PROCEEDINGS OF THE BALKAN SCIENTIFIC CONFERENCE OF BIOLOGY IN PLOVDIV (BULGARIA) FROM 19TH TILL 21ST OF MAY 2005 (EDS B. GRUEV, M. NIKOLOVA AND A. DONEV), 2005 (P. 63–70)

QUANTITATIVE ANALYSES OF HALOTHANE IMPACT ON PAXILLIN IN LUNG CARCINOMA CELL LINE A 549

R. Valtcheva*, K. Hristova, R. Pankov, E. Stephanova

Sofia University „St. Kl.Ohridski", Faculty of Biology, Department of Cytology, histology and embryology, 8, Dragan Tsakov str, 1164 Sofia, Bulgaria

Contact details for correspondening:
Assoc. Prof. Elena Stephanova, PhD
Head of Dept. Cytology, histology and embryology, Faculty of biology, Sofia University 8, Dragan Tsankov str., 1164 Sofia
Tel: + 359 (0) 2 8668501, Fax: + 359 (0) 2 865 66 41
E.mail: stephanova@biofac.uni-sofia.bg

ABSTRACT. Cell adhesion is one of the main characterictics of eukariotic cell viability. Immediately after adhesion, the cells form signal-transduction macromolecular complexes – focal adhesions when the cell adhesive receptors integrins are become clusterred in cell membranes, as a result of binding to extracellular matrix. The volatile anaesthetic halothane is known to cause changes in cell plasma membranes, both modulating lipid bilayer fluidity and/or directly interacting with membrane proteins, including integrin receptors.

Our previous study showed that halothane at concentration of 1.5 mM doesn't change the dynamic of A 549 adhesion to collagen IV. However the anaesthetic provoked vinculin and paxillin disappearance from the focal adhesion of the investigated lung carcinoma cell line.

Western blot analyses and densitometry measurements used in the present study revealed that the above-mentioned dose (1.5 mM) didn't change total paxillin quantity in A 549, but did inhibit the active form of paxillin, i.e. the phosphorylated on tyrosine 31 protein.

We conclude that the disruption of focal adhesions in A 549 in response to halothane treatment is accompanied with reduced activity of some proteins in A 549.

KEY WORDS. Cell adhesion, A 549, paxillin

INTRODUCTION

The main molecules responsible for cell adhesion in vitro are the integirin receptors, connecting the cellular cytoskeleton to the extracellular matrix (*1*). Integrin-ligand binding induces clustering in the cell plasma membrane, followed by formation of focal contacts, including various actin-binding (structural), adapter and signal proteins (Ser and Tyr kinases). The maturation of focal contacts enhances integrin affinity to their ligands and also controls the extracellular matrix fibrilogenesis (*2-7*).

One of the major focal adhesion constituents is the 68-kDa-protein paxillin, which is a typical phosphoprotein. Its molecule has a multiple binding sites for talin, tensin, vinculin, and the cytoplasmic domain of β1 integrin subunit, as well for some signal proteins, such as focal adhesion kinase (FAK), Src, Csk. (*8-10*). The requirement for focal adhesion targeting of paxillin was determined by transfection of paxillin constructs into CHO.K1 fibroblasts. Although, paxillin constructs containing both deletion and point mutations that abrogate binding of FAK and/or vinculin were found to target effectively to focal adhesions, mutations related to other regions including carboxyl-terminal half of paxillin was found to be important for this targeting. Transfection analyses of sequential carboxyl-terminal truncations of the four individual LIM motifs and site-directed mutagenesis, demonstrated that paxillin localizes to focal adhesions and functions as a primary determinant of protein subcellular localization (*11*). Generally paxillin phosphorylation is related to cell adhesion, actin cytoskeleton remodelling and cell growth control. One of the first sites, phosphorylated in paxillin molecule is Tyrosine 31. (*12-14*).

The volatile hydrocarbone halothane is widely used in medicine as an anaesthetic agent, because of immediate anaesthesia induction and rapid patient recovery after surgery intervention. (*15*). Mechanism of anaesthesia is related to ligand-gated ion channels modulation which could be based on changing membrane fluidity and/or direct interactions of anaesthetic with membrane proteins (*16, 17*). Halothane-membrane receptors interactions are proved also for other molecules like major histocompatibility complex (MHC) (*18*) and adhesive receptors in platelets, leukocytes and monocytes (*19, 20*). Inhibited adhesion has been reported, as well for two Escherichia coli strains - K88 and NG7C to Hep-2 epithelial cell surface (*21*) and for A 549 lung carcinoma to collagen IV (*22*).

