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Non-dioxin-like polychlorinated biphenyls induce a release of arachidonic acid in liver epithelial cells: A partial role of cytosolic phospholipase A₂ and extracellular signal-regulated kinases 1/2 signalling

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Abstract

Non-dioxin-like polychlorinated biphenyls (NDL-PCBs) have been shown to act as tumor promoters in liver; however, the exact mechanisms of their action are still only partially understood. One of the interesting effects of NDL-PCBs is the acute inhibition of gap junctional intercellular communication (GJIC), an effect, which has been often found to be associated with tumor promotion. As previous studies have suggested that NDL-PCB-induced disruption of lipid signalling pathways might correspond with GJIC inhibition, we investigated effects of PCBs on the release of arachidonic acid (AA) in the rat liver epithelial WB-F344 cell line, a well-established model of liver progenitor cells. We found that both 2,2',4,4'-tetrachlorobiphenyl (PCB 47) and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153), but not the dioxin-like, non-*ortho*-substituted, 3,3',4,4',5-pentachlorobiphenyl (PCB 126), induce a massive release of AA. The AA release, induced by PCB 153, was partially inhibited by extracellular signal-regulated kinases 1/2 (ERK1/2) signalling inhibitor, U0126, and by cytosolic phospholipase A₂ (cPLA₂) inhibitor, AACOCF₃. Although PCB 153 induced both ERK1/2 and p38 activation, the specific p38 kinase inhibitor, SB203580, had no effect on AA release. Inhibitors of other phospholipases, including phosphatidylcholine-specific phospholipase C or phosphatidylinositol-specific phospholipase C, were also without effect. Taken together, our findings suggest that the AA release, induced by non-dioxin-like PCBs in liver progenitor cell line, is partially mediated by cytosolic PLA₂ and regulated by ERK1/2 kinases. Our results suggest that more attention should be paid to cell signalling pathways regulated by AA or eicosanoids after PCB exposure, which might be involved in their toxic effects.

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Keywords

polychlorinated biphenyls; aryl hydrocarbon receptor; genotoxicity; gap junctional intercellular communication; contact inhibition; rat liver epithelial cells

1. Introduction

Arachidonic acid (AA) is an important precursor of bioactive lipid mediators, eicosanoids, which are formed upon the release of AA from membrane phospholipids (Larsson et al. 2004). However, AA itself, as well as some other polyunsaturated fatty acids, might also serve as second messenger, which is known to regulate a number of cellular functions (Brash 2001). The mechanisms controlling AA release have been previously shown to be targeted by numerous environmental pollutants, including polycyclic aromatic hydrocarbons (PAHs) or polychlorinated biphenyls (PCBs) (Brant and Caruso 2006; Forsell et al. 2005; Kodavanti and Derr-Yellin 2002; Olivero and Ganey 2000; Tithof et al. 2002; Tithof et al. 1998).

PCBs are a group of persistent environmental pollutants, widely distributed as complex mixtures. Non-*ortho*-substituted coplanar PCBs, sometimes termed as dioxin-like PCBs, have been shown to elicit a set of adverse effects associated with the activation of the aryl hydrocarbon receptor (AhR), resulting in liver damage, thymus atrophy, skin lesions, a wasting syndrome, and tumor promotion (Robertson and Hansen 2001; Safe 1994). The *ortho*-substituted PCBs, which tend to form noncoplanar structures of the biphenyl molecule that does not significantly activate AhR, exhibit a different spectrum of toxic modes of action, which have been also linked to neurotoxicity, immunotoxicity, endocrine disruption or tumor promotion (Glauert et al. 2001; Hansen 1998; Robertson and Hansen 2001; Pessah et al., 2006). Interestingly, these non-dioxin-like PCBs (NDL-PCBs), or their metabolites, have been found to inhibit gap-junctional intercellular communication (GJIC) and to activate extracellular-signal-regulated kinases 1/2 (ERK1/2) in rat liver epithelial WB-F344 cells, a well-established model of liver progenitor cells (Hemming et al., 1992; Machala et al. 2004; Machala et al. 2003; Shafritz and Dabeva 2002).

