EFFECT OF CIGARETTE SMOKE ON SUBLINGUAL GLAND AND LIVER PHOSPHOTYROSINE PROTEIN PHOSPHATASE AND ACID PHOSPHATASE ACTIVITIES

MODIFICAÇÕES NA ATIVIDADE FOSFOTIROSINA PROTEÍNA FOSFATASE E FOSFATASE ÁCIDA EM GLÂNDULA SUBLINGUAL E FÍGADO DE RATOS EXPOSTOS À FUMAÇA DE CIGARRO

Ana Paula BASSETO
Fernando Tozze Alves NEVES
Susana Farias TOLEDO
Students from University of Sacred Heart.

Thelma Lopes da SILVA
Biologist – Technician of Department of Biological Sciences – Bauru Dental School – University of Sao Paulo.

Marília Afonso Rabelo BUZALAF
Flávio Augusto Cardoso de FARIA
José Mauro GRANJEIRO
Assistant Professors of Department of Biological Sciences – Bauru Dental School – University of Sao Paulo.

Voluntary and involuntary smoking influenced general health. Links between parental smoking and caries experience in young children have been suggested. Recent studies have demonstrated an increase in oxidative stress in tissues of many individuals exposed to gas-phase cigarette smoke (CS). The aim of the present study was to analyze the effect of CS on specific activity of total acid phosphatase (TAP), inespecific acid phosphatase (IAP) and phosphotyrosine protein phosphatase (PTP) in tissues of rats exposed to CS. Forty-nine rats (Rattus norvergicus) were divided randomly into experimental and control (not exposed to CD) groups. The experimental group was exposed 3 times daily to CS from 10 cigarettes (1 cigarette: pitch=11mg; nicotine=0.9mg; CO=12mg), for 10 minutes. The animals were sacrificed after 0, 25, 50 and 75 days. The phosphatase activities were determined in acetate buffer pH 5 and 37ºC. After 25 days, in liver, TAP, IAP and PTP activities increased 500%, but were inhibited 75% after 50 days. A similar behavior was observed to sublingual gland after 25 and 50 days of CS exposure. However, after 75 days the enzymes levels increased slightly in liver but were inhibited in sublingual gland. These results indicate that phosphatase isoforms could be biomarkers of passive smoking induce alterations in some tissues. The early events associated to some diseases caused by CS could be associated to the inhibition of the PTP, enzymes involved in cell proliferation, growth and differentiation.

UNITERMS: Cigarette smoke; Phosphotyrosine protein phosphatase; Acid phosphatase; Sublingual gland, liver.

INTRODUCTION

In Europe, Japan, and United States of America, 80-90% and 55-80% of lung tumors in men and women, respectively, are attributed to cigarette smoking. In addition, 75-90% of esophagus, larynx and oral cancers are related to tobacco effects, acting alone or in conjugation to alcohol[8,17]. Moreover, smoking addiction has been associated to fertility decrease in women[12,13] and men[31].

Links between involuntary smoking and some diseases, especially in children, even in intra-uterine life are confirmed[22]. Involuntary smoker may have middle ear exudation[26], asthma exacerbation[4] and alterations in lung function[27]. Recent reports have also suggested an association between parental smoking and caries experience in young children[25,30]. Studies with osteogenic and pre-osteoblastic cell clones from rat calvaria indicate that nicotine affects osteoblastic differentiation[32]. On the other hand, the formation of multinucleated tartrate-resistant acid phosphatase-positive cells was inhibited by nicotine in a dose-response manner.

Recent studies have demonstrated that exposure to CS causes a significant decrease on the activity of some salivary enzymes, like amylase, lactic dehydrogenase and acid phosphatase[19]. As a big family of hydrolytic enzymes, acid phosphatases are ubiquitously distributed in nature. Human acid phosphatases constitute a group of 6 isoenzymes previously identified by electrophoresis[15]. Type 5-isoform differs from others because it is insensitive to tartrate and pHMB[16] (p-hidroximercuribenzoate), known as tartrate-resistant acid phosphatase (TRAP). Other kind of acid phosphatase (low-molecular weight, insensitive to tartrate, but completely inhibited by heavy metals and derivates) is now classified as phosphotyrosine protein phosphatase[24] and has been implicated on cell cycle control[10].

