The ACAT Inhibitor VULM1457 Significantly Reduced Production and Secretion of Adrenomedullin (AM) and Down-Regulated AM Receptors on Human Hepatoblastic Cells

J. Dřímal[1], V. Fáberová[2], L. Schmidtová[2], M. Bednáriková[2], J. Dřímal Jr.[3] and D. Dřímal[4]

[1] *Institute of Experimental Pharmacology, Slovak Academy of Sciences, Bratislava, Slovakia*
[2] *Drug Research Institute Modra, Slovakia*
[3] *Department of Information Technology, Slovak Gas Industry, Bratislava, Slovakia*
[4] *Faculty of Material Science and Technology, Slovak University of Technology, Trnava, Slovakia*

Abstract. Acyl-CoA:cholesterol acyltransferase (ACAT) is an important enzyme in the pathways of cholesterol esterification. It has been shown that new ACAT inhibitor 1-(2,6-diisopropyl-phenyl)-3-[4-(4'-nitrophenylthio)phenyl] urea (VULM-1457) significantly reduced atherogenic activity in animal experimental atherosclerosis. Proliferative hormone adrenomedullin (AM) has been shown to be released in response to hypoxia, however, its role in cellular protection has remained elusive. The effect of increased local production of AM in cells and resultant down-regulation of AM receptors has not been investigated yet. We hypothesized that increased expression of AM in hypoxic cells was the result of excessive AM production with resultant AM receptor down-regulation, surface-membrane protein degradation and that the new specific ACAT inhibitor would reduce AM induction in hypoxia and thus proliferation of cells. In order to investigate specific cellular AM signaling and protection induced by VULM1457, we characterized specific surface-membrane [^{125}I]AM receptors expressed on cells, evaluated AM secretion (RIA assays), AM mRNA expression in cultured cells (RT-PCR analysis) and proliferation (incorporation of [^{3}H]thymidine) in control, hypoxic and metabolically stressed human hepatoblastoma cell lines exposed to gradually increasing concentrations of VULM1457. The new ACAT inhibitor VULM1457 in concentration 0.03 and 0.1 μmol/l significantly down-regulated specific AM receptors on HepG2 cells, reduced AM secretion of HepG2 cells exposed to hypoxia. These results suggest that VULM1457, as new member of ACAT family of inhibitors could negatively regulate cell proliferation induced by AM, which may correlate with down-regulation of

Correspondence to: Ján Dřímal, Institute of Experimental Pharmacology, Slovak Academy of Sciences, Dúbravská cesta 9, 841 04 Bratislava 4, Slovakia
E-mail: exfadrim@savba.sk

membrane-bound AM receptors on HepG2 cells, and moreover, with the induction and expression of AM in hypoxia.

Key words: ACAT inhibitors — Adrenomedullin — Cells in culture — HepG2 cells — RIA assays — VULM1457 — Whole cell binding

Introduction

Antihypoxic and stimulatory peptide adrenomedullin (AM) has been identified in almost all human tissues and it has been suggested that AM may up-regulate AM message, tumor cell growth, angiogenesis, inhibition of other hormone release and of apoptosis survival (Hinson et al. 2000). AM gene expression was found to be triggered by hypoxia and shown to increase markedly in hypoxic human hepatocytes (Sonna et al. 2003). Elevated tissue AM concentrations were reported in patients with myocardial infarction and in congestive heart failure (Kato et al. 1996; Jougasaki et al. 1995). Analysis by reverse transcriptase-polymerase chain reaction was used to evaluate AM receptor mRNA in diverse human neoplastic cell lines (except for human hepatoblasts), (Miller et al. 1996). Evidence revealed that AM mRNA was overexpressed in most of cancer cell lines and AM acted as paracrine/autocrine growth factor in malignant cells (Takahashi et al. 2002). Dominant effect on AM stimulated cells, however, was the marked increase in intracellular cyclic-AMP (Yeung et al. 1996), and this effect was recently attributed mostly to agents inhibiting cell proliferation. AM may play also a role in hepatic cirrhosis leading to the formation of ascites (Kojima et al. 1998). Acyl-CoA:cholesterol acyltransferase (ACAT) is an important cellular protein in the pathways of cholesterol esterification. Pharmacological evidence showed that ACAT inhibitor F-1394 reduced atherosclerosis in apolipoprotein-E deficient mice (Kusuoki et al. 2001). A new specific ACAT inhibitor 1-(2,6-diisopropyl-phenyl)-3-[4-(4'-nitrophenylthio)phenyl] urea (VULM1457) (Fig. 1, Oremus et al. 1999) significantly reduced atherogenic activity in animal experimental model of atherosclerosis (Fáberová et al. 2002). Hypoxia was found to up-regulate AM expression in cells, while the desensitization of AM receptors has not been systematically studied yet. We hypothesized that increased AM expression in hypoxic cells was the result of excessive production of stimulating peptides and increased down-regulation of AM receptors with the

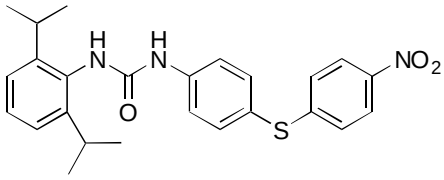

Figure 1. Structural formula of new ACAT inhibitor VULM1457.

