PUBLICATIONS
Differential effects of 15-HPETE and 15-HETE on BHK-21 cell proliferation and macromolecular composition

Y.V. Kiran Kumar, A. Raghunathan, S. Sailesh, M. Prasad, Mohan C. Vemuri and P. Reddanna

School of Life Sciences, University of Hyderabad, Hyderabad (India)

(Received 10 September 1992)

Key words: 15-Hydroperoxyeicosatetraenoic acid; Prostaglandin D₂; BHK-21 cell

Arachidonate and/or linoleate metabolites have been implicated in modulating cell growth, replication and cell transformations. In studies with BHK-21 cells, we found lipoxygenase and cyclooxygenase inhibitors (NDGA and indomethacin, respectively) to be antiproliferative. Studies on the metabolism of arachidonic acid in BHK-21 cells have demonstrated that prostaglandin D₂ is the major cyclooxygenase product, and 15-hydroxyeicosatetraenoic acid (15-HETE) is the major lipoxygenase product. Addition of D₂ showed a significant decrease in the BHK-21 cell number showing antiproliferative action. Addition of lipoxygenase products, on the other hand, showed differential effects in that 15-HPETE decreased the cell number while 15-HETE increased. NDGA and 15-HPETE decreased DNA, RNA and protein contents, while 15-HETE significantly increased them. 5-HPETE and 5-HETE also showed similar results but were less potent than 15-H(P)ETEs. The differential effects of 15-HPETE and 15-HETE could be due to the generation of free radicals by the hydroperoxide and mitogenic response by hydroxide.

Introduction

The metabolites of the arachidonic acid cascade are known to influence tumor growth. This was evident primarily through the studies using different inhibitors of cyclooxygenase and lipoxygenase pathways, the two major routes of the arachidonic acid cascade. The results of in vivo and in vitro studies are partly contradictory, with respect to cyclooxygenase inhibitors which decrease tumor growth in vivo [1,2] but enhance tumor cell proliferation in vitro [3]. However, lipoxygenase inhibitors are shown to inhibit tumor growth both in vitro and in vivo [4–6]. Besides, the cis-unsaturated fatty acids are shown to exhibit tumoricidal action by a free radical dependent process [7].

Prostaglandins, the metabolites of the arachidonate cyclooxygenase pathway are known to evoke species-specific and tissue-specific physiological responses [8]. Further, prostaglandins are also implicated in tumor cell growth. Prostaglandin D₂ inhibits the growth of cultured mastocytoma cells [9], and inhibits the synthesis of DNA, RNA and protein in L1210 cells [10]. However, information on lipoxygenase-mediated metabolites of arachidonic acid is very limited in tumor cells. Specific metabolites of arachidonic and linoleic acids via the lipoxygenase pathway have been shown to be exercising a regulatory function in epidermal growth factor signal transduction in fibroblasts [11]. Arachidonate 5-lipoxygenase an enzyme catalyzing the formation of 5-hydroxyeicosatetraenoic acid (5-HETE) was identified and purified in rat basophilic leukemia (RBL-1) cells [12]. The production of leukotrienes, which are formed by way of arachidonate 5-lipoxygenase was reported to increase in malignant cells like rat basophilic leukemia cells [13], But there is no substantial information on the identification of other lipoxygenase metabolites and their influence on tumor cell growth and development.

In the present study we have used suspension type BHK-21 cell lines. The cell growth and proliferation was monitored in the presence and absence of the inhibitors of cyclooxygenase and lipoxygenase pathways. Further, the metabolism of arachidonic acid via the cyclooxygenase and lipoxygenase pathways was monitored and the lipoxygenase products identified were screened for their influence on cell growth, DNA, RNA and protein composition. Since hydroperoxides (HPETEs) and hydroxides (HETEs) exhibit differential effects on free radical generation and lipid peroxidation, both compounds were employed in the present study.
Materials and Methods

Indomethacin, NDGA (nordihydroguaiaretic acid), arachidonic acid (99% pure), calcium ionophore A 23187, prostaglandin standards (PGE 2 , PGD 2 , PGF 2α ) were obtained from Sigma, St. Louis, USA. [3 H]Arachidonic acid was obtained from BARC, India (532 mCi/mmol). 15-HPETE and 15-HETE were synthesized and purified on HPLC in our laboratory by using soybean lipooxygenase.

