OLGA CAREVIĆ, D.Sc.
Zagreb

Traffic Safety and Ecology
Original scientific Paper
U.D.C. 656.13:614.71:504.054
Accepted: Apr. 21, 1998
Approved: Jul. 14, 1998

THE EFFECTS OF BENZO(A)PYRENE AND 2, 3, 7, 8-TETRACHLORODIBENZO-P-DIOXIN FROM AUTOMOBILE EXHAUST UPON MAMMALIAN CELL VIABILITY

SUMMARY

Incomplete combustion process is a potential source of benzo(a)pyrene (BaP) and tetrachlorodibenzo-p-dioxin (TCDD). These compounds have been detected in effluents of municipal incinerators, sewage sludge, cigarette smoke, automobile exhaust etc. Although BaP and TCD have carcinogenic potential, in recent years these agents have received great attention due to their environmental persistence and remarkably acute toxicity.

To assess health risks associated with human exposure to BaP and TCDD, it was of interest to evaluate their effects upon human polymorphonuclear leucocytes by measuring the release of lysosomal and cytoplasmic enzymes.

Suspensions of human polymorphonuclear leucocytes (PMNL) were treated with BaP, TCDD and BaP+TCDD at concentrations of 10^{-7}, 10^{-6}M. These agents provoked a progressive time- and dose-dependent release of lysosomal enzyme beta-N-acetyl-glucosaminidase and cytoplasmic enzyme lactate dehydrogenase, beta-GLM and LDH respectively. At concentrations listed TCDD was much more effective in realising both enzymes beta-GLM and LDH than BaP. In the experiments with the combination of BaP+TCDD, extracellular release of beta-GLM or LDH was significantly higher as compared to BaP or TCDD-treated samples. It seems possible that TCDD affected the solubility of beta-GLM and LDH to a greater extent than the activity of BaP.

The observations obtained in these studies suggest that BaP and TCDD damage the lysosomal and cellular membranes.

1. INTRODUCTION

An increasing contamination of the atmosphere, water and soil by potentially harmful substances characterises worldwide the state of environment today (Figure 1). The most important sources of air pollu-

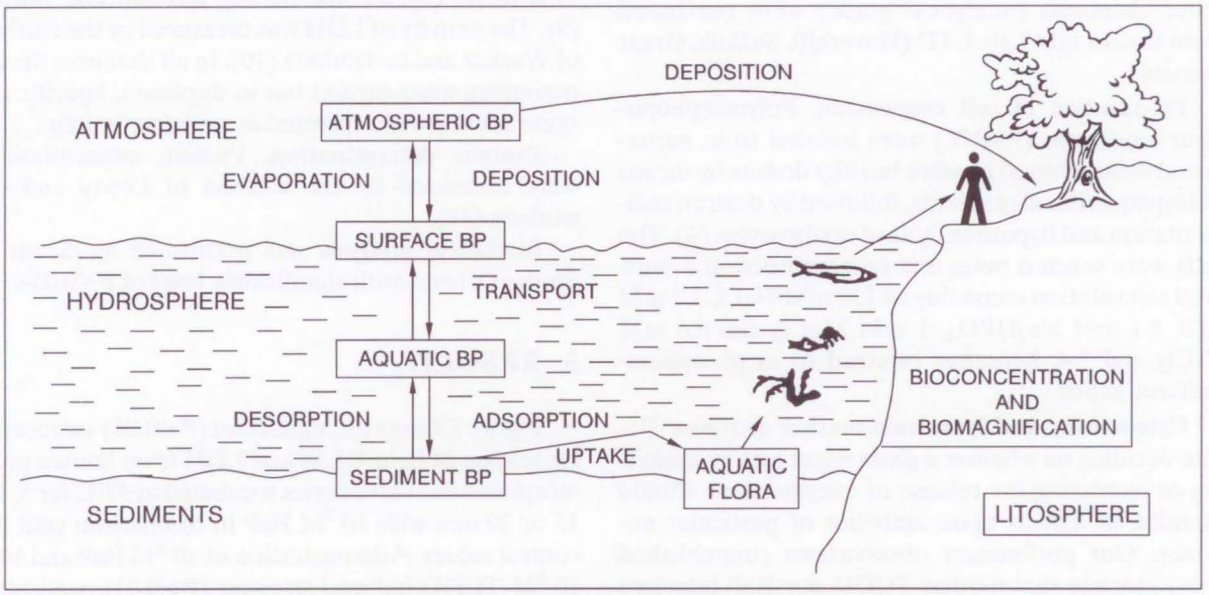

Figure 1- Distribution of the environment pollutante (BP) according to the scheme illustrated by S. Safe: Mammalian and Environmental Toxicology, Springer Verlag, Berlin 1987.

tion are combustion processes for energy generation in power plants, factories, and households, and last but not least, traffic automobile exhaust.