degradation of membrane proteins, and that VULM1457 would reduce AM induction, AM mRNA expression and thus proliferation of cells. Human hepatoblastic cells (HepG2) express AM receptors, produce and secrete AM in concentrations that may reach levels required for receptor activation. The surface membrane AM receptors are highly induced by reduced oxygen tension and expression of AM mRNA seems to be transcriptionally regulated (Garayoa et al. 2000), however, the role underlying autocrine/paracrine AM mechanism(s) in tumor cells remains undefined. The production and secretion of AM in hypoxic cells may be adaptive or inflammatory. It is clear that either activation or disruption of AM signaling may significantly contribute to the pathology of hypoxic cells. In this study we used the human hepatocyte cell line (HepG2) because HepG2 cells in culture are highly metabolically active and thus are a very suitable model mimicking surface membrane AM receptor regulations and AM secretion in hypoxia.

Materials and Methods

All experimental procedures with human cells were performed in accordance with the Helsinki Declaration of 1975 and studies that employed human cells in culture were performed in compliance with the Principles of Laboratory Care, formulated by the National Society for Medical Research. The experiments were realized evenly in the Laboratory of Cardiovascular Pharmacology at the Institute of Experimental Pharmacology of the Slovak Academy of Sciences and in the Laboratories of Pharmacology and Microbiology of the Drug Research Institute (Modra, Slovakia) and they were approved by the principles of Institutional Good Laboratory Praxis.

Human cells in culture, whole cell radio-ligand binding and assays have been described in detail previously (Dřímal et al. 2000). Human hepatoma cell line (HepG2) was obtained from the ECACC (Salisbury, UK). HepG2 cells were maintained in Dulbecco's modified Eagle's medium containing 10 % fetal calf serum. The cells were grown as monolayer cultures and maintained in Costar flasks containing 10 ml of a medium supplemented with 10% (v/v) fetal bovine serum non-essential amino acids, 100 units/ml penicillin, 100 ug/ml streptomycin under standard cell culture incubation conditions at 37℃ 20% O_2, 5% CO_2, 75% N_2 for normoxic conditions. To simulate hypoxia, the HepG2 cells were incubated in 1 % O_2, 4 % of carbon dioxide and 95 % N_2 balanced in a sealed box. The whole-cell binding assays were performed in triplicate with cells (5×10^5 cells/well) under polarized conditions (Buffer I, in mmol/l): NaCl 135, $MgCl_2$ 1.0, KH_2PO_4 0.44, NaH_2PO_4 0.34, $NaHCO_3$ 2.6, HEPES 20.0, glucose 5.6, pH 7.4. In equilibrium binding assays aliquots of HepG2 cells (0.8–1.1 mg of protein) were incubated with 10 increasing concentrations of human ([^{125}I]-iodotyrosyl)-AM (from 0.5 to 3.0 nmol/l). Nonspecific binding was routinely determined in the presence of 0.1 μmol/l of AM antagonist (human peptide fragment $AM_{(22\text{-}52)}$). After incubation (60 min), the mixtures were filtered over 25 mm Whatman GF/C glass-fibre filters and the bound radioligand was separated. RIA assay of AM: AM present in the culture media was extracted by using Sep-Pak C_{18} cartridges (Waters, Milford, MA, U.S.A.), eluted

with Buffer II (60 % acetonitrile, HPLC grade, in 1 % trifluoracetic acid) and the extract was reconstituted with RIA assay buffer (Buffer III). The concentrations of AM in the media were measured by commercial competitive AM RIA assay Kit (Peninsula Laboratories Inc., Belmont, CA., U.S.A.) with two overnight incubations. Cell proliferation was assessed by measurement of [^{3}H]-thymidine uptake. Cell viability was assessed by Trypan Blue Dye exclusion test.

The total RNA was extracted from cultured HepG2 cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) and subjected to Northern blot analysis as reported by Takahashi (2000). The probe for human AM mRNA was Hind III/EcoRI cDNA fragment of PBS-human AM-2. The β-actin mRNA expression was used for internal control. Radioactive signals were detected by exposing filters to X-ray films (Kodak). The intensity of hybridization signals was determined by photostimulated luminescence with Bioimage Analyzer. The intensity representing AM mRNA was normalized with the intensity of β-actin band. Protein concentration was determined by the method of Bradford (1976) using bovine serum as standard. Statistical analysis was performed by Student's t-test elaborating experimental data by means of Excel, Inplot and Origin programs. Data are expressed as the mean ± SEM, differences and correlations were considered significant at a value of $p < 0.05$.

Chemicals

$AM_{(1\text{-}52)}$, human angiotensin-II (AT-II; Calbiochem), BAY11-7082 (Calbiochem), human endothelin-1 (ET-1; Sigma), Sep-Pack C_{18} Cartridges (Amprep), tumor necrosis factor-α (TNF-α; Bachem), 13-O-tetrahydrodecanoyl-phorbol-13-acetate (Serva), ([^{125}I]-iodotyrosyl)-AM (Amersham), ([^{125}I]-iodotyrosyl)-AM human-RIA Kit (Peninsula Laboratories Inc.).