Our previous study revealed that halothane, applied at concentration of 1.5 mM disrupts the newley formed focal adhesion contacts, shown by immunofluorescence analyses for vinculin and paxillin (*23-26*). This dose, however doesn't change the percentage of A 549 adhesion, left to adhere for five hours without and in presence of halothane on collagen IV (*22*).

In the present study we evaluated the effect of 1.5 mM halothane on paxillin quantity in A 549. It is well known that multiple proteins in the focal adhesions are subjected to phosphorylation, among which are the cytoplasmic domains of the β integrin subunite, vinculin, FAK, SFK, paxillin etc (*1, 27, 28*). Western blot analyses and densitometry measurements of the bands, visualized by antibodies specifically recognizing total paxillin and paxillin phosphorylated on Tyr 31 showed that the

focal adhesion disruption is accompanied with inhibition of paxillin phosphorylation, but not with the diminished total paxillin quantity. This effect maybe due to the impact of the volatile anaesthetic on integrin-ligand affinity, which is inhibited thus causing reduction in phosphorylation of some proteins in focal adhesions, hence to the disruption of these cell-extracellular matrix contacts.

MATERIAL AND METHODS

Cell culture conditions

Human lung carcinoma cell line A 549 (ATCC No CCL-185) was grown in DMEM supplemented with 10 % FBS (HyClone) and antibiotic solution mixture of Ampicillin/Streptomycin (Sigma). Cells were maintained at 37^0 C in a humidified atmosphere of 5% CO_2. Cells were subcultured 24 hours before treatment.

Halothane solution preparation and exposure

DMEM supplemented with 10 % FBS was saturated with halothane added (Liceva) as 2 vol. %, by heating at the boiling point of the anesthetic (50^0 C) for 48 hours. UV-Vis spectral analysis measurement showed that the concentration of anesthetic in the saturated medium was 3 mM. The saturated solution was diluted with DMEM just before each treatment. Cells were treated with the indicated halothane concentrations for 2 hours at 37^0 C in a humidified atmosphere of 5% CO_2.

SDS PAAG elecrophoresys of total A 549 cell lysates

A 549 were subcultured in petry-dishes (60 mm/dm) until they reach 95 % confluence. Then cells were exposed to different halothane concentrations, ranging from 0.15 to 1.5 mM. After treatment, cells were lysed in RIPA buffer (150 mM NaCl, 2 mM EDTA, 1 % Na deoxycholate, 0.1 % SDS, 1 % Triton X – 100, 10 % glycerol, 50 mM Hepes, pH – 7.5) supplemented with coctail protease inhibitors (Boheringer) and phosphatase inhibitors 1 mM $NaVO_4$ and 1 mM NaF. Cell lysates were separated in 8 % SDS polyacril amide/bis gels, prepared according to the manufacturers instructions (Bio-Rad), using Tris-Glycine electrophoretic running buffer and SeaBlue molecular weight markers.

Western blot analysis and densitometry calculations of paxillin

For Western blot analysis, electrophoretically separated proteins from A 549 lysates were transferred on nitrocellulose membranes, and stained with Ponceau solution (SIGMA). Then membranes were incubated for one night with primary monoclonal anti-human paxillin and primary polyclonal anti-human phospho paxillin (Y-31) at 4^oC. Incubation with the secondary HRP-conjucted anti-mouse antibody was for 1.5 hours at room temperature. Peroxidase reaction was developed with 1.6 mg/ml diaminobensidine and 0.1 % H_2O_2. Densitometry measurements of the bands were performed by ScionImage software.

Statistical methods

Results were obtained from at least 3 repeatment of each experiment. The data were statistical processed by One-way Analysis of Variance and by Kruskal-Wallis Test (nonparametric) (ANOVA), using InStat software.

RESULTS AND DISCUSSION

Our previous studies on the effect of volatile anaesthetic halothane, applied at different doses to the lung carcinoma cells A 549, revealed inhibition of cell adhesive properties in response to 2 hours of exposure to 2.1 mM halothane. The lower dose of 1.5 mM doesn't affect these cells in the same way. Inhibition of A 549 adhesion hardly occurs after 5 hours exposure at lower concentration (*22*). Surprisingly 2 hours treatment with 1.5 mM caused vinculin and paxillin redistribution and their disappearance from the periphery localized dot-like focal adhesions in the lung cells. The lower dose of 0.9 mM didn't affect these structures.