Different phospholipases have been found to be targeted by PCBs or PAHs, thus suggesting that induction of AA release is both toxicant- and cell type-specific (Brant and Caruso 2006; Forsell et al. 2005; Tithof et al. 2002; Tithof et al. 1998). Both PAHs and PCBs have been suggested to perturb lipid signalling pathways in rat liver WB-F344 concomitantly with inhibition of GJIC (Machala et al., 2003; Upham et al. 2008). Hii and co-workers demonstrated that AA transiently inhibits GJIC and activates ERK1/2 in WB-F344 cells (Hii et al. 1995a; Hii et al. 1995b). However, nothing is currently known about the impact of PCBs on AA release (especially the role of individual phospholipases), or the relationship between the PCB-induced AA release and activation of mitogen-activated protein kinases (MAPKs), including both ERK1/2 and p38 kinases, in liver progenitor cells. A sustained activation of phospholipases and MAPKs might modulate cell proliferation or apoptosis (Choudhury et al. 2000; Mitchell et al. 1999; Tithof et al. 2002). Moreover, as activated MAPKs are known to regulate activity of phospholipases (Evans et al. 2002; Gijon et al. 2000; Gudmundsdottir et al. 2001; Hayama et al. 2002; Choudhury et al. 2000; Olivero and Ganey 2000; Upmacis et al. 2004; Xu et al. 2003), it is important to understand the relationship between effects of PCBs on AA release and other signalling pathways, as well as its possible impact on cellular growth and survival. It should be, nevertheless, also kept in mind that the effects of PCBs might differ in normal and transformed cells; effects of PCBs on non-transformed cells might provide information about their possible tumor-promoting effects.

In the present study, we investigated the effects of model dioxin-like and NDL-PCBs on AA release in the WB-F344 cell line, an in vitro model of rat liver progenitor cells. As only NDL-PCBs were found to induce a marked AA release in this cellular model, we, then, analyzed the role of MAPK activation and individual phospholipases in the observed release of AA, as well as the direct impact of AA on survival and proliferation of WB-F344 cells.

2. Materials and Methods

2.1. Chemicals

2,2',4,4'-Tetrachlorobiphenyl (PCB 47), 3,3',4,4',5-pentachlorobiphenyl (PCB 126) and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153) were purchased from Promochem (Wesel, Germany). Stock solutions were prepared in dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) and stored in the dark. Rabbit polyclonal antibodies directed against p44/42 MAP Kinase #9102, phosphorylated p44/42 MAP Kinase (Thr202/Tyr204) #9101, p38 MAP Kinase #9212 and phosphorylated p38 MAP Kinase (Thr180/Tyr182) #9211 were provided by Cell Signalling Technology (Beverly, MA, USA). Horseradish peroxidase-conjugated anti-mouse IgG (NA931) and anti-rabbit IgG (NA934), polyvinylidene difluoride (PVDF) membrane Hybond-P, and chemiluminescence detection reagents (ECL+Plus) were obtained from GE Healthcare (Little Chalfont, UK). Chemical inhibitors U0126, SB203,580, D609, U73122 and MAFP were from Calbiochem (San Diego, CA, USA) and BEL (bromoethanol lactone) was from Biomol (Hamburg, Germany). AA, butylated hydroxytoluene (BHT), AACOCF₃ and 2-propanol (HPLC grade) were purchased from Sigma-Aldrich (Sigma-Aldrich, Prague, Czech Republic) 1-pyrenyldiazomethane (PDAM) was obtained from Molecular Probes (Molecular Probes Inc, Eugene, OR, USA). Acetonitrile gradient grade for HPLC and n-hexane p.a. ACS were purchased from Merck (Merck, Ricany-Jazlovice, Czech Republic). Ultrapure water was obtained from a Milli-Q UF Plus water system (Millipore, Molsheim, France). All other chemicals were provided by Sigma-Aldrich.