Results

We employed cell synchronization in cultured HepG2 cells in order to minimize the risk of side effects related to the variability of indefinite phase of cell cycle (an approach with addition of growth factors that enabled us to manipulate the cell cycle). In the first group of experiments, human $AM_{(1\text{-}52)}$ was added to normoxic subconfluent cultures of HepG2 cells and its effect was measured by determination of cell number/dish (Fig. 2). AM in the concentration 10^{-11} mol/l significantly increased cell numbers after 12- and 24-h incubation periods. After 24-h incubation with the concentration 10^{-9} mol/l of AM, cell numbers almost doubled. In a parallel control group, the preincubation of cells with Buffer I solution containing 0.125 % of dimethyl sulfoxide did not affect the cell count. Proliferative peptides AT-II and ET-1 increased cell growth, while alkaloid vinblastine (10.0 μmol/l) induced apoptosis in cultured HepG2 cells and inhibited the response induced with proliferative peptides (not shown). Kinetic analysis of [^{125}I]AM specific binding was performed in six separate experiments (not shown) with two concentrations close to

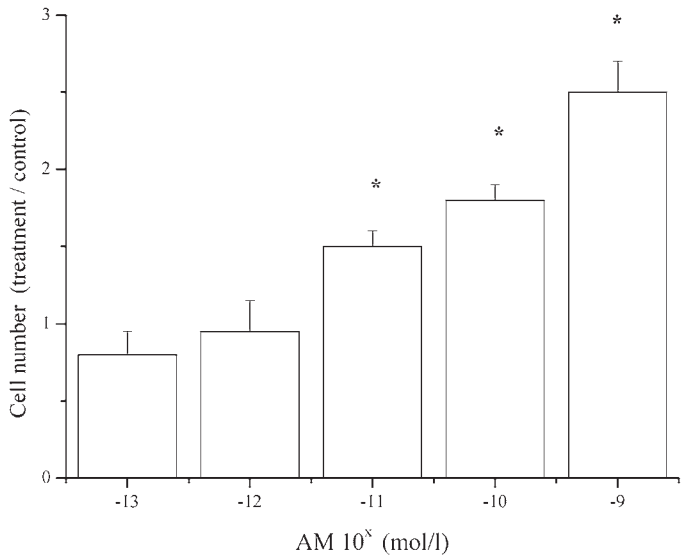

Figure 2. Stimulation of HepG2 cells proliferation by human $AM_{(1\text{-}52)}$. The increase in cell number was measured in one interval (24-h) in Dulbecoo's minimal essential medium without fetal calf serum, supplemented with the indicated concentrations (mol/l) of AM. The pepsidase inhibitors pepstatin-A (5 μg/ml), PMSF (10^{-4} mol/l) and aprotinin (0.4 μmol/l) added to the medium had no effect on the number of cells. Values are the mean $\pm$ SEM; * significant response when compared to control.

the before assigned dissociation constant K_D, (1/100 K_D and 10 $\times$ K_D). The maximum of specific binding was achievable within several minutes. The dissociation curves in kinetic experiments (not shown) were obtained in approximately 40–60 min after addition of nonradioactive AM (50 μmol/l). The dissociation curves were monoexponential with a dissociation constant $k_1 = 0.099 \pm 0.015$ min^{-1}. In preliminary studies accomplished in polarized HepG2 cells, aliquots of HepG2 cells (2.0–2.5 $\times$ 10 ^{5} cells) were incubated with increasing concentrations of [^{125}I]AM for 60 min. The course and characteristics of the saturation curves in our control experiments (Fig. 3) confirmed the presence of one type of binding sites (B_{max}), with the affinity constant K_D in nanomolar range (reasonably low for whole cell binding). The Hill plot (not shown) and Hill coefficient ($n_H = 1.0$) showed the existence of a homogenous group of sites identified on the HepG2 cells.

Specific surface-membrane [^{125}I]AM binding on cultured HepG2 cells

Radiolabeled AM bound in a saturable manner on HepG2 cells with K_D of 0.56 $\pm$ 0.2 nmol/l and at $B_{max} = 115 \pm 12$ fmol/mg of protein (Figs. 3 and 4). Preincubation of HepG2 cells with 0.1 μmol/l of VULM1457 significantly reduced the total number of specific [^{125}I]AM binding identified on cells ($B_{max} = 58 \pm 9$, $p < 0.05$) at untouched affinity. Preincubation of HepG2 cells with high concentrations