Phosphotyrosine protein phosphatases (PTP's) are a super family of enzymes that catalyze the hydrolysis of phosphate esterified both to free tyrosine and to peptides or proteins[29]. PTP's catalytic action contrasts to the activity of protein tyrosine kinases involved in the control of cell proliferation and differentiation. Due to PTP's antiproliferative action, these genes have been considered as tumor suppressor genes[14]. These enzymes share a limited motif (CXXXXXRS/T) that defines the region of the active site and the catalytic mechanism, which proceeds through the formation of a cysteinil-phosphate intermediary[33]. This is susceptible to heavy metals or free radicals, which interact directly to the cysteine residue that is essential to the active site, thus inhibiting enzyme activity.

Studies relative to kinetic, physico-chemical and structural properties of PTP's are available[7,9,24]. However, little is known about the profile of this enzyme during development of a pathology. In this work, we demonstrated that exposure of rats to CS caused a marked decrease on PTP activity in salivary sublingual glands and liver extracts.

MATERIALS AND METHODS

Forty-nine Wistar rats (weight around 200 g) were divided into 2 groups: control (not exposed to CS) and experimental. Experimental group was subjected 3 times daily to CS of 10 cigarettes (1 cigarette: nicotine 0.9 mg; pitch 11 mg; CO 12 mg) for 10 min, as described for Cendon et al.[3] All animals received water and pelleted diet "ad libitum".

Animals were sacrificed 0, 25, 50 and 75 days after daily exposure to CS, by excess ethyl ether inhalation. The sublingual and submandibular glands, liver, heart, spleen, kidney, lung and testicles were removed (afternoon). The organs were washed in saline solution (NaCl 0.9%), weighted and frozen (-20°C).

Enzyme extraction protocol

The removed tissues were homogenized individually in 100 mM acetate buffer, pH 5.0, containing EDTA and 1 mM b-mercaptoethanol, and centrifuged at 20,000 rpm for 30 min. The supernatant extract was used for protein, total acid phosphatase (TAP) and phosphotyrosine protein phosphatase activity (PTP) assays. The PTP assay was made through differential inhibition by pHMB, as previously described[1].

Enzyme Activity Assay

The enzyme activity was determined at 37°C in 100 mM acetate buffer, pH 5.0 and containing 5 mM p-NPP (p-nitrophenyl-phosphate) with a final volume of 1 mL. The reaction initiated by addition of enzyme, was terminated after 5 min by the addition of 1 mL of 1M NaOH. The formation of p-nitro-phenoxide (pNP) was determined spectrophotometrically through the reading of absorbance at 405 nm, using a molar extinction

coefficient of 18,000 M^{-1} cm^{-1}. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the hydrolysis of one mmol of pNPP per minute. Specific activity(SA) is defined as the number of units per milligram of protein, which was quantified by the Lowry's method, as described by Hartree[11], using BSA as standard.

Phosphatase activity in absence of inhibitors was considered as total acid phosphatase (TAP). Enzyme activity inhibited by 1mM pHMB corresponded to PTPs activity. Residual activity in presence of 1 mM pHMB corresponded to inespecific acid phosphatases (IAP, lisossomal acid phosphatase and TRAP).

RESULTS

The CS generator system permitted the complete burn of 10 cigarettes in 10 min, by a constant air flux. No animal died during the experimental period.

Specific activity of TAP, PTP and IAP in submandibular gland, heart, spleen, kidney, and testicle of animals exposed to CS didn't vary in relation to control group, in any of experimental periods.