2.2. Cells and treatment

Non-tumorigenic, diploid rat liver epithelial cells, WB-F344, were grown in modified Eagle's Minimum Essential Medium with 50 % increased concentrations of essential and nonessential amino acids, and supplemented with 1 mM sodium pyruvate, 10 mM HEPES, 5 % heat-inactivated fetal bovine serum (FBS) and gentamycin (50 mg/l). All tissue culture reagents were from Sigma-Aldrich. Cells were incubated in a humidified atmosphere of 5 % CO₂ at 37° C, and they were routinely maintained in 75 cm² flasks and subcultured twice a week. For detection of effects of PCBs on MAPK activation and AA release, cells were treated with PCBs for time intervals indicated in legends to Figures. The specific treatments with inhibitors are indicated below. Inhibition of MEK1/2 and p38 MAPK was performed by a 30-minute preincubation with 10 µM U0126 and 10 µM SB203580, respectively. Methyl arachidonyl fluorophosphonate (MAFP) and arachidonyl trifluoromethylketone (AACOCF₃), inhibitors of group IV4A-PLA₂ and group VIA-PLA₂, were used in 10 µM concentration. 2.5 µM BEL, a specific inhibitor of VI-PLA₂, 2.5 µM U73122, inhibitor of phosphatidylinositol-specific PLC (PI-PLC) and 40 µM xanthogenate tricyclodecan-9-yl (D609), which blocks phosphatidylcholine-specific PLC (PC-PLC) and sphingomyelinase activities, were used to selectively inhibit other major phospholipases. Inhibitor concentrations were selected based on our previous experiments with rat liver oval cells (Machala et al. 2003).

2.3. Determination of AA release

Confluent WB-F344 cells were washed three times with PBS and incubated with test compounds diluted in phenol red-free, serum-free RPMI-1640 medium for time period indicated in legends to Figures. Following the incubation, medium was removed and centrifuged at 800 g for 10 min in polypropylene 15 ml tubes (TPP, Trasadingen, Switzerland).

The supernatant was carefully removed and transferred into fresh 15-ml tubes and stored at -80°C until further analysis. A sensitive RP-HPLC method with fluorimetric detection was used for the determination of AA in cell culture medium. The method is based on liquid-liquid extraction of the analyte (Hara and Radin 1978) and its pre-column derivatization with 1-pyrenyldiazomethane (PDAM) (Brekke et al. 1997; Nimura et al. 1988). Liquid-liquid extraction was used to extract arachidonic acid from culture medium. Medium (2.8–3.0 ml) was acidified with acetic acid (12 μl), extracted twice with 1.3 ml of the mixture of hexane/isopropanol (5/1) and once with 2.0 ml of hexane. Combined organic layers were dried under a stream of nitrogen. Sample was redissolved in 100 μl of methanol with BHT (1 mg/ml) and derivatized with PDAM according to (Nimura et al. 1988). After 90 min of derivatization, the mixture of solvents was dried under a stream of nitrogen. The sample was redissolved in 100 μl of acetonitrile and aliquot of 5 μl was injected into the HPLC column. The HPLC system consisted of Waters 717plus autosampler, Waters 600E multisolvent delivery system and Waters 474 scanning fluorescence detector (Waters, Milford, MA, USA). A Nova-Pak 150 $\times$ 3.9 mm column with particle diameter 4 μm (Waters) was used. A gradient of water and acetonitrile was applied for elution of AA: 0–50 min. 75–100% of acetonitrile. The flow rate of the mobile phase was 1.0 ml/min, the column temperature was set at 40°C . The analyte was detected at $\lambda_{\text{ex}}=340\text{ nm}$ and $\lambda_{\text{em}}=395\text{ nm}$.

2.4. Western blotting

Confluent WB-F344 cells, grown in 6-well cell culture plates (TPP), were exposed to studied compounds or to 0.1 % DMSO (vehicle) for indicated time periods. Epidermal growth factor (EGF, Sigma-Aldrich) and anisomycin (Sigma-Aldrich) were used as a positive control for induction of ERK1/2 and p38 phosphorylation, respectively. The cultivation medium was replaced with a fresh medium without serum 24 h before exposition. Cell lysates were prepared using SDS sample buffer (1 % SDS, 10 % glycerol, 100 mM Tris, pH 7.4). Protein concentration was estimated using Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Inc.; Hercules, CA, USA). For Western blotting, equal amounts of total protein were subjected to 10% SDS PAGE, electrotransferred onto Hybond-P membrane, immunodetected using appropriate primary and secondary antibodies, and visualized by ECL+Plus reagent according to manufacturer's instructions. After immunodetection, each membrane was stained with amidoblack to confirm equal protein loading.