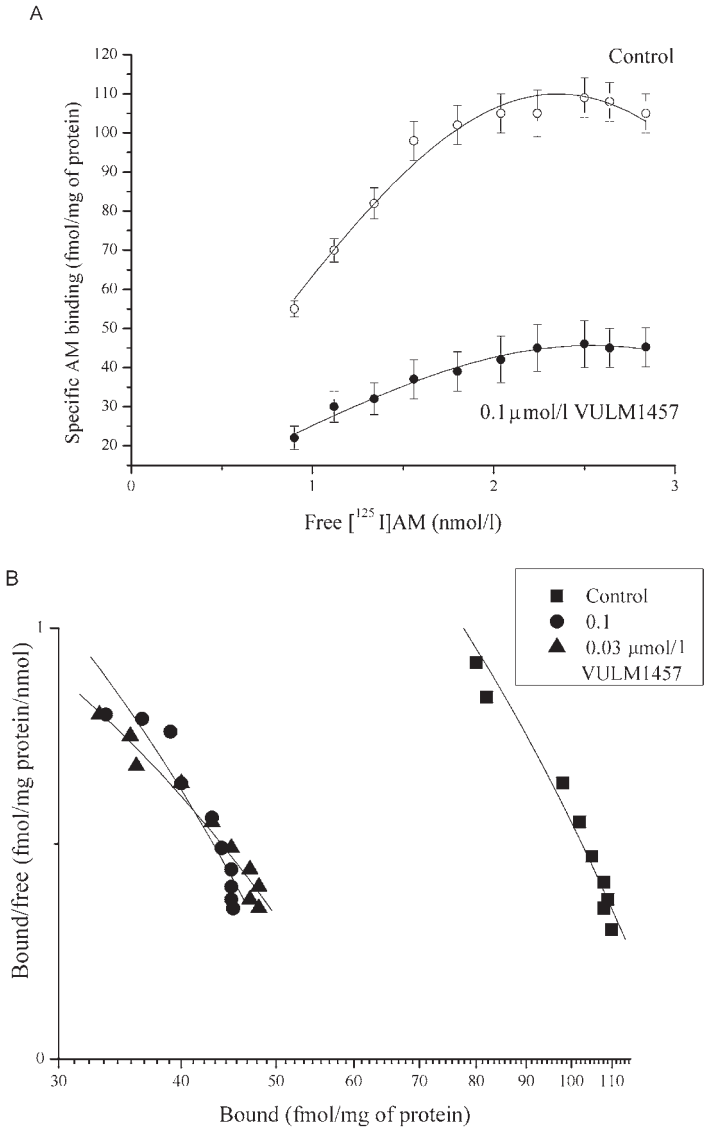

Figure 3. Effect of preincubation with VULM1457 on [^{125}I]AM binding on HepG2 cells. **A.** An example of the whole cell specific saturation binding isotherms of human [^{125}I]AM$_{(1\text{-}52)}$ in native HepG2 normoxic (control) cells and HepG2 preincubated (24 h) with 0.1 μmol/l of VULM1457. Range of free ligand employed was always in low nmolar concentration (0.15–3.0 nmol/l). Incubation was performed in the absence and in the presence of 10 μmol/l of cold human AM$_{(1\text{-}52)}$. Values are the mean ± SEM, $n = 24$, (experiments in triplicate). **B.** Scatchard analysis of [^{125}I]AM binding (bound/free/bound AM) after preincubation of 0 (control), 0.03 and 0.1 μmol/l of VULM1457. Each set represents six experiments performed. Characteristics of the dissociation constant (K_D) and the total number of sites (B_{max}) of [^{125}I]AM binding are presented in text.

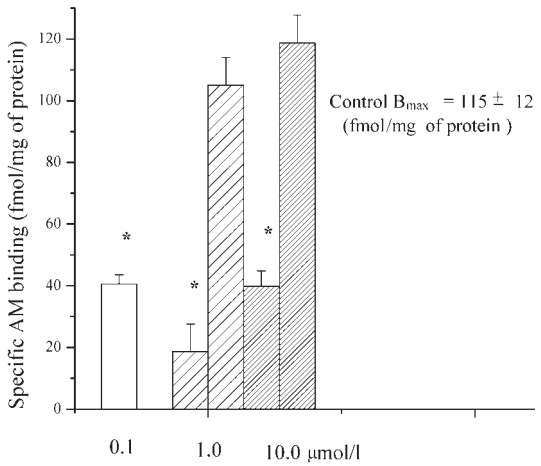

Figure 4. Reduction in the total number of specific [^{125}I]AM$_{(1\text{-}52)}$ binding sites ($B_{\max}$) after preincubation of HepG2 cells with low concentration of VULM1457. (Control $B_{\max}$ = 115 ± 12 fmol/mg of protein) Note: down-regulation of specific high affinity AM binding ($B_{\max}$) after 0.1 μmol of VULM1457 and the dissociation of specific binding sites (to high and low affinity of binding, B_{lmax} and $B_{2\max}$) identified on HepG2 cells after both high concentrations of VULM1457 (1.0 μmol/l (sparse columns) and 10.0 μmol/l (dense columns)). Values are the mean ± SEM (n = 48); * statistical significance compared to control; $p < 0.01$.

of VULM1457 (1.0 and 10.0 μmol/l) significantly modified the characteristics of binding of AM, i.e. AM bound to more group of binding sites, with the proportion of high and low affinity of binding sites in favor of low affinity binding (not shown).