The automobile motor vehicles discharge significant quantities of carbon monoxide, oxides of nitrogen, smoke, lead compounds, fuel impurities and unburned hydrocarbons especially benzo(a)pyrene (BaP). Among these agents, 68 various chemical compounds are also identified in the automobile exhaust particulates by gas chromatography included carcinogenic and mutagenic 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) (1,2).

Although the mechanisms of BaP and TCDD actions are not fully understood, major toxic effects seem to be mediated by cytosolic protein, aryl hydrocarbon receptor (3). This receptor has been identified in organs and tissues of several animal species as well as in mammalian cultured, and isolated cells, such as human fibroblasts and lymphocytes. However, there are also other types of BaP and TCDD effects which are related to their lipophilic properties. One of such effects is the interaction with biological membranes. Since our previous investigations have shown that BaP and TCDD partially concentrate in lysosomes, it was of interest to compare their effects upon integrity of lysosomal and cellular membranes by monitoring the extracellular release of lysosomal and cytoplasmic enzymes from human poliymorphonuclear leucocytes.

2. MATERIALS AND METHODS

Chemicals. TCDD was graciously provided by Dow Chemical (Midland, MI, USA), while BaP was obtained from Fluka AG (Buchs, Switzerland). All the other chemicals (analytical grade) were purchased from Koch-Light Lab. LTD (Haverhill, Suffolk, Great Britain).

Preparation of cell suspension. Polymorphonuclear leucocytes (PMNL) were isolated from heparinized venous blood of adult healthy donors by means of Hypaque/Ficoll gradients, followed by dextran sedimentation and hypotonic lysis of erythrocytes (4). The cells were washed twice and re-suspended in a buffered salt solution consisting of 138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1 mM $MgCl_2$ and 0.6 mM $CaCl_2$, pH 7.4, hereafter referred to as phosphate-buffered saline.

Cytoplasmic and lysosomal marker enzymes. Before deciding on whether a given agent acts by inhibiting or increasing the release of enzymes, one should examine its effects upon activities of particular enzymes. Our preliminary observations (unpublished data) indicate that neither TCDD nor BaP interfere with activities of beta-N-acetylglucosaminidase (beta Glm, EC 3.2.1.30) and lactate dehydrogenase (LDH, EC 1.1.1.27). These enzymes were therefore chosen as lysosomal and cytoplasmic marker enzymes.

Test for the extracellular enzyme release. Washed human PMNL (5×10^6 in 0.9 ml phosphate-buffered saline) were incubated at 37°C for 5, 10, 15 and 20 min. with 0.1 ml TCDD, BaP and combination of BaP and TCDD dissolved in dimethylsulphoxide (DMSO) used as a solvent. Finally, equal-volume doses of DMSO were included in the experiments as control samples.

The concentrations of the agents were chosen on the basis of the preliminary observations (unpublished data).

At the defined time, the cell suspensions were removed and centrifuged at 1500 x g at 4°C for 20 min. The resulting supernatants were assayed for the released beta-GLM and LDH activities. Total activities of these enzymes were measured in selected reaction mixtures after the cells had been lysed by addition of 0.1% (v/v) Triton X-100. Appropriate control experiments were performed by measuring the release of enzymes tested in the specimens incubated with equal-volume doses of DMSO, or without TCDD or BaP and even without DMSO at 37°C for time defined and concentrations used.

The enzyme activities determined in the supernatants were expressed as percentages of their total activities in cell suspensions as described previously (5-7). This parameter served as a measure for the in vitro release of beta-GLM and LDH under experimental conditions described. Also, the LDH release from PMNL served as an indicator of cell viability in the investigations **in vitro.**

Enzyme assays. The activity of beta-GLM was determined according to the procedure of Sellinger and co-workers (8), as modified by Baccino and Zuretti (9). The activity of LDH was measured by the method of Wacker and co-workers (10). In all instances the experiments were carried out in duplicate. Specific enzyme activity was expressed as units/mg protein.

Protein determination. Protein concentrations were measured by the method of Lowry and co-workers (11).