Liver extracts of animals exposed to CS for 25 days showed a 5-fold increase in TAP, PTP and IAP activities (Figure 1A). After 50 days of exposition, the activity of these enzymes was reduced in 60% in respect to control. The rate among the activity of these enzymes in treated and control groups after 75 days was 1 for TAP and PTP and around 2 for IAP.

In sublingual gland (Figure 1B), there was an increase in TAP, IAP and PTP activities after 25 days of treatment of 2.25, 2.0 and 3.5-fold. However the activity of these enzymes was reduced in more than 50%, after 50 and 75 days of exposition to CS.

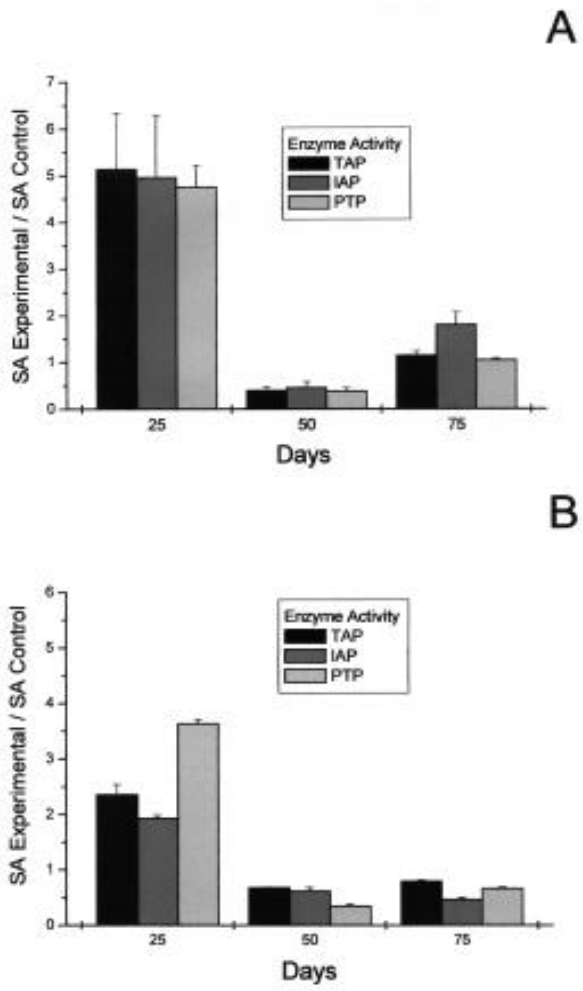

FIGURE 1- Relative anzimatic activity (SA Experimental/ SA control) in function of the period of exposition to cigarette smoke in Liver (A) and in salivary sublingual galns (B). Enzyme activity was determined in triplicate in the tissues removed from 7 rats, as described in Materials and Methods

DISCUSSION

Cigarette smoking contains more than 4,000 different chemical compounds. Of these, 400 are carcinogens. Moreover, CS contains oxidants, as oxygen free radicals[23] and volatile aldehydes[21]. These would be, probably, the main responsible for the injuries caused to the biomolecules exposed to CS. Exposure of plasma to CS results in an increase of carbonilated proteins[6] and in the selective disappearing of endogenous plasma antioxidants[5]. Nagler et al.[19] demonstrated that the reduction in salivary amylase and lactic dehydrogenase activities after exposure to CS could be partially prevented by addition of antioxidants. However, the level of salivary acid phosphatase activity was not affected by the tested antioxidants.

We verified in vivo that CS, after 50 and 75 days of exposition, can selectively affect some tissues, causing a decrease in phosphatase activity. This observation is particularly relevant to PTP's, because they are involved in the control of cell division, growth and differentiation.

The initial contact of hepatocytes and sublingual gland cells with the compounds present in CS or derived from it could cause initially the recruitment of defense cells (rich in lisossomal acid phosphatase,

TRAP and also containing PTP's). This could explain the increase in enzyme activity observed after 25 days of exposure to CS.