Cell culture

BHK-21 cells (suspension type, passage 14) were procured from NFWATCC Pune, India. They were maintained in Glastow's modified Eagle's medium with l-glutamine, tryptose (Hi media, India) and 8% FCS (Gibco) under 37°C in a 5% CO 2 95% humid air chamber. Cells were passed every alternate day. At specific intervals of time as designed in the experiment the cells were collected by centrifugation at 400 rpm and the cell pellet was washed twice with fresh medium and were used for further experiments. Cell viability was examined by the trypan blue dye exclusion method.

Identification of prostanoids and HETEs

5 • 10 5 cells were suspended in 50 mM potassium phosphate buffer pH 7.4 and were sonicated at 20 Hz four times with 15-s pulses. The sonicate was centrifuged at 10000 × g for 10 min. The supernatant was again centrifuged at 105 000 × g for 1 h. All these centrifugations were carried out at 4°C. To the supernatant 2 mM CaCl 2 , 2 mM ATP and 10 μCi of 25 μM arachidonic acid were added and incubated at room temperature for 2 min with continuous shaking. The reaction was terminated by acidifying the reaction mixture with 1 M HCl. The products were extracted into hexane/ether (1:1, v/v) and evaporated to dryness on a vacuum rotary evaporator. The dried products were dissolved in 1 ml of methanol and stored at -20°C for further studies. The process was repeated for the extraction of cyclooxygenase products without the addition of CaCl 2 and ATP.

Prostaglandins were identified by separation on TLC where 10 μl of standard PGs (1 μg each of PGD 2 , PGE 2 , PGF 2α ), endogenous products and incubated products were spotted on whatman silica gel G TLC plates and were resolved with a mobile phase of chloroform/methanol/acetic acid (100:5:1, v/v) at 4°C. Plates were sprayed with 50% sulfuric acid and were heated at 120°C for 3 minutes and the spots were visualized by fluorescence.

An aliquot of the products was reduced with sodium borohydride and evaporated under nitrogen and redissolved in n-hexane/2-propanol/acetic acid (1000:13:1, v/v). The reduced products were separated on HPLC using straight phase column (Shimpak CLC-SIL 0.5 x 25 cm) with the same solvent system at a flow rate of 1 ml/min. The eluant was continuously monitored at 235 nm and the individual peaks were collected separately. The peaks were identified based on their retention times, co-chromatography with appropriate standards, radioactivity and GC-MS analysis.

GC-MS analysis

GC-MS analysis of 15-HETE was carried out at Pennsylvania State University, University Park, PA with the facilities of Dr. C. Channa Reddy. Hewlett Packard 5890 series II gas chromatograph coupled to a Hewlett Packard 5971 mass spectrometer was used. The separation conditions were 15 m fused silica column, 0.20 mm internal diameter with 0.20 μm film thickness, temperature program 3 min/70°C, then 10°C/min to 240°C. The gas carrier was helium, 2 ml/min. The samples (15-HETE) were methylated and silylated using BSTFA of Supelco, Bellefonte, PA.

Treatment with inhibitors

BHK-21 cells (10 5 cells/ml of medium or 2 x 10 4 cells/well) were incubated at a final concentration with each of the following: (i) Ethanol, 0.05%; (ii) NDGA, 2.5 μM; (iii) 15-HETE, 1 μM; (iv) 15-HPETE, 1 μM; (v) indomethacin, 10 μM; (vi) PGD 2 , 5 μM and (vii) control (without any addition) for 24 h at 37°C and 5% CO 2 . Viable cells were counted.