Statistical analysis was performed by means of Student's t-test with significance level of $P<0.05$).

3. RESULTS

Figure 2 shows the significant ($P<0.05$) extracellular release of beta-GLM and LDH from human polymorphonuclear leucocytes incubated at 37°C for 5, 10, 15 or 20 min with 10^{-7}M BaP in comparison with the control values. Administration of 10^{-6}M BaP and 10^{-7}, 10^{-6}M TCDD induced stronger ($P>0.01$) concentration-related leakage of both enzymes studied at time intervals indicated in Figure 2, as compared with

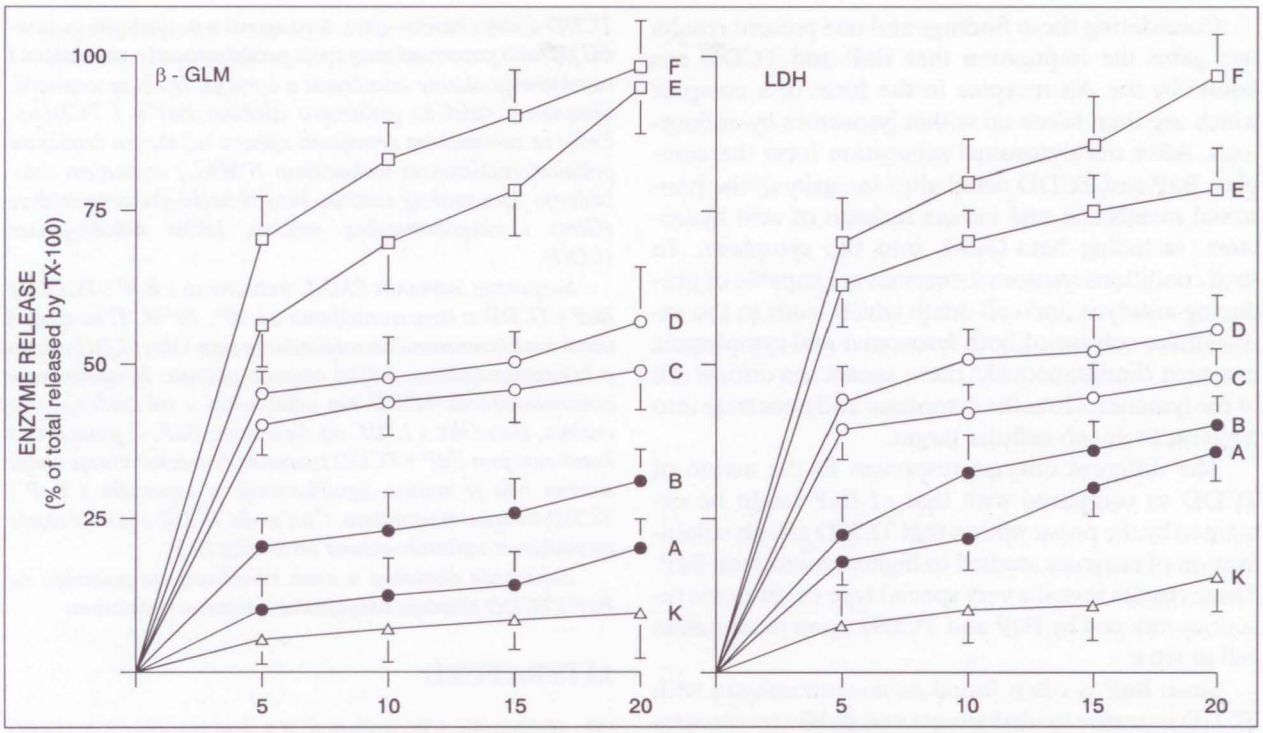

Figure 2 - Time – and dose – dependent release of beta–G1m and LDH from human polymorphonuclear leucocytes after incubation at 37°C for 5, 10, 15 and 20 min. with DMSO (K), 10^{-7}M BaP (A); 10^{-6}M BaP (B), 10^{-7}M TCDD (C),10^{-6}M TCDD (D), 10^{-7}M BaP + 10^{-7}M TCDD (E) and 10^{-6}M BaP + 10^{-6}M TCDD (F). Point represent the mean ± standard error of the mean of three experiments

DMSO or 10^{-7}M BaP. However, TCDD was more effective in releasing beta-GLM and LDH than BaP using equimolar concentrations.