2.5. Determination of cell numbers and apoptosis

Cell proliferation assessment was performed as described previously (Umannová et al. 2007). Cells were incubated with indicated concentrations of free or BSA-bound AA for 24–72 h. Following the exposure, medium was removed, cells were harvested by trypsinization and counted with a Coulter Counter (Model ZM, Coulter Electronics, Luton, UK). Apoptosis induced by tested compounds was assessed using morphological criteria (nuclear fragmentation). Cells (including floating cells) were harvested and fixed in 70 % ethanol, as described above. The fixed cells were centrifuged (200g, 5 min) and resuspended in 200 μl of supernatant. Aliquot of the suspension was mixed with Mowiol-DAPI solution (10% Mowiol solution was prepared in 25 % glycerol, 100 mM Tris-HCl, pH 8.5 and DAPI 1 $\mu\text{g}/\text{ml}$) and mounted for observation with a fluorescence microscope Olympus IX70. A minimum of 200 cells was counted per sample.

2.6. Statistical analysis

The data were expressed as means $\pm$ S.D. and analyzed by ANOVA followed by Tukey range test. When the variances were not homogenous, nonparametric Mann-Whitney U-test was used. A *P* value of less than 0.05 was considered significant.

3. Results and Discussion

A number of studies have suggested that liver progenitor cells, or oval cells, could participate in liver carcinogenesis (Dumble et al. 2002; Knight et al. 2000; Roskams 2006). Both dioxin-like PCB congeners and NDL-PCBs have been proposed to act as tumor promoters in liver tissue (Glauert et al. 2001). However, while in the former case, the tumor-promoting activity of PCBs is attributed to activation of AhR, far less is known about the mechanisms underlying tumor promoting effects of NDL-PCBs. It has been previously shown that NDL-PCBs are efficient GJIC inhibitors in a well-defined model of liver progenitor cells (Machala et al. 2003; Shafritz and Dabeva 2002). Similar results were observed in other liver-derived cell model systems in which NDL-PCBs, and not the dioxin-like PCBs, inhibited GJIC (Swierenga et al. 1990; Hemming et al. 1992). Decreased GJIC and expression of connexins was also observed in liver slices obtained from rats treated with PCBs, thus indicating that inhibition of GJIC by PCBs is relevant to in vivo systems (Krutovskikh et al. 1995).

Our studies have suggested that the inhibition of GJIC might be associated with disruption of lipid signalling pathways in WB-F344 cells (Machala et al. 2003), and more recently, we have also found that methylated PAHs inhibiting GJIC induce a rapid release of AA in the same cell line, suggesting a possible link between those events (Upham et al. 2008). As other studies suggested that PCB mixtures, such as Aroclor, might also induce AA release in various cell types (Olivero and Ganey 2000; Tithof et al. 1998), we first investigated the effects of selected di-*ortho* (PCB 47, PCB 153) and non-*ortho* (PCB 126) congeners on AA release. In order to avoid pre-treatment of cells with the radioactively-labelled AA, we employed a sensitive, non-radioactive, HPLC-based method for this analysis. As shown in Figure 1, both NDL-PCBs induced a release of AA in a time dependent manner. The initial limited release of AA after 30 min was followed by a massive release of AA observed after 3 h of incubation. This is in a sharp contrast with the effects of PAHs, which induce a significant AA release within a few minutes (Upham et al. 2008). The di-*ortho* PCB 153 induced AA release in a dose-dependent manner, while dioxin-like PCB 126 did not change AA levels in medium up to 3 h incubation and up to 50 μ M concentration. Several NDL-PCBs have been recently shown to induce AA release in human platelets (Forsell et al. 2005). This, together with the present results, suggest that this type of activity, like the acute inhibition of GJIC, might be unique for NDL-PCBs.

In our previous study, an inhibitor of PC-PLC and sphingomyelinase, D609, has been found to prevent GJIC inhibition by PCB 153 (Machala et al. 2003). However, other phospholipases, namely some PLA₂s have been implicated in the PCB- or PAH-induced AA release (Forsell et al. 2005; Tithof et al. 2002; Tithof et al. 1998). Therefore, we next tested the effects of a series of phospholipase inhibitors on the release of AA in response to PCB 153. We found that only AACOCF₃, which is a specific inhibitor of cytosolic PLA₂, partially inhibited AA release at 3 h (Figure 2). Similar to PAHs (Upham et al. 2008), PC-PLC does not seem to be involved in the observed effects of di-*ortho* PCBs on AA release in WB-F344 cells (Figure 2).