Time course incorporation of 15-HPETE and 15-HETE

2 • 10 5 cells in 20 ml of medium were incubated with 15-HPETE or 15-HETE (each at 1 μM final concentration). At specific intervals of time (0, 8, 16 and 24 h) 5 ml of the incubation mixture was taken out, the cells were sonicated and the contents were extracted as above. The extract was separated on straight phase HPLC with a solvent of hexane/2-propanol/acetic acid (1000:15:1). Individual peaks were collected and 15-HPETE and 15-HETE were identified based on their retention times and cochromatography with standards.

Estimation of DNA, RNA and Protein

Estimation of DNA and RNA was done by the method of Schmidt [14] while the protein was estimated according to Lowry et al. [15].

Estimation of cellular peroxidase activity

Selenium-dependent glutathione peroxidase (EC 1.11.1.9, Se-GSH Px) activity levels in the cells were estimated [16] after incubation with 15-HPETE and 15-HETE. Cells were pelleted and suspended in 50 mM Tris-HCl pH 7.6 and were assayed for enzyme activity.
Separation of arachidonic cyclooxygenase and lipooxygenase metabolites on thin-layer chromatography. Lane 1, prostaglandins; lane 2, endogenous arachidonate metabolites; lane 3, prostaglandins F2α, D, and E, after incubation with BHK-21 cells. F2α, E, D, prostaglandins F2α, D, and E, HP. HETEs: AA, arachidonic acid.

TABLE I
Effects of inhibitors and metabolites of cyclooxygenase and lipooxygenase pathways of arachidonic acid on BHK-21 cell proliferation

The experiments were started with 2 × 10⁴ cells/well (initial cell number). Cells were counted after 24 h treatment with the respective compounds. Each value is the mean of six individual observations. N.S., not significant.

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>Cell number</th>
<th>Percent change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.70 ± 1.74</td>
<td></td>
</tr>
<tr>
<td>Ethanol (0.05%)</td>
<td>7.60 ± 1.60</td>
<td></td>
</tr>
<tr>
<td>NDGA</td>
<td>5.90 ± 0.90</td>
<td>- 22.30</td>
</tr>
<tr>
<td>15-HETE</td>
<td>(P &lt; 0.05)</td>
<td></td>
</tr>
<tr>
<td>15-HPETE</td>
<td>4.27 ± 0.75</td>
<td>- 43.80</td>
</tr>
<tr>
<td>15-HETE</td>
<td>(P &lt; 0.01)</td>
<td></td>
</tr>
<tr>
<td>Indomethacin (10 μM)</td>
<td>4.73 ± 0.04</td>
<td>- 37.76</td>
</tr>
<tr>
<td>(N.S.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostaglandin D2</td>
<td>4.34 ± 0.53</td>
<td>- 42.80</td>
</tr>
<tr>
<td>(5.0 μM)</td>
<td>(P &lt; 0.001)</td>
<td></td>
</tr>
</tbody>
</table>

Effect of selected inhibitors and products of lipooxygenase and cyclooxygenase pathways

After incubation of BHK-21 cells with NDGA (2.5 μM) 15-HPETE (1 μM), 15-HETE (1 μM), indomethacin (10 μM) and PGD (5 μM) for 24 h, the viability of the cells was found to be about 98% sug-

TABLE II
Concentrations of 15-HPETE and 15-HETE remaining after respective time periods of incubation with BHK-21 cells

Cells were incubated with 1 μM 15-HPETE or 1 μM 15-HETE. At specific time intervals an aliquot of the incubation mixture was taken for the extraction of products. Products were analyzed on HPLC and quantified by their peak areas. Each value is the mean of three individual observations.