When the suspension of PMNL was incubated with both compounds, BaP and TCDD, either in concentrations of 10^{-7}M, especially of 10^{-6}M the enzyme release of beta-GLM and LDH was significantly ($P>0.01$) higher as compared to the values obtained in BaP or TCDD treated samples. Also, there were no special differences in the extracellular release between beta-GLM and LDH following application of BaP, TCDD or BaP+TCDD.

Data obtained with specimens which were incubated in the same manner, but without agents or DMSO, were used as background levels. These data were subtracted from the results of DMSO or agents-treated patterns and are not included in Figure 2.

4. DISCUSSION

It is well documented that automobile discharges significant quantities of many toxic compounds including carcinogenic BaP and TCDD. Our previous investigations indicated that BaP and TCDD interfere with human PMNL (12,13). The present studies compare the effects of BaP and TCDD upon integrity of human PMNL by measuring the extracellular release of lysosomal (beta-GLM) and cytoplasmic (LDH) enzymes.

A striking observation in the present studies was the time- and dose-dependent extracellular release of beta-GLM and LDH from human PMNL treated with BaP, TCDD of BaP+TCDD. However, the discharge which occurs in TCDD-treated suspensions was found to be significantly increased as compared to the effects of BaP. In the experiments of combined exposure to BaP+TCDD the extracellular release of beta-GLM and LDH was by far more significant than that of TCDD, especially BaP-treated specimens.

Because of the very complex nature of the factors influencing extracellular enzyme release the present results are not sufficient to explain the mechanisms by which BaP and TCDD enter the cell and induce leakage of beta-GLM and LDH. However, it is widely accepted that BaP and TCDD bind Ah-receptor and enter the nuclear fraction of the target cell. On the other hand, it has been shown that in some cell types the complex formed between drug and receptor follows rapid internalisation into endosomes via coated pits. Once internalised, most such complexes are transported to lysosomes where the leakage between drug and receptor can be recycled back to the cell surface, while the drug liberated from complex is set free to exert its effects upon lysosomal membrane.

Considering these findings and our present results one gains the impression that BaP and TCDD are bound by the Ah-receptor in the form of a complex which are then taken up within lysosomes by endocytosis. After intralysosomal separation form the complex, BaP and TCDD could alter integrity of the lysosomal membrane and induce leakage of acid hydrolases, including beta-GLM, into the cytoplasm. In such conditions lysosomal enzymes are capable of producing autolysis and cell death which leads to the extracellular release of both lysosomal and cytoplasmic enzymes. Simultaneously, these agents can diffuse out of the lysosomes into the cytoplasm and penetrate into nucleus, their sub-cellular target.

The different enzyme-responses to the action of TCDD as compared with that of BaP might be explained by the presumption that TCDD affects solubilisation of enzymes studied to higher extent than BaP. These results reveal a very special type of cytotoxic reactions induced by BaP and TCDD upon mammalian cell **in vitro.**

Since BaP is often found as co-contaminant with TCDD in traffic loaded streets and public transportation system as well as other areas in the city where people spend several hours a day, the particulates of automobile exhaust should get the main concern.

5. CONCLUSION

The effects of BaP and TCDD upon integrity of human polymorphonuclear leucocytes are important toxicological problems mediated by Ah receptor, lysosomal and cytoplasmic enzymes, their release, autolysis and cell death. The results of these studies indicate stronger cytotoxic effects of TCDD in comparison to the values obtained in BaP-treated samples. On the other hand, the effects of BaP in combination with TCDD were by far greater than those observed in BaP or TCDD experiments. At equimolar concentrations of 10^{-7}, 10^{-6}M, BaP, TCDD or BaP+TCDD induced time- and dose-dependent effects.

The present model system may yield information important to our understanding of organismal responses to environmental pollutant, especially traffic automobile exhaust.

SAŽETAK

UČINCI BENZO(A)PIRENA I 2, 3, 7, 8-TETRAKLORIDBENZO-P-DIOKSINA IZ ISPUŠNIH PLINOVA AUTOMOBILA NA ŽIVOTNU SPOSOBNOST STANICA SISAVACA

Potencijalno podrijetlo benzo(a)pirena (BaP) i tetraklordibenzo-p-dioksina (TCDD) je u nepotpunom izgaranju raznih goriva. Ti su spojevi nađeni pri spaljivanju otpada, u dimu cigareta, u ispušnim plinovima automobila itd. Iako BaP i TCDD djeluju karcinogeno, ti su agensi u posljednjim godinama privukli pozornost zbog svoje perzistentnosti u ekosustavu i uzrokovanja akutne toksičnosti u čovjeka. Da bi se ustanovili zdravstveni rizici za pučanstvo izloženo BaP-u i TCDD-u, činilo se zanimljivim procijeniti njihove učinke na humanim polimorfonuklearnim leukocitima (PMNL) mjerenjem oslobađanja lizosomskog enzima, beta-N-acetil-glukozaminidaze (Glm) i citoplazmatskog enzima, laktat dehidrogenaze (LDH).