This could be confirmed through the microscopic evaluation of the tissues, after treatment with CS at the same experimental conditions of this work, what is currently being done in our laboratory, aiming to evaluate the correlation between phosphatase activity and possible cell or structural alterations.

The exact mechanism involved in the final reduction of the PTP's activity was not determined yet. It's known that PTP's are especially sensitive to heavy metals and biologic oxidants, as free radicals[2], in function of the formation of complexes or oxidation of Cys-12 and Cys-17 (present in the active site of the enzyme). As free radicals present in CS[28] are potential PTP's inhibitors, the enzyme activity decrease could be due to oxidation of the essential cysteine residue present in the active site. The 50 days of CS exposition required to induce loss of PTP activity may be necessary for antioxidant system depletion in treated animals. It is also possible that the long and repeated exposure to CS causes a reduction in the tissue antioxidant capacity, thus exposing sensitive proteins to oxidant agents present in CS.

An intriguing result was the absence of CS effect on submandibular gland (that produces protein-rich saliva), while the sublingual gland (that produces mucin-rich saliva) was markedly affected. Additional studies should be done to clarify this effect. Ma et al.[18] demonstrated that CS promotes a reduction in epidermal growing factor (EGF) produced by salivary glands, resulting in a smaller production of gastric mucous. Nagler et al.[19] related that saliva poor in proteins was less efficient as antioxidant. Since parotid and submandibular glands produce a protein-rich saliva, they would be more resistant to the effect of CS. Possibly the time of exposition and/or dose of smoke used in our work may have been insufficient to cause the depletion of antioxidant system of submandibular gland.

This work showed that the activity of different isoforms of acid phosphatase, especially PTP's, can be used as a biomarker to monitor the effect of cigarette smoking in different tissues. Since PTP's participate in the control of cell cycle, growth and differentiation, more detailed studies should be conduced to bring new information in respect of their exact biologic role, as well as their involvement in the development of various pathologies.

RESUMO

O fumo voluntário e involuntário influencia a saúde geral. Têm-se sugerido ligações entre o hábito de fumar dos pais e a experiência de cáries em crianças. Estudos recentes demonstraram um aumento do estresse oxidativo em diversos tecidos de indivíduos expostos à fumaça do cigarro (FC). Neste trabalho analisamos o efeito da FC na atividade específica da fosfatase ácida total (FAT), fosfatase ácida inespecífica (FAI) e proteínas tirosina fosfatase (PTP) em diversos tecidos de ratos expostos à FC ambiental. Foram utilizados 49 ratos Wistar (Rattus norvergicus) machos adultos (200g), divididos aleatoriamente em grupo controle (não exposto à FC) e experimental. O grupo experimental foi exposto 3 vezes ao dia à fumaça produzida por 10 cigarros (1 cigarro: alcatrão=11mg; nicotina=0,9mg; monóxido de carbono=12mg), durante 10 minutos. Os animais foram sacrificados após 0, 25, 50 e 75 dias. A atividade fosfatásica (nmol/min mg) foi determinada no extrato solúvel dos tecidos analisados, utilizando 5 mM de pNPP como substrato no pH 5 e 37ºC. Na glândula sublingual ocorreu um aumento de cerca de 2x na FAT e FAI e 3,5 vezes na PTP após 25 dias; após 50 dias a atividade FAT e PTP foi menor que 50% em relação ao grupo controle, mantendo-se neste patamar após 75 dias. No fígado, após 25 dias, ocorreu um aumento de cerca de 500% na FAT, PTP e FAI; após 50 dias houve marcante diminuição (75%) na atividade dessas enzimas. A sensibilidade das diferentes isoformas da fosfatase ácida à FC a coloca como potencial biomarcador das alterações induzidas pela exposição à fumaça de cigarro ambiental. Concluímos que a exposição crônica à FC promove marcante diminuição da atividade fosfatase ácida e PTP na glândula salivar sublingual e no fígado de ratos. A inibição da PTP poderia ser o início de modificações moleculares relacionadas a algumas doenças associadas ao cigarro uma vez que essa classe de enzimas desempenha papel fundamental no controle da proliferação, crescimento e diferenciação celular.