<table>
<thead>
<tr>
<th>Incubation period (h)</th>
<th>15-HPETE remaining (μM)</th>
<th>15-HETE formed (μM)</th>
<th>15-HETE remaining (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.000</td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>4</td>
<td>0.982</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>0.978</td>
<td>-</td>
<td>0.55</td>
</tr>
<tr>
<td>32</td>
<td>0.975</td>
<td></td>
<td>0.48</td>
</tr>
</tbody>
</table>
Fig. 2. HPLC separation of metabolites generated by incubating enzyme with $[^3]H$Arachidonic acid. Products were generated by incubating the enzyme preparation as described in Materials and Methods. Each peak was collected individually and the radioactivity in them was counted in a liquid scintillation counter. The major peak at 14.76 min was coinjected with standard 15-HETE (inset).

Fig. 3. GC-MS analysis of 15-HETE. 14.76 min peak obtained from HPLC separation was collected. The peak was derived as described in Materials and Methods and analyzed on a Hewlett Packard GC-MS system.
inhibitors and metabolites of cyclooxygenase and lipoxygenase pathways of arachidonic acid on the macromolecular composition of BHK-21 cells

* expressed in jug/ml. Each value is the mean of four experiments.

<table>
<thead>
<tr>
<th></th>
<th>DNA</th>
<th>RNA</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.350 ±0.980</td>
<td>2.615 ±0.080</td>
<td>6.745±0.070</td>
</tr>
<tr>
<td>5-HPETE</td>
<td>3.225±0.954</td>
<td>2.750 ±0.210</td>
<td>6.566±0.088</td>
</tr>
<tr>
<td>15-HETE</td>
<td>0.830±0.076</td>
<td>2.589 + 0.200</td>
<td>5.100 ±0.140</td>
</tr>
<tr>
<td>15-HPETE</td>
<td>0.583±0.050</td>
<td>2.090 ±0.090</td>
<td>4.585 ±0.040</td>
</tr>
<tr>
<td>NDGA</td>
<td>4.95 ±0.220</td>
<td>5.495 ±0.440</td>
<td>11.325 ±0.049</td>
</tr>
</tbody>
</table>

...that the compounds used are not cytotoxic (1). NDGA, an inhibitor of the lipoxygenase inhibited (-22%) the cell proliferation mod-15-HPETE, the lipoxygenase product eradicated a higher antiproliferative effect. 15-HETE, a reduced product of 15-HPETE other hand showed a seemingly opposite effect: change with 15-HETE was not stastically significant as observed from the data that indomethacin Δ, appeared to be antiproliferative in action at 1 and 5 μM, respectively. The inhibition of cell proliferation was more with PGD₂ (43%) than with indomethacin (38%).

**use levels of 15-HPETE and 15-HETE**

Levels of 15-HPETE and 15-HETE remaining 8, 16, and 24 h of incubation were determined by fractionation and separation on SP-HPLC. As shown in Table II 15-HPETE content mostly remained unchanged even after 24 h of incubation. In 15-HETE-treated cells a rapid decrease of 15-HETE was observed between 0 h and 8 h. Decrease of 15-HETE continued up until 24 h but at a lower rate as compared with the initial rate of decrease.

**Changes in macromolecular composition after treatment**

A study of the DNA replication, transcription and translational abilities of the cell will confirm the nature of the compound with reference to its proliferative or antiproliferative properties. Accordingly, the DNA, RNA and protein levels after treatment have been assayed and are shown in Table III. Incubation of BHK-21 cells with NDGA, the lipoxygenase inhibitor, resulted in a drastic decrease in DNA content with no change in RNA content. The protein content also was decreased significantly. 15-HPETE, the lipoxygenase product of arachidonic acid, also decreased DNA, RNA and protein contents significantly, the extent of decrease being maximum in DNA content. 15-HETE, the reduction product of 15-HPETE however, showed the exactly opposite trend in that it increased DNA, RNA and protein contents significantly.

**Changes in cellular peroxidase activity levels**

Se-GSH Px activity was detected in the 10000×g supernatant fraction of the BHK-21 cells and the same was determined in control and experimental cells (Table IV). Increased Se-GSH activity was observed in the cells treated with 15-HPETE as well as 15-HETE. But in cells incubated with NDGA, there was no apparent change in the peroxidase activity observed.