Specific [^{125}I]AM binding on hypoxic cells

Aliquots of HepG2 cells (2.5×10^5 cells, stabilized in serum-free medium) were incubated 24 h in hypoxic conditions. Marked increase in the total number of sites specifically labeled with [^{125}I]AM was observed in hypoxic cells (Fig. 5). Significant increase in B_{maxHypox} = 196 ± 20 fmol/mg of protein, ($p < 0.05$) was achieved at K_{D} values similar to normoxic conditions ($K_{\mathrm{D\,Hypox}}$ = 0.66 ± 0.2 nmol/l). Preincubation of HepG2 cells with 0.1 μmol/l VULM1457 significantly reduced the specific [^{125}I]AM binding on hypoxic cells with B_{maxHypox} being 127 ± 10, ($p < 0.05$) and K_{D} 0.06 ± 0.11 nmol/l. Preincubation of cells with 0.1 μmol/l VULM1457 significantly enhanced the number of cells (24.2 ± 6 %, $p < 0.05$) and higher concentrations of VULM1457 (l.0 and 10.0 μmol/l) reduced the total number of cells (Fig. 6).

We examined also the presence of AM in conditioned medium of HepG2 cells (Fig. 7). We demonstrated the effect of hypoxia in significant increasing of the total number of AM receptors identified on HepG2 cells and significant down-regulating the AM message in HepG2 preincubated with the lowest concentration of AM

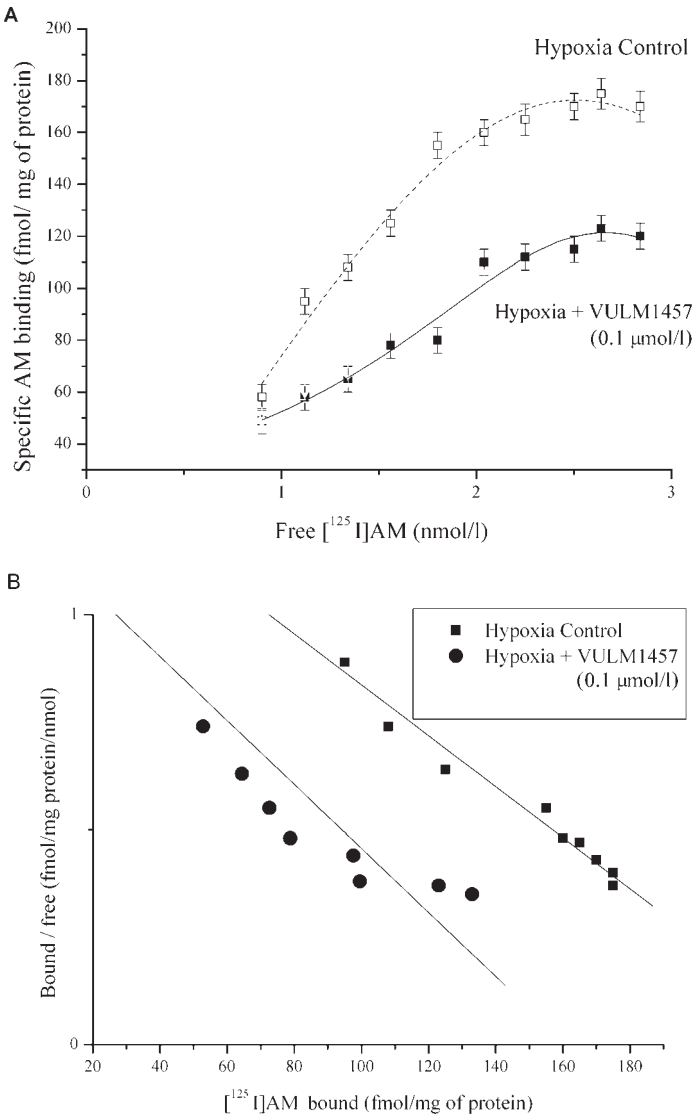

Figure 5. A. Specific whole cell saturation isoterms of human $[^{125}I]AM_{(1\text{-}52)}$ binding in cultured HepG2 cells exposed to hypoxia (24 h, 1% O_2, 4% CO_2 and 95% N_2) and preincubated with concentration 0.1 μmol/l of VULM1457. B. Scatchard analysis of $[^{125}I]$AM binding after preincubation of hypoxic HepG2 cells with 0.1 μmol/l of VULM1457. Each set represents six experiments performed

(Fig. 5). With the high concentrations of VULM1457 (1.0 and 10.0 μmol/l), the reductions in $[^{125}I]$AM specific binding on HepG2 cells was markedly attenuated

