Activation by sodium fluoride of drug-metabolizing enzymes in rat hepatoma-derived Fa32 cells

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Abstract Protection against xenobiotic insult, including cancer chemoprotection, can be achieved by a variety of natural and synthetic compounds belonging to over 20 different classes of chemicals. They all induce or activate drug-metabolizing enzymes. The discovery of a new class of activator is currently reported. Sodium fluoride activated the phase I ethoxyresorufin-*O*-deethylase (to 240%) and pentoxyresorufin-*O*-dependentylase (to 156%), and the phase II glutathione transferase to 120% of the basal activities in rat hepatoma-derived Fa32 cells. It is, therefore, a bifunctional enzyme activator. A time- and concentration-dependent activation was observed. A possible impact of the daily fluoride uptake from drinking water is suggested.

Key words: Fa32 cell; Sodium fluoride; Glutathione transferase; Ethoxyresorufin-*O*-deethylase; Pentoxyresorufin-*O*-dependentylase; Activation

1. Introduction

Protection against toxic insult, including cancer chemoprevention, can be achieved by a wide variety of compounds belonging to over 20 different classes of chemicals [1]. They include both natural and synthetic compounds such as polycyclic aromatic hydrocarbons, flavonoids, azo dyes, isothiocyanates, diterpenes, phenolic antioxidants, indoles, unsaturated lactones, 1,2-dithiol-3-thiones, and thiocarbamates [1]. All of these chemicals share the property of inducing electrophile-processing phase II enzymes, e.g. glutathione transferase (GST), which is a major protective mechanism [2–4]. Phase I enzymes, mainly cytochrome P450-dependent enzymes, are often activated by the same chemicals. Talalay et al. have defined monofunctional inducers as compounds that selectively induce only phase II enzymes and bifunctional inducers that induce both phase I and phase II enzymes [5,6].

Metabolic activity includes the biotransformation and detoxification of xenobiotics and is one of the major functions of the liver. Reduced glutathione (GSH) and phase II enzymes play an important role in these phenomena. During a series of cytotoxicity assays it was observed that sodium fluoride became nine times more toxic in GSH-depleted cells (unpublished). The influence of sodium fluoride on drug-metabolizing enzymes was, therefore, further investigated in Fa32 cells. Sodium fluoride is used for several purposes: as an insecticide and in many pesticide formulations, as a steel degassing agent, as a disinfectant for fermentation apparatus, and so on [7]. Humans are mainly exposed to fluorides by the fluoridation of drinking water and by dental products. This sometimes causes problems, especially in children [8].

Established Fa32 cells are derived from a rat hepatoma [9]. We previously demonstrated that the endogenous GSH content interacts with the cytotoxicity of metals in Fa32 cells, and deduced the presence of the GSH biosynthetic enzyme γ-glutamylcysteine synthetase from the effect of the specific inhibitor L-buthionine-*S*,*R*-sulfoximine on the endogenous GSH content [10]. Earlier, the cytosolic GSTs in Fa32 cells were shown to be comparable to those in rat liver, except that the hepatoma-specific GST 7-7 is an additional enzyme in the cultured cells [11]. Since also the GST activation by phenobarbital and butylated hydroxyanisole in Fa32 cells closely resembles that in rat liver (unpublished), these cells are considered a valuable alternative model for the investigation of GST-dependent metabolic interactions in rat liver.

2. Materials and methods

Established Fa32 cells were provided by Prof. Szpirer (Université Libre de Bruxelles, Brussels, Belgium). The cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (complete medium).

The cytotoxicity was measured using the slightly modified [12] neutral red uptake inhibition assay [13]. Briefly, 6×10^4 cells were seeded in each of the 64 wells of a microtiter plate (Nunc, Roskilde, Denmark). After 24 h the cells were treated with different concentrations of freshly prepared sodium fluoride in complete medium (8 wells/concentration) for 24 h or 72 h. The toxicity was established by the determination of the NI_{50}, i.e. the concentration of test compound required to induce a 50% inhibition in neutral red uptake compared to control cells. The 50% inhibitory concentrations were extrapolated from dose-response effect curves by linear regression analysis.