**Discussion**

The association of eicosanoids with multi-stage carcinogenesis arises from a number of experimental observations centering around eicosanoid production in tumors and correlation between inhibitors of eicosanoid biosynthesis and tumor growth. Cell culture systems...
have been extensively employed for analyzing the role of eicosanoids in either tumor promotion or inhibition. Detection of various arachidonate metabolites in tumor cells is one of the crucial steps in understanding the tumor growth. Tumor cells were shown to have an elevated concentration of prostaglandins [17,18]. Of all the prostaglandins that are detected in cancer cells PGD, was shown to be present in the highest concentration [9] and was considered as a potential anti-neoplastic agent in cultured cells [10]. Inhibitors of prostaglandin synthase or lipoxygenase were reported to inhibit the growth of some cells [5] and there is increasing evidence that metabolites produced by these enzymes affect the growth of cells [19]. We have analyzed the metabolism of arachidonic acid in BHK-21 cells and the data suggest PGD, to be the major cyclooxygenase metabolite of arachidonic acid. No other prostanooids were found in detectable amounts. Incubation with PGD, significantly decreased the BHK-21 cell proliferation supporting the antiproliferative properties of PGD.

The analysis of the lipoxygenase products of arachidonic acid revealed 15-HETE as the major product. Inhibition of the cyclooxygenase pathway with indomethacin enhanced the production of 15-HETE, suggesting that 15-HETE is formed via the lipoxygenase pathway only. In our study no other lipoxygenase metabolites were detected in substantial amounts.

To obtain the information on the possible role of the lipoxygenase pathway in BHK-21 cell growth, the cells were incubated with NDGA, the selective inhibitor of lipoxygenase pathway. NDGA significantly inhibited the cell proliferation, suggesting the possibility of involvement of lipoxygenase metabolites in the growth of BHK-21 cells. Similar antiproliferative effects of NDGA on a variety of cell lines were reported [5,20,23]. In order to explore the need of arachidonic acid lipoxygenase metabolites to sustain the growth of BHK-21 cells, the cells were incubated with 15-HPETE and its reduction product, 15-HETE. It is interesting to note that 15-HPETE showed antiproliferative effects, while 15-HETE stimulated cell proliferation. These results indicate that the lipoxygenase inhibitors substantially reduce cell proliferation, presumably by inhibiting the production of hydroxyl metabolites of arachidonic acid but not the hydroperoxides. Recent studies have indicated that polyunsaturated fatty acids can be employed as anticancer drugs [20]. Such anticancer properties can be explained in terms of the formation of hydroperoxides (which exhibit antiproliferative properties) upon incubation of cells with unsaturated fatty acids. However, the ultimate effects of the lipoxygenase pathway appear to depend on the relative reduction of HPETEs to HETEs by cellular peroxidases. Recent studies by Glassgow et al. [11] have shown that the mitogenic response of the epidermal growth factor can be modulated differentially by linoleic and arachidonic acid metabolites in Syrian hamster embryo fibroblasts.

(Sc-GSH P+X) is the major cellular peroxidase involved in the reduction of organic peroxides [24]. In the present study detectable activity of Sc-GSH P+X was observed in BHK-21 cells and the same was increased in the presence of 15-HPETE. However, the externally added 15-HPETE levels were not altered substantially in these cells, even after 24 h of incubation, which could be responsible for the observed antiproliferative effects of 15-HPETE. The low Sc-GSH P+X observed in these cells may not be efficient in the conversion of 15-HPETE to 15-HETE. Also, the K+ reported for 15-HPETE was 12 µM [25], which is very high especially when the 15-HPETE added in the present study was 1 µM. Further studies on cellular peroxidases, lipoxygenases and peroxide tone could clarify their role in cell proliferation. The rapid decrease in the concentration of 15-HETE upon incubation with BHK-21 cells could be due to its selective incorporation into cellular phospholipids [26].