Other authors have proved inhibitory effects of volatile anesthetics on cell adhesive properties. Halothane exposure at duration of 3, 6, 12 or 24 hours leads to lowered intercellular adhesion molecule-1 (ICAM-1) expression in human melanoma cells SK-MEL-37 hence to diminished adhesion (*29*). Redistribution of the platelet integrin - GPlb is seen after exposure to 0.5 MAC (minimal alveolar concentrations) sevoflurane, 1.0 MAC halothane and 2.0 MAC isoflurane. (*20*). Halothane also inhibits stimulated by thrombin receptor agonist protein - 6 (TRAP-6) platelets adhesion to monocytes, and this has been mediated by interaction of anaesthetic with surface adhesion molecule CD62P (*30*). Similar effects has been proved in studies on adhesive cell cultures, such as reduction in fibronectin affinity of the human epithelial cell line HEp-2, after exposure for 2 h to 2 % halothane (*21*).

In the present study, using Western blot, followed by densitometry measurements we showed that the total quantity of paxillin in A 549 didn't change, when cells were treated with different halothane concentrations up to 1.5 mM (Fig.1). As it was shown previously reorganization of paxillin and its disappearance from the focal adhesions was observed when the cells were exposed to 1.5 mM. Paxilin proteolysis in comparison to the non-treated control cells was not detected, when the anaesthetic was applied in both lower non-toxic and higher toxic concentrations (results not shown). These results suggest that focal adhesion proteins, like paxillin are still present in A 549 cytoplasm after 2 hours exposure to halothane but are not localized in the focal contacts.

Paxillin phosphorylation is related to the integrin activation. We found that 1.5 mM halothane caused statistically significant ($P<0.05$) reduction in phosphopaxillin in comparison both to total cellular proteins (Fig.2) and to the level of total paxillin (Fig.3). Other authors revealed that sequential truncations in some paxillin molecules affects its subcellular localization to focal adhesions (*11*). One of the first sites, phosphorylated in paxillin molecule is Tyrosine 31, which further regulates cell adhesion, actin cytoskeleton remodelling and cell growth control. (*12-14*). From results obtained in the present study we could summarise, that halothane reduced A 549 integrin-ligand binding affinity applied at doses equal and upper than 1.5 mM,

hence disrupting the focal contacts and inhibiting activation (phosphorylation) of focal contacts proteins.

ACKNOWLEDGEMENTS

The authors thank Ministry of education and sciences of Bulgaria for the financial support (Grant No 1301/03 - 2375).

REFFERENCES:

GEIGER, B., BERSHADSKY, A., PANKOV, R., and YAMADA, K. M. 2001. *Nat Rev Mol Cell Biol 2*, 793-805.

HENRY, M. D., and CAMPBELL, K. P. 1998. *Cell 95*, 859-70.

PANKOV, R., and YAMADA, K. M. 2002. *J Cell Sci 115*, 3861-3.

PANKOV, R., CUKIERMAN, E., KATZ, B. Z., MATSUMOTO, K., LIN, D. C., LIN, S., HAHN, C., and YAMADA, K. M. 2000. *J Cell Biol 148*, 1075-90.

ALTANKOV, G., GRINNELL, F., and GROTH, T. 1996. *J Biomed Mater Res 30*, 385-91.

ALTANKOV, G., and GROTH, T. 1996. *J Biomater Sci Polym Ed 8*, 299-310.

AVNUR, Z., and GEIGER, B. 1981. *Cell 25*, 121-32.

TURNER, C. E., GLENNEY, J. R., JR., and BURRIDGE, K. 1990. *J Cell Biol 111*, 1059-68.

MAZAKI, Y., HASHIMOTO, S., and SABE, H. 1997. *J Biol Chem 272*, 7437-44.

NAKAMURA, K., YANO, H., UCHIDA, H., HASHIMOTO, S., SCHAEFER, E., and SABE, H. 2000. *J Biol Chem 275*, 27155-64.

BROWN, M. C., PERROTTA, J. A., and TURNER, C. E. 1996. *J Cell Biol 135*, 1109-23.

TURNER, C. E. 1994. *Bioessays 16*, 47-52.