For measuring the phase II GST activity nearly confluent Fa32 monolayers were treated with sodium fluoride in complete medium in 10 cm Petri dishes for 3 days, using 11 ml per Petri dish. Homogenization and cytosol preparation were carried out as described [11]. The GST activity was measured with 1-chloro-2,4-dinitrobenzene as the second substrate [14]. The specific activity is expressed in nmol/min/mg protein. The proteins were measured by the Lowry method. For the determination of the GST subunit pattern, GST in the cytosol obtained from six Petri dishes was purified by GSH affinity chromatography [11]. The GST subunit composition was analyzed by using HPLC on a narrow-bore Vydac 30 nm C18 reverse-phase column (250×2.1 mm), eluted at a flow rate of 0.25 ml/min [10].

In order to measure the phase I cytochrome P450 enzymes P450IA1 (EROD; 7-ethoxyresorufin-*O*-deethylase) and P450IIB1 (PROD; 7-pentoxyresorufin-*O*-dependentylase) [15], 60 000 cells/well were seeded in six wells of five rows (A–E) of a titer plate. Two additional wells in each row received only 200 μl complete medium without cells and were used for background subtraction later on. After 24 h the five wells in the first three columns received fresh complete medium and the cells in the remaining wells were treated for 24 h or 72 h with

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Abbreviations: EROD, ethoxyresorufin-*O*-deethylase; GSH, reduced glutathione; GST, glutathione transferase; PROD, pentoxyresorufin-*O*-dependentylase

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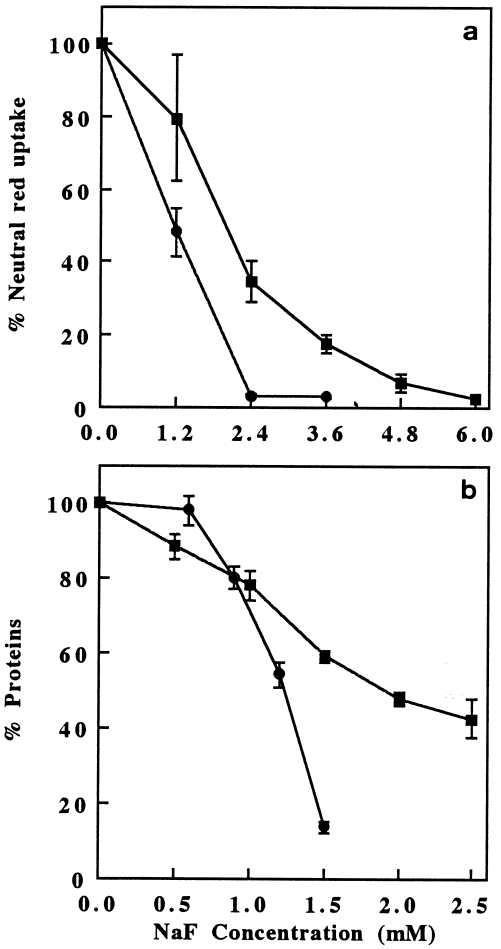

Fig. 1. Cytotoxicity of sodium fluoride in Fa32 cells, as measured by neutral red uptake (a) and total protein content (b). The cells were treated for 24 h (■) or 72 h (●).

different concentrations of sodium fluoride. EROD and PROD activities were further measured essentially as described [16]. The cells were washed with 200 μl PBS/well. The assay was started by adding 100 μl/well of complete medium containing 8 μM 7-ethoxyresorufin or 15 μM 7-pentoxyresorufin, supplemented with 10 μM dicoumarol to prevent further metabolism of the resorufin formed by the cytosolic enzyme diaphorase [17]. After 3 h at 37°C, a 75 μl aliquot of cell medium was transferred to another titer plate. At this stage 75 μl of a 0–0.6 μM resorufin standard concentration range, also prepared in complete medium, was added in the eight wells of rows F–H; 25 μl with 15 Fishman units of β-glucuronidase and 120 Roy units of arylsulfatase (Boehringer, Mannheim, Germany) in 0.1 M sodium acetate buffer, pH 4.5, was added to both the samples and the standards. The plates were covered with aluminum foil and shaken at room temperature. After 2 h, 200 μl ethanol were added to each well, and the plates were centrifuged at 3000 rpm for 10 min. After transfer of 200 μl supernatant to a new titer plate, the fluorescence units (FU) were measured at 600 nm emission with excitation at 535 nm using a Dynatech Fluorolite 1000 fluorescence microplate reader. Higher, deviating fluorescence values were measured for the wells in the first row (row A); these were therefore not used. The fluorescence was linear within the used standard range.

