown that the cysteine moiety in glutathione in not able to reactivate the oxidized ALDH *in vitro*, although it effectively protects the enzyme from air-oxidation (11). The most likely explanation of the Fluimucil® experiment is therefore protection from ALDH oxidation, presumably *via* the radical scavenging activity.

CONCLUSION

No statistically significant correlation (α = 0.05) was detected between ALDH3A1 oxidation status [%] and antioxidant capacity of saliva in patients following high or low antioxidant diet. There is also no such correlation in saliva of non smoking volunteers not following any diet limitations.

Statistically significant correlation between the degree of ALDH3A1 activity and antioxidant capacity was revealed for a group of 14 heavy smokers.

Antioxidant-containing drug, Fluimucil®, markedly reduces salivary ALDH oxidation degree, but this effect disappears after several hours.

The general conclusion of this paper is that the mechanism of salivary ALDH3A1 inactivation must be related to free radical activity, presumably in the salivary gland, but it is not simply correlated with antioxidant capacity of saliva.

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