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THE TUMOR PROMOTER TETRADECANOYL-PHORBOL-ACETATE (TPA) ELICITS THE REDISTRIBUTION OF ZINC IN SUBCELLULAR FRACTIONS OF RABBIT THYMOCYTES MEASURED BY X-RAY FLUORESCENCE

Péter Csermely[1], Sándor Gueth[2], and János Somogyi

[1]Inst. Biochem.I., Semmelweis Univ. Sch.Med.,
POB 260 Budapest , H-1444 Hungary

[2]Dept. of Atomic Physics, Eötvös Loránd Univ.,
Fac. Natl. Sci. , Budapest , Hungary

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Prolonged (90 minutes) incubation with tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA) elicits the redistribution of zinc from the nuclear fraction and mitochondria to the cytosol and microsomes in rabbit thymocytes. The zinc redistribution might play a role in the TPA-caused desensitization of T lymphocytes towards calcium and in the TPA-induced inhibition of G1 → S phase transition and cytotoxicity of T lymphocytes.

The tumor promoter TPA has a mitogenic, co-mitogenic effect on T lymphocytes (1,2). As a potent activator of protein kinase C (3) TPA is thought to accomplish its effects via the "protein kinase C pathway" (4-7). However the mode of action of TPA seems to be more complex, since a. depending on the duration of the TPA incubation an effective inhibition of the proliferation of T lymphocytes can also be achieved (7-9); b. there are several reports describing differences in the effects of TPA and the endogen activators of protein kinase C, diacylglycerols (5,10), or presenting other evidences that the "protein kinase C pathway" might be not the only mechanism of the action of TPA (11).

Besides the co-internalization (down-regulation) of essential receptors (T3,T4,transferrin, etc.) with the phorbol ester receptor (5-7,9) the inhibitory effects of TPA might be explained with the attennuation, block of calcium signals by TPA (12). In our previous work we have investigated the effect of TPA on the Ca-ionophore induced rise in the intracellular Ca^{2+} concentration (13). We have found that prolonged incubation with TPA sharply

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decreases the ionophoretic activity of Ca-ionophores. The optimal conditions of this TPA-induced desensitization needed a 9o minutes preincubation with TPA concentrations higher than lo nM. After demonstrating the insufficiency of several possible explanations (immobilization of ionophores, activation of Ca-extrusion systems, etc.) we have observed that the intracellular heavy metal chelator, TPEN restores the ionophoretic activity of Ca-ionophores after TPA treatment. This observation led to the hypothesis that TPA may cause changes in the heavy metal content of different subcellular fractions. To test this hypothesis and to examine which heavy metal ion(s) are responsible for the diminuation of the effect of Ca-ionophores in our present report we investigate the changes in heavy metal content of subcellular fractions upon previously optimalized (9o minutes, 2o nM) TPA treatment by X-ray fluorescence.

MATERIALS AND METHODS

Rabbit thymocytes were isolated by the method of Kleiman et al (14). The cells were incubated with 2o nM TPA (Sigma) or the solvent dimethyl-sulfoxide (control cells) at 37^{o}C for 9o minutes at a cell density of lo cells/ml in Eagle's Minimum Essential Medium supplemented with lo mM Hepes (pH 7.4,Sigma), essential amino acids, streptomycin and penicillin (loo-loo U/ml) (Gibco). The viability of the cells was never below than 95 % as judged by trypan blue exclusion. After incubation the cells were resuspended in the isolation buffer containing 14o mM KCl, o.25 mM $MgCl_2$ and 2o mM Hepes pH 7.o at a cell density of lo^8 cells/ml. The isolation of four subcellular fractions (nuclear pellet, mitochondria, microsomes and cytosol) was performed as described earlier (15). For the background measurements appropriate "blank" samples were prepared. The protein content of subcellular fractions from lo^9 rabbit thymocytes was determined (16), the samples were dried with lyophilisation and their dry weight was measured. Finally the samples were placed on a o.1 mm thick polypropilene foil.

The X-ray fluorescence spectra were measured using ^{125}I radiocative isotope as gamma-photon source with an activity of 2oo MBq. The spectra were measured by a semiconductor Si(Li) detector and the data were collected with an NTA lo24 multichannel analyzer (EMG, Budapest Hungary). The acquisition time was usually 24 hours. The peaks observed were identified using repetitively recorded calibration curves of an appropriate standard containing the metals examined.