It is interesting to note that both indomethacin and PGD, suppressed cell proliferation without any cell death, suggesting that the dosage of these compounds was not cytotoxic. On the whole these results are in agreement with the earlier findings [5,10]. However, it is not clear how both indomethacin (inhibitor of PGH synthase) and PGD, show antiproliferative properties. It is known that inhibition of the cyclooxygenase pathway actually stimulates the lipoxygenase pathway. In the present study the production of 15-HPETE also increased when the cells were incubated with indomethacin, suggesting the possible stimulation of the lipoxygenase pathway (control 100%, indomethacin 112%). This increased amount of 15-HPETE could be responsible for the overall antiproliferative effects observed in the presence of indomethacin. The effects of the lipoxygenase pathway on BHK-21 cells were also reflected in changes in the macromolecular composition. Both NDGA, the lipoxygenase inhibitor and 15-HPETE, the immediate lipoxygenase metabolite, showed significant reduction of DNA content supporting their antiproliferative roles. 15-HETE, on the contrary, increased the DNA content. In transformed erythroleukemia cells 15-HETE was detected and was shown to increase DNA synthesis in differentiating cells but not in proliferating cells [21]. However, our results indicate that 15-HETE increases DNA synthesis in proliferating cells too. The stimulation of cell proliferation and DNA synthesis by 15-HETE may be brought about by inhibition of diacylglycerol kinase leading to an increase of the cellular diacylglycerol level [22]. Diacylglycerol activates protein kinase C, which stimulates DNA synthesis presumably by phosphorylation of nuclear proteins. Our preliminary stud-
otein phosphorylation indicate that 15-HETE cally involved in phosphorylation of the 54 ein (data not shown). NDGA and 15-HPETE lecreased the RNA and protein levels, while drastically increased the RNA and protein these results indicate that 15-HETE is relaore involved in translational activities, while nd 15-HPETE affect the transcriptional activ-

BHK-21 cells. These differential effects of and HETEs might be responsible for the g and contradictory reports in the literature e of lipoxigenases and eicosanoids in cancer rion of BHK-21 cells with 5-HPETE and 5-
so showed similar results to those observed in nce of 15-HPETE and 15-HETE, the extent ich less (Table V). These compounds appear potent as they are not the principle metabo-rachidonic acid in BHK-21 cells.

clusion, this study demonstrates that arachi-
id is metabolized by BHK-21 cells by both enase and lipoxigenase pathways and the e of these pathways are involved in the reg-

The interesting f the present study is the differential effects of E and 15-HETE on the cell proliferation, tion and translational activities. The peroxi-

cuivity coupled to lipoxigenase seems to be an t determinant of cell proliferaton.

dgements

dork is supported by grants from the Depart-
Biotechnology, New Delhi, India (grant No. 03/25/007/89). M.C.V. is a receipient of entist B award. The authors gratefully ac-
ge Dr. C. Channa Reddy for providing GC-MS nd to Mr. Chris for analyzing the samples.

MULTIPLE GELS IN 2DE – BETTER RESOLUTION

Mohan C. Vemuri, N. Naga Raju, A. Raghunathan and P. Prakasa Babu
School of Life Sciences, University of Hyderabad, Hyderabad – 500 134, India.

1 INTRODUCTION

Identification and characterization of proteins was made easier by high resolution two-dimensional electrophoresis developed by O'Farrell [1]. This popular technique involves isoelectrofocussing (IEF) in the first dimension and SDS-PAGE in the second dimension. Visualization of polypeptides resolved by 2-DE by ultra sensitive silver staining [2], has a considerable contribution in improving the sensitivity of 2-DE technique, allowing screening and detection of rare proteins in diseases of clinical and biological samples. There have also been many modifications such as addition of SDS in IEF [3], Non-equilibrium pH gradient gel electrophoresis (NEPHGE) involving a refinement in the resolution of basic proteins [4], development of "giant gels" to analyse expression of protein-gene products [5] and computerised scanning of 2-DE gels to make protein catalogs [6]. Most of these modifications are in the first dimension run or take advantage of the excessive protein loading as in "giant gels" which significantly increased the resolution and utility of 2-DE gels. Despite these improvements, there are still minor draw backs, in the second dimension such as the limitation of running only two gels at a time, leading to 'batch-to-batch and run-to-run variations especially when large number of samples have to be subjected to 2-DE. Simple visual protein pattern recognition itself could be confusing when more than two samples have to be analysed for protein changes using 2-DE. In order to overcome this difficulty, we have attempted to improve the second dimension SDS-PAGE run by transferring at least eight IEF tube gels at a time on to the second dimension slab gels allowing resolution of eight 2-DE gels, which we call "multiple gels".
This paper is an attempt to describe the simple fabrication of "multiple gel" unit and to document the utility of multiple gels in protein pattern analysis.