The protein content was measured after the 3 h incubation period with the phase I substrates (see above). The wells were rinsed twice with 200 μl PBS, and treated with 100 μM 0.1% Triton X-100 in 50 mM sodium borate buffer, pH 9.0. After incubation for 1 h at 37°C, 30 μl freshly prepared 1 mM 3-(4-carboxybenzoyl)-quinoline-2-carboxaldehyde (CBQCA)/6.7 mM KCN in 50 mM sodium borate buffer, pH 9.0, was added to each well [18]. CBQCA is virtually non-fluorescent in aqueous solution and reacts with protein amines in the presence of cyanide [19]. The titer plates were covered with aluminum foil and shaken at room temperature for 1 h. The fluorescence was measured at 530 nm emission with excitation at 485 nm using a Dynatech Fluorolite 1000 fluorescence microplate reader. To obtain sample protein concentrations the FU were calibrated against a bovine serum albumin standard curve prepared and assayed as the samples, using the wells of the three bottom rows (F–H). These protein measurements also allowed us to establish the relative toxicity of sodium fluoride by the determination of the PI_{50}. This is the concentration of test compound required to induce a 50% reduction of total protein content, as compared to the control cells.

The experiments were performed three times. Mean values ± standard deviations are given.

3. Results

The sensitivity of the Fa32 cells towards sodium fluoride was first examined by the neutral red uptake inhibition assay (Fig. 1a). After 24 h a NI_{50} of 2.00 ± 0.09 mM sodium fluoride was observed, and after 72 h a NI_{50} of 1.20 ± 0.06. These profiles were afterwards compared with the total protein contents, measured for the calculation of the specific enzyme activities (Fig. 1b). The PI_{50} values thus obtained were 1.96 ± 0.05 mM after 24 h and 1.21 ± 0.04 mM after 72 h. Consequently, the cytotoxicity values of both methods corresponded very well.

The influence of sodium fluoride on the phase II GST activity was then investigated. A concentration-dependent activation was observed (Fig. 2), with a maximal activation of 20% above the control at a concentration of 1.20 mM sodium fluoride. At higher concentrations sodium fluoride became too toxic. Since Fa32 cells have several GST isoenzymes, as is the case in rat liver [11], the GST subunit composition was analyzed by HPLC. However, no specific activation of any GST subunit was observed (results not shown). This means that all GST isoenzymes were activated to the same degree.

The cytochrome P450-dependent EROD and PROD were measured directly in the tissue culture plates. A maximal, concentration-dependent, stimulation of 240% of the control EROD was observed after 24 h sodium fluoride treatment (Fig. 3a). PROD was stimulated less, to 156% of the control. After 72 h EROD was poorly but significantly enhanced to 111% of the control, PROD being increased more, to 135% (Fig. 3b). Also for the phase I enzymes sodium fluoride be-

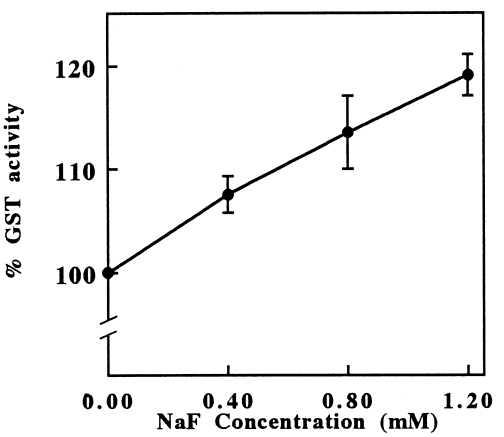

Fig. 2. The influence of sodium fluoride on the specific glutathione transferase activity in Fa32 cells after 72 h. Specific activity in the control cells: 448 ± 51 nmol/min/mg protein.