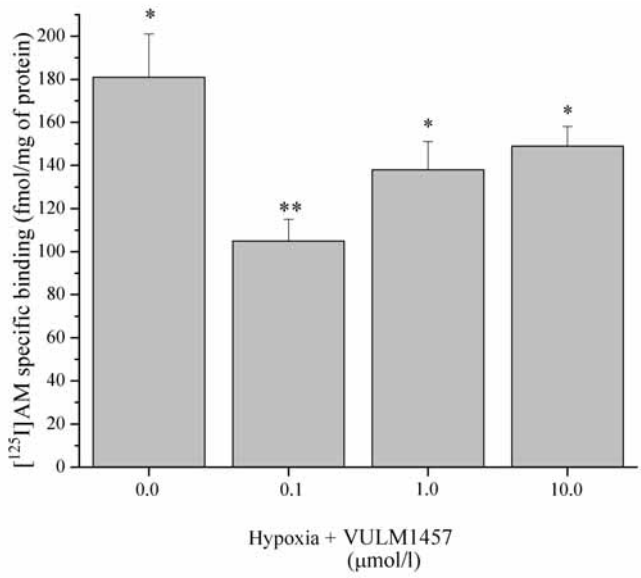

Figure 6. Total numbers of specific [^{125}I]AM binding sites identified on HepG2 cells exposed to hypoxia (24 h, 1 % O_2, 4 % CO_2 and 95 % N_2) and preincubated with VULM1457. Values are mean ± SEM ($n = 16$); * response after preincubation with high concentrations of VULM1457 ($p < 0.05$).

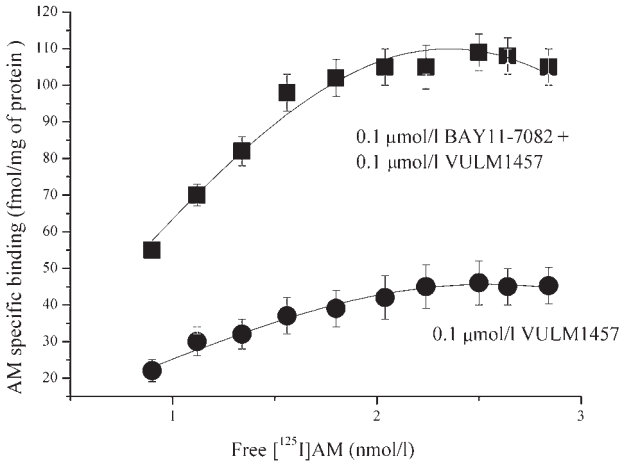

Figure 7. Equilibrium binding data for human [^{125}I]AM specific binding on HepG2 cells preincubated (24 h) with 0.1 μmol/l of VULM1457 and the effect of BAY11-7082. Saturation isotherm of human [^{125}I]AM specific binding in the presence 0.11 μmol/l of BAY11-7082 (squares) significantly attenuated the reduction in total number of AM receptors induced by VULM14547 (circles). $n = 12$, $p < 0.05$.

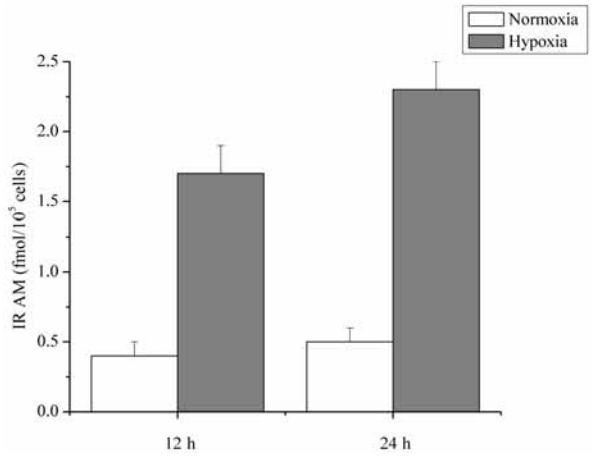

Figure 8. RIA assays of immunoreactive (IR) AM in the conditioned media of HepG2 cells cultured 24 h under normoxia or 1% O_2 atmosphere (hypoxia).

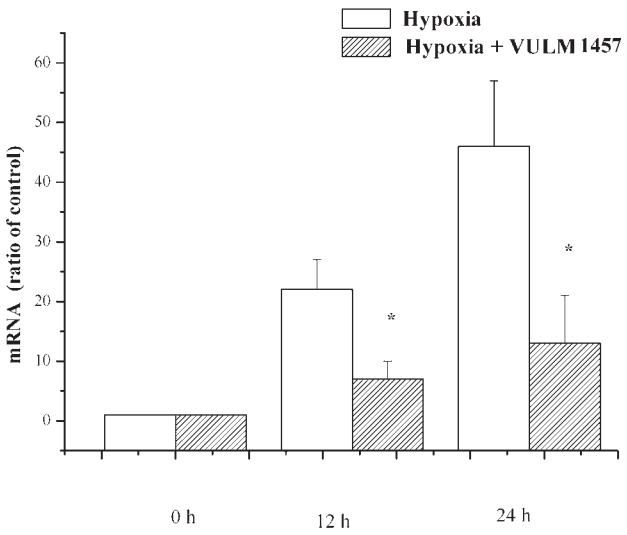

Figure 9. Time course of AM mRNA expression in HepG2 cells exposed to hypoxia for the period indicated (0 – control; 12 and 24 h) and cells permanently preincubated with 0.1 μmol/l of ACAT inhibitor VULM1457. The relative AM mRNA levels were obtained from 5 separate experiments. * statistically significant response when compared to control, $p < 0.01$.