UNITERMOS: Cigarro; Fosfotirosina proteína fosfatase; Fosfatase ácida, Glândula Salivar; Fígado.

ACKNOWLEDGEMENT

The authors thank to Prof. Dr. Rumio Taga for the critical review of this manuscript and also to the

FOB-USP technicians Ovídio dos Santos Sobrinho, Gilmar Vicente da Silva, Luiz Carlos da Silva, Erasmo Gonçalves da Silva, for the technical assistance. This work was supported by FAPESP, FOB-USP, PRP-USP.

REFERENCES

1- BUZALAF, M.A.R. et al. Kinetic characterization of bovine lung low-molecular-weight protein tyrosine phosphatase. **Exp. Lung. Res**., v.24, n.3, p.269-72, May/June 1998.

2- CASELLI, A. The inactivation mechanism of low molecular weight phosphotyrosine-protein phosphatase by H_2O_2. **J. Biol. Chem.**, v.273, n.49, p.32554-60, Dec. 1998.

3- CENDON, S.P. Pulmonary emphysema induced by passive smoking: an experimental study in rats. **Braz. J. Med. Biol. Res**., v. 30, n.10, p.1241-7, Oct 1997.

4- CHILMONCZYK B.A. et al. Association between exposure to environmental tobacco smoke and exacerbations of asthma in children. **N. Engl. J. Med**., v.328, n.23, p.1665-9, June 1993.

5- CROSS, C.E. et al. Cigarette smoke oxidation of human plasma constituents. **Ann. N. Y. Acad. Sci**., v.686, p.72-89; discussion 89-90, May 1993.

6- EISERICH, J.P. et al. **Biothiols in health and disease** . s.l., Marcel Dekker, 1995. p. 437-53

7- FAUMAN, E.B.; SAPER, M.A. Structure and function of the protein tyrosine phosphatases. **Trends Biochem. Sci**., v.21, n.11, p.413-7, Nov 1996.

8- FRANCESCHI, S. Comparison of cancers of the oral cavity and pharynx worldwide: etiological clues. **Oral Oncol**., v.36, n.1, p.106-5, Jan 2000.

9- GRANJEIRO, J.M.; TAGA, E. M.; AOYAMA, H. Purification and characterization of a low-molecular-weight bovine kidney Acid phosphatase. **An. Acad. Bras. Ci**., v.69, n.4, p.451-60, 1997a.

10- GRANJEIRO, J.M. et al. Bovine kidney low molecular weight acid phosphatase: FMN-dependent kinetics. **Biochem. Mol. Biol. Int**., v.41, n.6, p.1201-6, May 1997b.

11- HARTREE, E.F. Determination of proteins: a modification of the Lowry method that give a linear photometric response. **Anal. Biochem**., v.48, n.2, p.422-27, 1972.

12- HOWE, G. et al. Effects of age, cigarette smoking, and other factors on fertility: findings in a large prospective study. **Brit. Med. J.** (Clin Res Ed), v.290, n.6483, p.1697-700, June 1985.

13- JENSEN, T.K. et al. Adult and prenatal exposures to tobacco smoke as risk indicators of fertility among 430 Danish couples. **Amer. J. Epidemiol**., v.148, n.10, p.992-7, Nov. 1998.

14- LA FORGIA, S. Receptor protein-tyrosine phosphatase gamma is a candidate tumor suppressor gene at human chromosome region 3p21. **Proc. Natl. Acad. Sci**. USA, v.88, n.11, p.5036-40, June 1991.

15- LAM., W. K. et al. Biochemical properties of tartrate-resistant acid phosphatase in serum of adults and children. **Clin. Chem**., v.24, n.7, p.1105-8, July 1978.