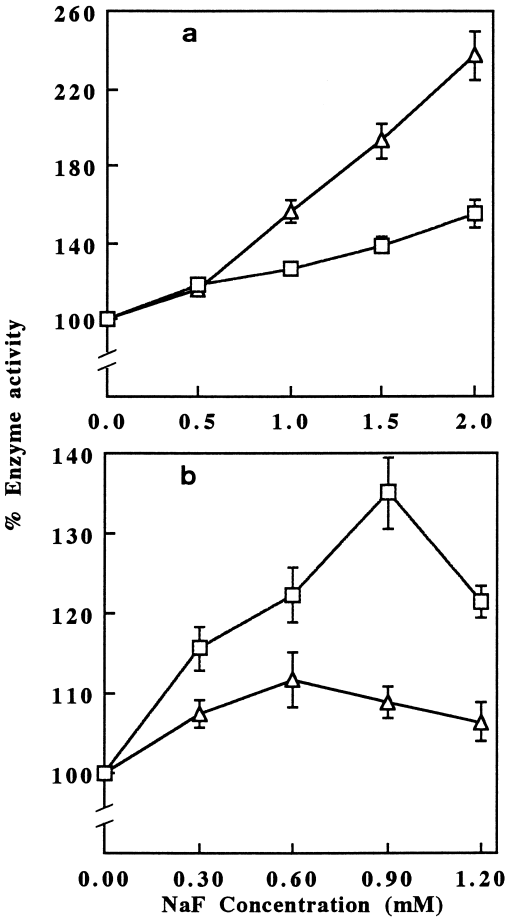

Fig. 3. The influence of sodium fluoride on the specific EROD (△) and PROD (□) activity in Fa32 cells after 24 h (a) and 72 h (b). Specific activities in the control cells were for EROD: 3.07 ± 0.09 nmol/mg protein/3 h and for PROD: 1.42 ± 0.16 nmol/mg protein/3 h.

came too toxic at the highest concentrations, as reflected by the declining EROD and PROD activities in Fig. 3b.

4. Discussion

The activation of phase II GST is much lower than that by the strongest GST activator *trans*-stilbene oxide in mouse liver [20]. Using the same substrate as in our experiments GST increases of 171% with the classical activator phenobarbital, 145% with 2-methylcholanthrene, and 130% with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin were found in rat liver [21]. Sulforaphane, an aliphatic isothiocyanate from broccoli, was described as an activator of phase II detoxification enzymes [22] and was shown to significantly inhibit 7,12-dimethylbenz[*a*]anthracene-induced mammary carcinogenesis in rats [23]. This activator and its recently described synthetic analogue, sulforamate [(±)-4-methylsulfinyl-1-(*S*-methyldithiocarbamyl)-butane], increase the GST activity in mouse liver to respectively 114 and 111%, but have a higher effect in mammary glands [24]. Two other recently described phase II drug-metabolizing enzyme activators, quinoline and isoquinoline, enhance the GST activity in rat liver to 120% of that in control rats [25]. The sodium fluoride activation of GST we found in Fa32 cells (Fig. 2) is very well comparable with the latter one [25].

Fa32 cells have a higher EROD than PROD activity, as is the case in rat hepatocytes [16]. A specific EROD activity of 0.13 ± 0.05 nmol/mg protein/3 h was found in human hepatoma-derived HepG2 cells [26]. About twice this activity was found in Fa32 cells (Fig. 3). Benzo[*a*]anthracene, 2,3-benzofluorene, benzo[*a*]pyrene, 7-methylbenzo[*a*]anthracene, and pyrene increased the concentration-dependent EROD activity in HepG2 cells, but other polycyclic aromatic hydrocarbons and organochlorine compounds, which have mutagenic or carcinogenic activity, did not [26]. Phase I enzymes are always more activated than phase II enzymes [2–5] which was also the case for sodium fluoride in Fa32 cells (Figs. 2 and 3). Sodium fluoride behaves as a bifunctional activator [5,6] in Fa32 cells, since both phase I and phase II enzymes are activated.

Two differences between sodium fluoride and the known activators have to be mentioned. First, the inorganic compound sodium fluoride has a chemically very simple structure, in contrast to the other known activators [1,24–26]. Secondly, sodium fluoride is used on a large scale, albeit in low concentrations. Because of its ability to reduce the development of dental caries, the fluoridation of drinking water is a general practice in the USA. This measure is accepted and recommended by health authorities and dentists [27]. Exposure to other inducers and/or activators is rather occasional or accidental. It would be interesting to investigate the impact of the daily fluoride consumption on the levels of phase I and II enzymes, and hence on the probably higher resistance against xenobiotic insult.