(Fig. 6). BAY11-7082, selective and irreversible inhibitor of I_κ B phosphorylation, and TNFα-induced surface expression of the cell adhesion molecules significantly

attenuated reduction in B_{max} of [^{125}I]AM induced by VULM1457 (Fig. 7). The production of AM (level of AM in hypoxic medium) with hypoxic HepG2 cells was 3–4-fold elevated in the present study (Fig. 8). However the AM mRNA levels in HepG2 cells exposed to 12- and 24-h hypoxia were increased from 6.7- to 15-fold and preincubation of HepG2 cells with 0.1 μmol/l VULM1457 significantly reduced AM mRNA in hypoxic HepG2 cells from 44 to 50 % (Fig. 9). The expression of AM mRNA was low in control samples and high in hypoxic HepG2 cells.

Discussion

The first major finding of this study was the demonstration that preincubation of the ACAT inhibitor VULM1457 in low μmolar concentration significantly down-regulated the total number of specific [^{125}I]AM binding sites identified on HepG2 cells, reduced AM secretion into media of HepG2 cells in culture and down-regulated [^{125}I]AM receptors on HepG2 cells exposed to hypoxia. The second finding was that the ACAT inhibitor VULM1457 in low μmolar concentration modified the time course and significantly reduced AM mRNA expression (from 44 to 50 %) in hypoxic human hepatoblastic cells without reoxygenation. Recent study with the GeneChip micro-array technique (Sonna et al. 2003) showed more than 40 other genes were upregulated by the effect of hypoxia in human hepatocellular carcinoma cell line (most in the range from 2- to 15-fold). Our finding of increased AM mRNA in hypoxic HepG2 cells (4.7 $\pm$ 0.8-fold) is also within the frame of these observations. Some of the heat shock proteins in cells were found to be markedly activated by hypoxia-inducing factor-1 (Semenza 1999). Physical fragility of the plasma membrane due to insertion of lipids, namely oxidized lipids (ox-LDL) may lead to cell membrane lysis. The reduced esterification of cellular cholesterol with fatty acids to form cholesterol esters after VULM1457 may alter cholesterol levels in cells and thus membrane-physical properties, and membrane-vesicle cycling, cell-membrane receptor down-regulation which may in turn affect cell at very specific points in the cell function. Indirect evidence for such speculation can be found in studies also on hepatoma cells (Quintart et al. 1979). Since lipid-mediated toxicity to certain tumor cells is dependent on the proliferation of target cell (Keller et al. 2005), it is possible that the protection induced by ACAT inhibitor VULM1457 and observed in this study is dependent on the proliferation of HepG2 cells and on the inhibition of microsomal cholesterol. So the reduction in the cholesterol esterification may explain why new ateroprotective ACAT inhibitorVULM1457 positively modified function of hypoxic human hepatoblastic cells. It has been found that antitumor alkaloid vinblastine induced apoptosis in cultured hepatocytes and human lymphoma cells (Tsukidate et al. 1993). Inductor of apoptosis vinblastine in the present study inhibited proliferation of cells induced by proliferative peptides AM, ET-1 and AT-II. Peptides AM and ET-1 and possibly also AM and AT-II appear to act as physiological antagonists. AM inhibits the synthesis and release of ET-1 (Kohno et al. 1996). Conversely, ET-1 appears to augment the production of AM in

cells (Sugo et al. 1995). As far as we are aware, the present study is the first addressing the induction of AM mRNA in human hepatoblastic cells. In the present study increase in mRNA was 4.2 ± 0.5-fold in normoxic HepG2 cells and slightly more (4.7 ± 0.8-fold) in hypoxic cells. A steeper induction of AM mRNA over 24 h exposure to 1% O_2 and more dramatic increases between the basal and maximal induction of AM mRNA (more than 25-fold) were reported in human tumorigenic MCF7 cells (Garayoa et al. 2000). The fact that potential anti-inflammatory agent, selective and irreversible inhibitor of TNFα-inducible phosphorylation, an agent that freely penetrate the cellular membrane, and selectively and irreversibly inhibits the TNFα inducible phosphorylation of I_κ B, and furthermore it inhibits also cellular adhesion proteins, resulting in decreased expression of NF_κ B in cells (Pierce et al. 1997). In the present study, BAY11-7082 significantly attenuated down-regulation of AM receptors induced by VULM1457 and this may indicate the participation of nuclear translocation in AM cellular signaling. We have demonstrated that with the exception of induction produced by AM and increased production of AM in hypoxic HepG2 cells, human AM receptor undergoes down-regulation after prolonged exposure to low μmolar concentrations of ACAT inhibitor VULM1457. The intensity of down-regulation of surface-membrane AM receptors in normoxic hepatoblastic cells is higher in normoxic (down-regulation to an approximately 50%) than in hypoxic cells (to 64.8%). We conclude that increased AM induction, increased production of AM in hypoxic cells and resultant down-regulation of surface-membrane AM receptors and reduced expression of AM mRNA after preincubation withlow μmolar concentration of VULM1457, observed in the present study, confirmed the protective effects of new ACAT inhibitor VULM1457.

Acknowledgements. We are grateful to Andrea Hyrava of Institute of Experimental Pharmacology, Slovak Academy of Sciences, for expert technical assistance. This study was supported in part by Ministry of Education of the Slovakia, grants VEGA No. 4025, 2/5007/25 and by Grant-in-Aid for Scientific Research APVT No. 20-20802.