cPLA₂ has been shown to be regulated by activated MAPK in response to various treatments (Evans et al. 2002; Gijon et al. 2000; Gudmundsdottir et al. 2001; Hayama et al. 2002; Choudhury et al. 2000; Upmacis et al. 2004). We have previously observed that di-*ortho* PCBs induce ERK1/2 activation in WB-F344 cells, which is, nevertheless, not associated with gap junction closure (Machala et al. 2003). As shown in Figure 3A, PCB 153 induced both p38 and ERK1/2 activation in WB-F344 cells, prior to the observed massive release of AA, thus suggesting that these kinases might modulate cPLA₂ activity. Therefore, we next examined the effects of specific inhibitors of p38 (SB203580) and ERK1/2 (U0126) activation on the PCB 153-induced release of AA. As shown in Figure 3B, U0126 partially inhibited AA release in a manner similar to AACOCF₃. Therefore, we also combined the two inhibitors, in order to see if their effects are additive, which would indicate independent effects. However, the

combination of both inhibitors yielded similar results, as either compound alone (data not shown), thus suggesting that both ERK1/2 and cPLA₂ might act within the same signalling pathway. Our results are similar to the reported involvement ERK1/2 in Aroclor 1242 mixture effects in human neutrophils (Olivero and Ganey 2000), thus suggesting that activation of MAPKs could be a common step being partially involved in the effects of PCBs on AA release. However, a different kinetics of AA release in neutrophils and WB-F344 cells seem to suggest that different phospholipases might be activated by PCBs in a cell-specific manner.

As AA is a bioactive molecule modulating cell survival and proliferation (Brash 2001), we also examined effects of free AA or BSA-bound AA on apoptosis and proliferation of WB-F344 cells. We found that AA had no effect either on cell numbers or on percentage of cells entering apoptosis (Table 1). Nevertheless, given the magnitude of AA release, more attention should be paid to effects of AA and AA-derived compounds on intracellular signalling. Eicosanoids (especially prostaglandins) play numerous roles in regulation of liver physiology and pathophysiology, including initiation of liver regeneration or liver tumor development (Casado et al. 2001; Hu 2003; Leng et al. 2003; Rudnick et al. 2001; Sirica et al. 2001; Wu et al. 2002). The model cell line used in the present study, WB-F344 cells, can be differentiated both into biliary epithelial cells and into hepatocytes, and transformed WB-F344 cells have been shown to give rise to liver cancer (Presnell et al. 1998). There are currently only limited data available about formation of prostaglandins in WB-F344 cells. While non-transformed WB-F344 cells may produce only a limited amount of prostaglandin E₂ (PGE₂) (Lee et al. 2006), expression of cyclooxygenase-2 (COX-2) rises sharply in transformed WB-F344 cell variants, as well as the PGE₂ production (Sirica et al. 2001; Lee et al. 2006). Activation of cPLA₂ might thus provide increased amounts of AA as a substrate for COX-2. Indeed, activation of the cPLA₂/COX-2/PGE₂ signalling has been shown to provide a growth promoting stimuli, which might play a role in hepatocarcinogenesis (Leng et al. 2003; Wu et al. 2002; Xu et al. 2006). Another major group of AA metabolites are products of cytochrome P450 enzymes, which have been shown to play a role in cPLA₂ and AA mediated cytotoxicity in liver cells (Caro and Cederbaum 2006). However, as no cytotoxicity of AA to WB-F344 cells has been observed, further studies seem necessary to establish the levels and activities of specific CYP isoforms, which might contribute to cytotoxic effects of AA in liver cells, in the present cell model. As it has been proposed that unesterified AA induces apoptosis (Cao et al. 2000), AA metabolism might be also protective, mainly by lowering levels of unesterified AA and by production of metabolites activating anti-apoptotic pathways, such as MAPKs (Hii et al. 1995a).

The present communication summarizes our findings suggesting that, unlike in the case of PAHs, the AA release induced by NDL-PCBs in rat liver epithelial cells is at least partially mediated by cPLA₂, and it is partially regulated by ERK1/2 kinases. The early low AA release is followed by an extensive release of AA after several hours. Although it might not directly affect cell viability or cell growth, as AA itself did not produce any signs of toxicity in WB-F344 cells, more attention should be paid to cell signalling pathways regulated by AA or eicosanoids, as the deregulation of AA signalling has been reportedly shown to contribute to carcinogenesis and it might take part in toxic effects of non-dioxin-like PCBs, including tumor promotion (Marks et al. 2000; Telliez et al. 2006). Therefore, modulation of AA signalling pathways by PCBs in epithelial cells deserves further attention.