Suspenzije humanih PMNL tretirane su s BaP i TCDD te BaP+TCDD u koncentracijama od 10^{-7}, 10^{-6}M. Ti su spojevi uzrokovali izvanstanično oslobađanje beta-Glm i LDH ovisno o koncentracijama i duljini trajanja pokusa. U ispitanim je koncentracijama TCDD bio učinkovitiji u oslobađanju oba enzima, beta-Glm i LDH, od djelovanja BaP. U pokusima s kombinacijom BaP+TCDD izvanstanično oslobađanje obaju enzima bilo je znatno signifikantnije u usporedbi s BaP i TCDD tretiranim uzorcima. Čini se da TCDD snažnije utječe na topljivost ispitanih enzima od učinka BaP.

Zapažanja dobivena u ovim istraživanjima pokazuju da BaP i TCDD oštećuju lizosomsku i staničnu membranu.

LITERATURE

[1] **Hochstein, J.R., Aulerich, R.J. and Bursian, S.J.** (1988) *Acute toxicity of 2,3,7,-tetrachlorodibenzo-p-dioxin to mink. Arch. Environ. Contam.* Toxicol. 17: 33-37.

[2] **Foo,S.C., Chin,K.K., Ong,S.L. and Chow,P.W.** (1989) *Analysis of incineration ashes for 2,3,7,8-tetrachlorodibenzo-p-dioxin, In: Brasser, L.J., Mulder, W.C. (eds).* Elsevier Science Publ., Amsterdam, pp 143-145.

[3] **Silkworth,J.B., Lipinskas, T. and Stoner,C.R.** (1995) *Immunosuppressive potential of several polycyclic aromatic hydrocarbons found at a Superfund site: new model used to evaluate additive interactions between benzo(a)pyrene and TCDD.* Toxicology 105: 375-386.

[4] **Smolen,J.T. and Weissmann, G.** (1982) *The effects of various stimuli and calcium antagonists on the fluorescence response of chlorotetracycline-loaded human neutrophils. Biochim. Biophys.* Acta 720:172-180.

[5] **Carević,M. and Carević,O.** (1982) *The effects of benzo(a)pyrene on rat liver lysosomes.* Toxicol. Lett.:10: 41-49.

[6] **Carević,O.** (1977) *The effect of gamma-hexachlorcyclohexane on rat liver lysosomes.* Biochem. Pharmacol. 26:1377-1381.

[7] **Carević,O.** (1986) *Effect of sodium thiomalate on immune complex-induced release of lysosomal enzymes from human poliymorphonuclear leucocytes.* Immunopharmacology 11: 7-11.

[8] **Sellinger,O.Z., Beaufay,P., Jacques,P., Doyen,A. and de Duve C.** (1960) *Tissue fractionation studies. 15. Intracellular distribution and properties of beta-N-acetylglucosaminidase and beta-galactosidase in rat liver.* Biochem. J. 74:450-456.

[9] **Baccino,F.M. and Zuretti, F.M.** (1975) *Structural equivalents of latency for lysosome function.* Biochem. J. 146: 97-108.

[10] **Wacker,W.E.C., Ulmer,D.D. and Vallee, B.L.** (1956) *Metaloenzymes and myocardial infarction. II Malic and*

lactic dep-dioxin on human polymorphonuclear leucocytehydrogenase activities and zinc concentration in serum. New Engl. J.Med. 255:449-56.

[11] **Lowry,O.H., Rosenbrough,N.J., Farr, A.L. and Randall,R.J.** (1951) Protein measurements with Folin phenol reagent. J.Biol.Chem.193: 265-275.

[12] **Carević,O.** (1990) *Kardiotoksičnost benzo(a)pirena, kancerogenog atmosferskog polutanta u urbanim sredinama.* Čovjek i promet 16:31-39.

[13] **Carević,M. and Carević,O.** (1994) *The effects of 2,3,7,8-dichlorodibenzo-s.* Arh. hig.rada i toksikol. 45: 161-166.