References

Bradford M. M. (1976): A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248—253

Dřímal J., Dřímal J. Jr., Dřímal D. (2000): Enhanced endothelin ET_B receptor down-regulation in human tumor cells. Eur. J. Pharmacol. **396,** 19—22

Fáberová V., Kakalík I., Juranová D., Schmidtová L., Sadloňová I., Žigová J., Zemánek M., Bezek S. (2002): VULM1457 – a novel hypocholesterolemic and antiatherosclerotic agent. Bratisl. Lek. Listy **103,** 312—315

Garayoa M., Martinez A., Lee S., Pio R., An W. G., Neckers L., Trepel J., Montuenga L. M., Ryan H., Johnson R., Gassmann M., Cuttitta F. (2000): Hypoxia-inducible factor-1 (HIF-1) up-regulates adrenomedullin expression in human tumor cell lines during oxygen deprivation: a possible promotion mechanism of carcinogenesis. Mol. Endocrinol. **14,** 848—862

Hinson J. P., Kaspas S., Smith D. M. (2000): Adrenomedullin, a multifunctional regulatory peptide. Endocr. Rev. **21,** 138—167

Jougasaki M., Wei C. M., McKinley L. J., Burnett J. C. Jr. (1995): Elevation of circulating and ventricular adrenomedullin in human congestive heart failure. Circulation **92,** 286—289

Kato J., Kobayashi K., Etoh T., Tanaka M., Kitamura K., Imamura T., Koiwaya Y., Kanagawa K., Eto T. (1996): Plasma adrenomedullin concentration in patients with heart failure. J. Clin. Endocrinol. Metab. **81,** 180—183

Keller J. N., Schmitt F. A., Scheff S. W., Ding Q., Chen Q., Butterfield D. A., Markesbery W. R. (2005): Evidence of increased oxidative damage in subjects with mild cognitive impairment. Neurology **60,** 1152—1156

Kohno M., Yasunari K., Yokokawa T., Horio M., Ikeda H., Kano M., Minami T., Hanehira T., Yoskikawa J. (1996): Interaction of adrenomedullin and platelet-derived growth factor on rat mesangial cell production of entothelin. Hypertension **27,** 1309—1313

Kojima H., Tsujimoto T., Uemura M., Takaya A., Okamoto S., Ueda S., Nishio K., Miyamoto S., Kubo A., Minamino N., Kangawa K., Matsuo H., Fukui H. (1998): Significance of increased plasma adrenomedullin concentration in pacients with cirrhosis. J. Hepatol. **28,** 840—846

Kusuoki J., Hansoty D. K., Aragane K., Fallon J. T., Badimon J. J., Fisher E. A. (2001): Acyl-CoA:cholesterol acyltransferase inhibition reduces atherosclerosis in apolipoprotein E-deficient mice. Circulation **103,** 2604—2609

Miller J. M., Martinez A., Unsworth E. J., Thiele C. J., Moody T. W., Elsasser T., Cuttitta F. (1996): Adrenomedullin expression in human tumor cell lines. J. Biol. Chem. **271**, 23345—23356

Oremus V., Šmahovský V., Fáberová V., Kakalík I., Schmidtová L., Zemánek M. (1999): 1,3-disubstituted ureas as ACAT inhibitors and method preparing thereof. WO – 9932437, Ca., U.S.A. Pat. **131,** 73411c

Pierce J. W., Schoenleber R., Jesmok G., Brest J., Moore S. A., Collins T., Gerritsen M. E. (1997): Novel inhibitors of cytokine-induced IκB phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects *in vivo.* Biol. Chem. **272,** 21103—21109

Quintart J., Leroy-Houyet M., Trouet A., Baudhuin P. J. (1979): Endocytosis and chloroquine accumulation during the cell cycle of hepatoma cells in culture. Cel. Biol. **82,** 644—653

Semenza G. L. (1999): Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1. Annu. Rev. Cell Dev. Biol. **15,** 551—578

Sonna L. A., Cullivan M. L., Sheldon H. K., Pratt R. E., Lilly C. M. (2003): Effects of hypoxia on gene expression by human hepatocytes (HepG2). Physiol. Genomics **12,** 195-207

Sugo N., Minamino H., Shoji K., Kanagawa K., Matsuo H. (1995): Effects of vasoactive substances and cAMP related compounds on adrenomedullin production in cultured vascular smooth muscle cells. FEBS Lett. **369,** 311—314

Takahashi K., Nakayama M., Totsune K., Murakami O., Sone M., Kitamuro T., Yoshinoya A., Shibahara S. (2000): Increased secretion of adrenomedullin from cultured human astrocytes by cytokines. J. Neurochem. **74,** 99—107

Tsukidate K. (1993): Microtubule antagonist activate programmed cell death in cultured rat hepatocyte. Am. J. Pathol. **143,** 918—925

Yeung V. T., Ho S. K., Nicholls M. G., Cockram C. S. (1996): Adrenomedullin, a novel vasoactive hormone, binds to mouse astrocytes and stimulates cyclic AMP production. J. Neurosci. Res. **46,** 330—335

Final version accepted: September 30, 2005