16- LAU, K. H. et al. Evidence that tartrate-resistant acid phosphatases from osteoclastomas and hairy cell leukemia spleen are members of a multigene family. **Int. J. Biochem**., v.23, n.11, p.1237-44, 1991.

17- LEVI, F. Câncer Prevencion: Epidemioly and Perspectives. **Eur. J. Cancer**, v.35, n.7, p.1046-58, Apr 1999.

18- MA, L.; et al. Cigarette smoke increases apoptosis in the gastric mucosa: role of epidermal growth factor. **Digestion**, v.60,n.5, p.461-8, Sept./Oct. 1999

19- NAGLER, R.M.; KITROSSKY, N.; CHEVION, M. Antioxidant activity of rat parotid saliva. **Arch. Otolaryng. Head Neck Surg**., v.123, n.9, p.989-93, Sept. 1997.

20- NAGLER, R. et al. Effect of cigarette smoke on salivary proteins and enzyme activities. **Arch. Biochem. Biophys**., v.372, n.2, p.229-36, July 2000.

21- O'NEILL, C.A. et al. Aldehyde-induced protein modifications in human plasma: protection by glutathione and dihydrolipoic acid. **J. Lab. Clin. Med**., v.124, n.3, p.359-70, Sept. 1994.

22- PICHINI, S. et al. The analysis of nicotine in infants' hair for measuring exposure to environmental tobacco smoke. **Forensic Sci. Int**., v.84, n.1-3, p.253-8, Jan. 1997.

23- PRYOR, W.A. et al. Electron-spin resonance study of mainstream and sidestream cigarette smoke: nature of the free radicals in gas-phase smoke and in cigarette tar. **Environ. Hlth. Perspect**., v.47, p.345-55, Jan. 1983.

24- RAMPONI, G.; STEFANI, M. Structural, catalytic, and functional properties of low Mr phosphotyrosine protein phosphatases. Evidence of a long evolutionary history. **Int. J. Biochem. Cell Biol**., v.29, n.2, p.279-92, Feb. 1997.

25- SCHOU, L.; UITENBROEK, D. Social and behavioural indicators of caries experience in 5 year-old children. **Comm. dent. oral Epidemiol**., v. 23, p. 276-81, 1995.

26- STRACHAN, D.P. et al. Passive smoking, salivary cotinine concentrations, and middle ear effusion in 7 year old children. **Brit. Med. J**., v.298, n.6687, p.1549-52, June 1989.

27- STRACHAN, D. P. et al. The relationship of salivary cotinine to respiratory symptoms, spirometry, and exercise-induced bronchospasm in seven-year-old children. **Amer. Rev. Resp. Dis**., v.142, n.1, p.147-51, July 1990.

28- VALKONEN, M.M.; KUUSI, T. Vitamin C prevents the acute atherogenic effects of passive smoking. **Free Radic. Biol. Med**., v. 28, n.3, p.428-36, Feb. 2000.

29- WALTON, K. M.; DIXON, J. E. Protein tyrosine phosphatases. **Ann. Rev. Biochem**., v.62, p.101-20, 1993.

30- WILLIAMS, S.A.; KWAN, S.Y.L.; PARSONS, S. Parental smoking practices and caries experience in pre-school children. **Caries Res**., v. 34, p. 117-22, 2000.

31- WONG, W.Y. et al. Cigarette smoking and the risk of male factor subfertility: minor association between cotinine in seminal plasma and semen morphology. **Fertil. Steril**., v.74, n.5,p.930-5, Nov. 2000.

32- YUHARA, S. et al. Effects of nicotine on cultured cells suggest that it can influence the formation and resorption of bone. **Eur. J. Pharmacol**., v.383,n.3, p.387-93, Nov 1999.

33- ZHANG, Z. -Y.; Wang, Y.; Dixon, J. E. Dissecting the catalytic mechanism of protein tyrosine phosphatases. **Proc. Natl. Acad. Sci**. U.S.A., v.91, n.5, p.1624-8, Mar. 1994.