RESULTS

In Fig.1. the X-ray fluorescence spectra of the nuclear fraction of lo^9 rabbit thymocytes can be seen. The very low resolution of the low energy (2-3 keV) X-ray photons is due to their absorption (mainly in the air). This is the reason why we could not detect any other elements with low atomic number except Cl. Among the heavy metals the K_α peaks of Fe, Cu and Zn can be detected. The less intensive K_β peak of Zn can also be seen. The K_α peak of Mn (5.9 keV) and the K_β peak of Fe (7.o keV) is very close to the detection limit in most of the cases. The K_β peak of Cu is overlapping with the K_α peak of Zn in our conditions. The L_α and L_β peaks of Pb are resulted from the Pb collimator hence they appear in the background spectrum too. Since there was no significant difference in the dry weight and protein content of the subcellular

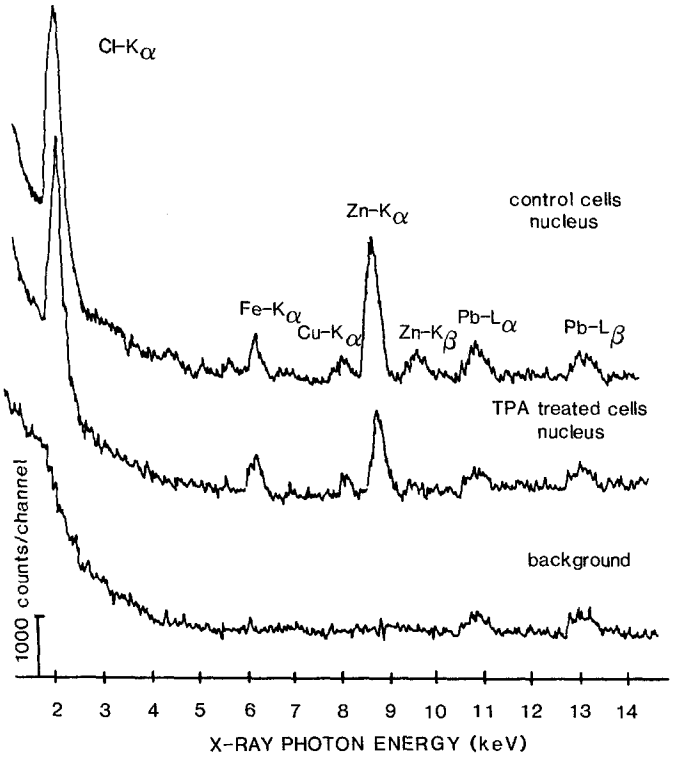

Figure 1. X-ray fluorescence spectrum of the nuclear fraction of rabbit thymocytes. The isolation of the cells, the incubation with 2o nM TPA (37°C, 9o minutes), the isolation of the nuclear fraction and the recording of the X-ray fluorescence spectra was done as described in Materials and Methods. The spectra are representatives of three separate experiments.

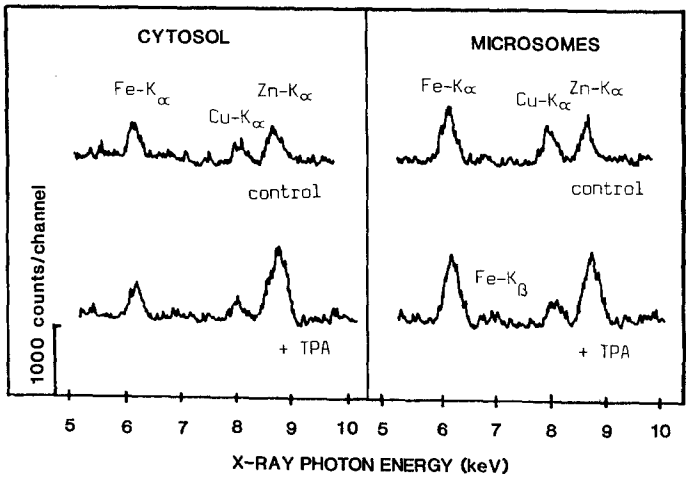

Figure 2. X-ray fluorescence spectrum of the cytosol and microsomes of rabbit thymocytes. The cells were incubated with 2o nM TPA at 37 °C for 9o minutes. The isolation of subcellular fractions and the recording of the X-ray fluorescence spectra was done as described in Materials and Methods. The spectra are representative of three separate experiments.

Table 1

RELATIVE AMOUNTS OF HEAVY METALS IN SUBCELLULAR FRACTIONS OF CONTROL AND TPA-TREATED RABBIT THYMOCYTES

Heavy metal	Normalised K_α peak areas							
	nucleus		mitochondria		cytosol		microsomes	
	control	+TPA	control	+TPA	control	+TPA	control	+TPA
Fe	4.8 (o.3)	4.8 (o.5)	3.6 (o.2)	2.o (o.5)	1.6 (o.1)	1.8 (o.1)	3.6 (o.2)	4.o (o.5)
Cu	1.6 (o.4)	1.6 (o.6)	1.1 (o.2)	o.4 (o.1)	o.4 (o.3)	o.6 (o.4)	1.6 (o.4)	1.4 (o.4)
Zn	1o.1 (o.2)	7.o** (o.3)	3.o (o.3)	1.2* (o.4)	1.4 (o.2)	3.8** (o.2)	2.4 (o.3)	3.8* (o.2)