TURNER, C. E. 1998. *Int J Biochem Cell Biol 30*, 955-9.

BROWN, M. C., PERROTTA, J. A., and TURNER, C. E. 1998. *Mol Biol Cell 9*, 1803-16.

O'KEEFFE, N. J., and HEALY, T. E. 1999. *Pharmacol Ther 84*, 233-48.

DILGER, J. P. 2002. *Br. J. Anaesth. 89*, 41-51.

LALCHEV Z., C. Y., TODOROV R., ALEXANDROV V., STOICHEV P., PETKOV R. 1992. *Applied Cardiopulmonary Pathophysiology 4*, 315-322.

DZOLJIC, M., BENE, L., KRASZNAI, Z., DAMJANOVICH, S., and VAN DUIJN, B. 2000. *J Photochem Photobiol B 56*, 48-52.

HEINDL, B., BECKER, B. F., ZAHLER, S., and CONZEN, P. F. 1998. *Acta Anaesthesiol Scand 42*, 995-1003.

FROHLICH, D., ROTHE, G., SCHMITZ, G., and HANSEN, E. 1998. *Eur J Anaesthesiol 15*, 641-8.

BATAI, I., and KERENYI, M. 1999. *Acta Anaesthesiol Scand 43*, 760-3.

VALTCHEVA, R., STEPHANOVA, E., JORDANOVA, A., PANKOV, R., ALTANKOV, G., and LALCHEV, Z. 2003. *Chem Biol Interact 146*, 191-200.

VALTCHEVA, R., E. STEPHANOVA, Z. LALCHEV, R. PANKOV. 2004.

VALTCHEVA et al b, R. V., ELENA V. STEPHANOVA, ROUMEN G. PANKOV, GEORGE P. ALTANKOV, ZDRAVKO I. LALCHEV,. 2003. *Turkish Journal of Biochemistry*, 166-167.

VALTCHEVA et al a, R. V., R. B. DAMIANOVA, Z.I. LALCHEV, G. P. ALTANKOV, R.G. PANKOV, E. V. STEPHANOVA. 2003. *X Jubilee Session of Faculty of Biology, Sofia University "St. Kliment Ohridski"*, 62.

VALCHEVA, R., E. STEPHANOVA, Z. LALCHEV, R. PANKOV. 2004. *Acta morphologica et antropologica*, in press.

KANO, Y., KATOH, K., MASUDA, M., and FUJIWARA, K. 1996. *Circ Res 79*, 1000-6.

TURNER, C. E., KRAMARCY, N., SEALOCK, R., and BURRIDGE, K. 1991. *Exp Cell Res 192*, 651-5.

AZUMA, K., MIKE, N., FUJIWARA, Y., SHIMADA, Y., and WATANABE, T. 1993. *J Anesth 7*, 442-6.

DE ROSSI, L. W., HORN, N. A., HECKER, K. E., ROBITZSCH, T., HUTSCHENREUTER, G., and ROSSAINT, R. 2002. *Anesthesiology 96*, 117-24.

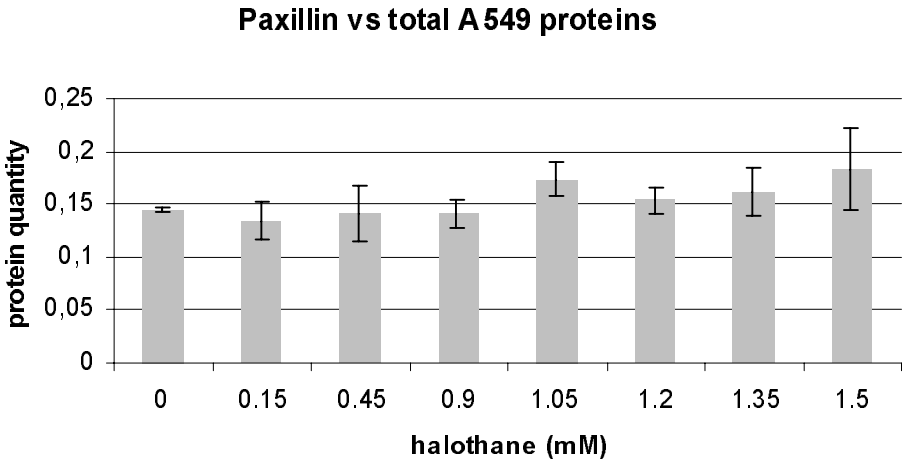

Fig. 1. *Paxillin quantification.*
Paxillin quantity (Y-ax) in A 549 cells treated with halothane at concentration range from 0 – 1.5 mM (X ax). Not statistically significant change in quantity has been observed. Results were obtained from three indipendent experiments. Standard deviations are shown as Y-bars.