References

[1] Wattenberg, L.W. (1985) Cancer Res. 45, 1–8.
[2] Benson, A.M., Batzinger, R.P., Ou, S.-Y.L., Bueding, E., Cha, Y.-N. and Talalay, P. (1978) Cancer Res. 38, 4486–4495.
[3] Fujita, S., Matsunaga, T., Matsubuchi, Y. and Suzuki, T. (1988) Cancer Res. 48, 254–259.
[4] Manoharan, T.H., Puchalski, R.B., Burgess, J.A., Pickett, C.B. and Fahl, W.G. (1987) J. Biol. Chem. 262, 3739–3745.
[5] Prochaska, H.J., De Long, H.J. and Talalay, P. (1985) Proc. Natl. Acad. Sci. USA 82, 8232–8236.
[6] Prochaska, H.J. and Talalay, P. (1988) Cancer Res. 48, 4776–4782.
[7] The Merck Index, 9th edn. (1976) p. 1141, Merck and Co., Rahway, NJ.
[8] Augenstein, W.L., Spoerke, D.G., Kulig, K.W., Hall, A.H., Hall, P.K., Riggs, B.S., El Saadi, M. and Rumack, B.H. (1991) Pediatrics 88, 907–912.
[9] Deschatrette, J. and Weiss, M.C. (1974) Biochemie 56, 1603–1612.
[10] Dierickx, P.J. (1996) ATLA 24, 399–403.
[11] Dierickx, P.J. (1989) Cell Biol. Int. Rep. 13, 967–969.
[12] Brandao, J.C., Bohets, H.H.L., Van de Vyver, I.E. and Dierickx, P.J. (1992) Chemosphere 25, 553–562.
[13] Borenfreund, E. and Shopsis, C. (1985) Xenobiotica 15, 705–711.
[14] Habig, W.H., Pabst, M.J. and Jakoby, W.B. (1974) J. Biol. Chem. 249, 7130–7139.
[15] Burke, M.D., Thompson, S., Elcombe, C.R., Halpert, J., Haapparanta, T. and Mayer, R.T. (1985) Biochem. Pharmacol. 34, 3337–3345.
[16] Donato, M.T., Gomez-Lechon, M.J. and Castello, J.V. (1993) Anal. Biochem. 213, 29–33.
[17] Lubet, R.A., Nims, R.W., Mayer, R.T., Cameron, J.W. and Schechtman, L.M. (1985) Mutat. Res. 142, 127–131.
[18] Molecular Probes (1996) Manual for CBQCA Protein Quantitation Kit. Leiden.
[19] Liu, J., Hsieh, Y.-Z., Wiesler, D. and Novotny, M. (1991) Anal. Chem. 63, 408–412.

[20] Guthenberg, C., Morgenstern, R., DePierre, J.W. and Mannervik, B. (1980) Biochim. Biophys. Acta 613, 1–10.
[21] Mukhtar, H., Baars, A.J. and Breimer, D.D. (1981) Xenobiotica 11, 367–371.
[22] Zhang, Y., Talalay, P., Cho, C.G. and Posner, G.H. (1992) Proc. Natl. Acad. Sci. USA 89, 2399–2403.
[23] Zhang, Y., Kensler, T.W., Cho, C.G., Posner, G.H. and Talalay, P. (1994) Proc. Natl. Acad. Sci. USA 91, 3147–3150.
[24] Gerhäuser, C., You, M., Liu, J., Moriarty, R.M., Hawthorne, M., Mehta, R.G., Moon, R.C. and Pezzuto, J.M. (1997) Cancer Res. 57, 272–278.
[25] Le, H.T. and Franklin, M.R. (1997) Chem. Biol. Int. 103, 167–178.
[26] Nakahama, A., Kuroda, K. and Yamada, A. (1995) Biochem. Pharmacol. 50, 1407–1412.
[27] World Health Organization (1984) Fluorine and Fluorides, Environ. Health Crit. 36, pp. 1–136, WHO, Geneva, Switzerland.