*significantly different $p < o.oo5$

**significantly different $p \lesssim o.ool$

The figures represent the K_α peak areas of Fe,Cu and Zn corrected to the background, sample geometry and the intensity of the gamma-photon source. The Zn-K_α peak areas are corrected to the overlapping Cu-K_β peaks too. Data are means of three separate experiments with the corresponding SD-s in parentheses. The p values were calculated using the Student's t test.

fractions of control and TPA treated cells and since the area under the peaks is roughly proportional with the amount of the appropriate element in the sample the decrease of the Zn K_α and K_β peaks after TPA incubation represent a significant decrease of the Zn content of the nucleus during incubation.

In Fig.2. the X-ray fluorescence spectra of the cytosol and microsomal fraction show just the opposite change than those of the nuclear pellet: the Zn content is significantly increased after incubation with TPA. Table 1. shows the semi-quantitative data of the K_α peak areas which are roughly proportional with the heavy metal content of the sample. The significant decrease of Zn content in the nuclear fraction (3o%) and mitochondria (6o%) is accompanied by a sharp increase in the Zn content of the cytosol (2.7-times) and microsomes (1.6-times). The other two heavy metals Fe and Cu, detected also show similar (though non-significant) tendencies.

DISCUSSION

The results presented in this report show a significant decrease in the Zn content of the nuclear fraction and mitochondria, and a concominant increase in the Zn content of cytosol and microsomes of rabbit thymocytes after TPA incubation. Taking into account our additional evidence that TPA causes no change in the total heavy metal content of T lymphocytes (measured by plasma emission spectroscopy; Csermely,P. and Somogyi,J. unpublished results) we may conclude that an intracellular zinc redistribution occurs upon TPA incubation.

This finding is in good agreement with our previous results (13) which indicate an increased availability of heavy metals surrounding the plasma membrane after prolonged incubation with TPA. The "Zn-depletion" from the nucleus by incubation with the tumor promoter TPA resembles to the results of Yarom et al (17) demonstrating diminished nuclear Zn content in malignant lymphocytes compared to normal ones.

The mechanism of this action of TPA is not known. The obvious explanation would include the phosphorylation of several proteins by the TPA-activated protein kinase C. These phosphorylations may result affinity changes of the occupied or unoccupied putative Zn-binding sites. However our previous observations show that the amount of "free" heavy metal ions in the cytosol increases very slowly reaching its maximum after 9o minutes of incubation (13). This observation suggests that the mechanism may be more complex than a simple single-step phosphorylation.

This type of Zn redistribution (nucleus, mitochondria → cytosol, microsomes) would occur if the overall affinity of the nuclear and mitochondrial Zn-binding sites decreased and/or the overall affinity of the cytosolic and microsomal Zn-binding sites increased during TPA incubation. Since our previous results (13) demonstrate an increased availability of "free" heavy metal ions in the cytosol and microsomes, i.e.: a "looser" attachment, the affinity decrease should predominate from the two above mentioned processes. If the affinity decrease predominates, than the affinity changes being mainly responsible for the zinc redistribution should occur in the nucleus. This conclusion also argues against a single-step phosphorylation mechanism via protein kinase C because the nucleus has practically no protein kinase C content (5).

What can be the consequences of the TPA-induced zinc redistribution? The decrease of the Zn content in the nucleus and mitochondria may cause significant changes in the activity of several enzymes. Zn seems to be essential for the activity of RNA, DNA polymerases, thymidine kinase (18) and protein transcription factor IIIA (19). Therefore the TPA induced depletion of zinc from the nucleus might be related to its antiproliferative activity, the arrest of $G_1 \rightarrow S$ phase transition (7-9).

On the other hand the zinc content of the cytosol and microsomal fractions increases upon TPA incubation. The primary structure of several receptor proteins e.g. that of cortisol, oestradiol, thyreotrop hormone (2o) or protein kinase C itself (21) shows the existence of putative Zn-binding sites. The increased availability of Zn in the cytosol might change the behaviour of these proteins. Heavy metals exhibit a higher affinity for a number of calcium-binding sites (22). Therefore an excess of heavy metal ions in the cytosol and microsomal fraction might occupy some of the calcium

binding sites, channel attennuating the calcium fluxes essential in signal transduction. In this way the TPA-induced heavy metal redistribution might be linked to the TPA-induced block of the intercellular communication (23), cytotoxicity (24,25) and the desensitization of TPA-treated cells towards extracellular calcium (12,26). Hence both sides of the TPA-induced zinc redistribution (decrease in the nucleus, mitochondria and increase in the cytosol, microsomes) may have a siginificant influence on the function and status of a living cell. The examination of the possible consequences enumerated is under current investigation in our laboratory.

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