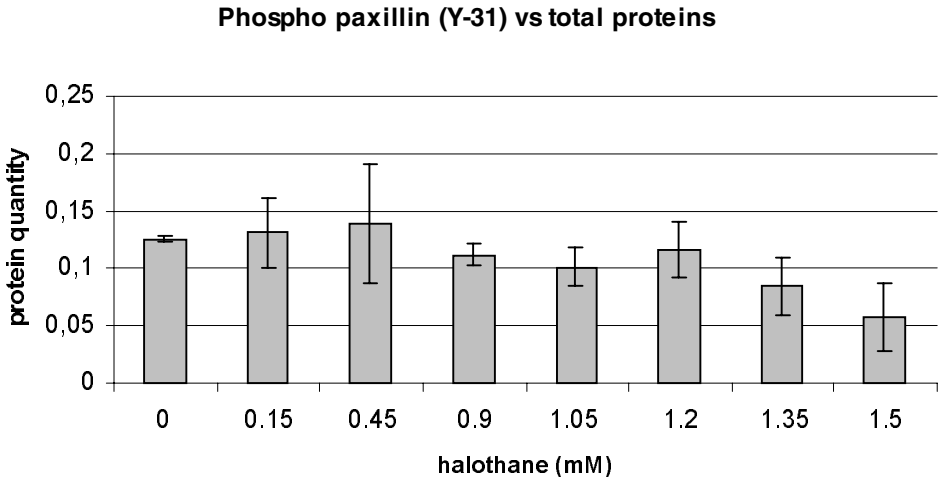

Fig. 2. *Quantification of paxillin phosphorylation in comparison to total proteins, extracted from A 549.*
*Quantity of Tyrosine phosphorylated (Y-31) paxillin (Y-ax) in A 549 treated with halothane at concentration range from 0 to 1.5 mM (X ax). Statisticaly significant inhibition in paxilline phosphorylation is seen in cells, exposed to 1.5 mM halothane is shown with *** ($p<0.001$). Results were obtained from three indipendent experiments. Standard deviations are shown as Y-bars.*

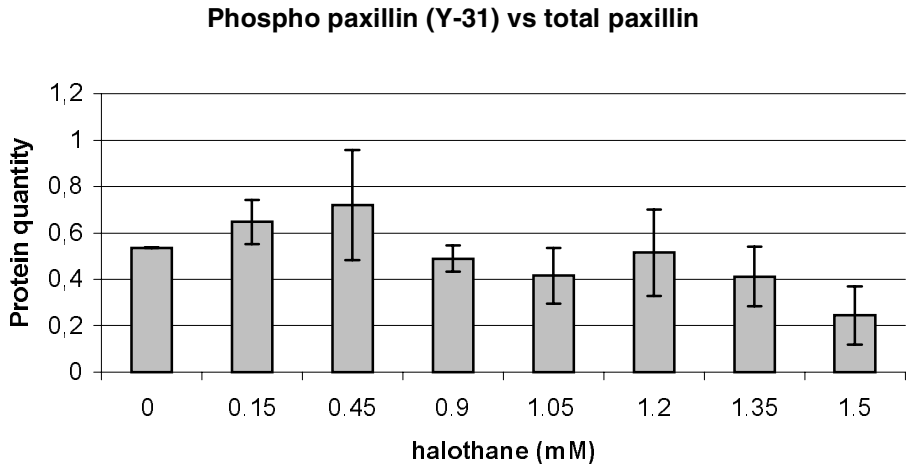

Fig. 3 *Quantification of paxillin phosphorylation in comparison to total paxillin in A 549. Quantity of Tyrosine phosphorylated (Y-31) paxillin (Y-ax) in A 549 treated with halothane at concentration range from 0 to 1.5 mM (X ax). Statisticaly significant inhibition in paxilline phosphorylation versus total paxillin is seen in cells, exposed to 1.5 mM halothane is shown with *** ($p<0.001$). Results were obtained from three indipendent experiments. Standard deviations are shown as Y-bars.*