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Figure 1.

Effects of PCB 47, 153 and 126 on AA release from WB-F344 cells. Cells were exposed to indicated concentrations of PCBs for 30 min or 3 h, and AA levels in medium were determined by HPLC as described in Materials and Methods. Data are expressed as means $\pm$ S.D. of three independent experiments. * A significant difference between control (DMSO) and treated samples ($p < 0.05$). ** A significant difference between control (DMSO) and PCB-treated samples ($p < 0.01$).

Figure 2.

AACOCF₃, inhibitor of cPLA₂, partially inhibits the PCB 153-induced release of AA. Cells were pre-treated with inhibitors and then exposed to 50 μ M PCB 153 for 3 h, and AA levels in medium were determined by HPLC as described in Materials and Methods. The concentrations and respective specificities of inhibitors are summarized in Materials and Methods. Data are expressed as means $\pm$ S.D. of three independent experiments. * A significant difference between PCB 153 and PCB 153 + inhibitor ($p < 0.05$).

Figure 3.

PCB 153 induces release of AA partly through activation of ERK1/2 signalling. (A) Rapid induction of ERK1/2 and p38 activation by PCB 153. Cells were treated with 50 μ M PCB153, DMSO (control), or with EGF as a positive control for ERK1/2 activation. Cell lysates were prepared and Western blotting performed as described in Materials and Methods. The results shown here are representative of three independent experiments. Detection of total ERK1/2 and p38 expression has been performed as described in Materials and Methods to verify equal loading – no differences were observed (data not shown). (B) U0126, inhibitor of ERK1/2 activation, partially inhibits the PCB 153-induced release of AA. Cells were pre-treated with U0126 (10 μ M) or SB203580 (10 μ M) and then exposed to 50 μ M PCB 153 for 3 h, and AA

levels in medium were determined by HPLC as described in Materials and Methods. Data are expressed as means $\pm$ S.D. of three independent experiments. * A significant difference between PCB 153 and PCB 153 + inhibitor ($p < 0.05$).

Table 1Exogenous AA^a does not affect either cell proliferation or apoptosis in WB-F344 cells

Treatment		Exponentially-growing cells ^b	Confluent cells ^c
Control	Cell numbers ($\times 10^3/\text{cm}^2$)	250.6 $\pm$ 53.5	297.5 $\pm$ 58.5
	Apoptosis (%)	0.8 $\pm$ 0.58	1.3 $\pm$ 0.29
AA 20 μM	Cell numbers ($\times 10^3/\text{cm}^2$)	247.2 $\pm$ 13.1	319.4 $\pm$ 32.3
	Apoptosis (%)	1.4 $\pm$ 0.45	1.3 $\pm$ 0.35
AA 50 μM	Cell numbers ($\times 10^3/\text{cm}^2$)	237.6 $\pm$ 16.2	309.6 $\pm$ 51.7
	Apoptosis (%)	1.6 $\pm$ 0.75	2.0 $\pm$ 0.87
AA 100 μM	Cell numbers ($\times 10^3/\text{cm}^2$)	258.6 $\pm$ 20.3	317.4 $\pm$ 45.1
	Apoptosis (%)	1.0 $\pm$ 0.71	1.8 $\pm$ 1.14

^a – The experiments were performed with free AA; in parallel, AA was applied in BSA-bound form as well – this form of AA had no effect on proliferation and apoptosis of WB-F344 cells either.

^b – Freshly-seeded cells (30,000 cells/cm²) were treated with indicated concentrations of AA or ethanol as a solvent control for 72 h.

^c – Cells were cultured for 72 h prior to exposition, and then treated for another 72 h with AA or ethanol.

For cell number determination, cells were trypsinized and counted with Coulter Counter, as described in Materials and Methods. For detection of apoptosis, both floating and adherent cells were collected and stained with DAPI as described in Materials and Methods, and analysed by fluorescent microscopy. A minimum of 200 cells were counted per each sample.

All data are expressed as means $\pm$ S.D. of three independent experiments.