2 MATERIALS AND METHODS

ISO ELECTROFOCUSING:
Protein samples from plasma membranes of neurons and glia of rat brain were subjected to IEF in tube gels as described earlier [7].

MULTIGEL UNIT:
The apparatus "Multigel unit" for running eight slabs at a time is essentially similar, but a scaled-up version of the unit used by Reid & Kieleski [8] and O'Farrel [1]. Apparatus dimensions are 54 x 7 x 17 cm (l x w x h). The lower perspex chamber is of 62 x 13 x 8 cm size (Fig.1C).

GEL CASTING AND ASSEMBLY:
Glass plate of 5 mm thickness, 54 x 17 cm was cut into rectangulars and used in making the slab gels. The outer plate was used without any further modification (Fig.1B), while the inner plate was notched 3.5 mm deep and 48 cm long leaving 3 cm

---

Fig 1: Multigel Unit and its components.
on either side of the plate (Fig 1A). The gels were cast in the same way as in conventional slabs. But during the polymerization of stacking gel the surface of each gel is overlaid with one clean glass rod of 2.5 mm diameter (48 cm) or four rods of 12 cm one beside the other. This results in the formation of a smooth gel surface on which the first dimension IEF gel can be placed.

IEF GEL TRANSFER AND SECOND-DIMENSION RUN:

When the gel casts were assembled and clamped on to the unit, it results in the formation of an upper buffer chamber (Fig 1D). The assembly is placed into lower chamber carefully from one end to avoid trapping of air bubbles beneath the gel. The glass rods on the top of the stacking gels were removed and IEF gels were transferred into the smooth groove made by the glass rod. The IEF gel was annealed to the stacking gel with hot agarose. By this method eight IEF gels can be placed, four on each side of the multigel unit. After the addition of upper chamber buffer, the gels were electrophoresed at 120 mA in the stacking gel region and 160 mA in the resolving gel region, for 4 to 5 hours till the dye front reaches bottom. The electrodes were disconnected after the completion of the run and the glass plates were pried apart with a kitchen knife. Since the IEF gel still remains stuck to the stacking gel, each gel was sliced vertically into four gels guided by the position of IEF gel. The gels were fixed as described earlier [7].

DETECTION BY SILVER STAINING:

Visualization of proteins was by the method of Merrill et al., [2] as modified by Blum et al., [9].

3 RESULTS

2 DE pattern of plasma membrane proteins of neurons and glia obtained by means of separation on multigel unit are shown in Figs 2-3. An example of only two protein patterns is shown although similar protein separation pattern was achieved in all eight samples using multigel unit. By comparison of the separation pattern from samples A and B in each figure as indicated, the position of proteins (numbers) can be clearly
identified. In all the eight gels, the proteins were identified at the same position of x-y coordinates, resulting in highly reproducible protein separation pattern.

4 DISCUSSION
Two dimensional gel electrophoresis is a most efficient analytical method for separating protein samples. The method described by O'Farrel [1] fifteen years ago has been taken over virtually unchanged in the following work, and with a minor modification in the second dimension, better and consistent resolution is achieved. The simple modification we made, is to fabricate an apparatus to run eight conventional slab gels (9 x 11 cms) as two large gels. The reproducibility and resolution of protein is shown in Fig.2-3. This modification allows certain advantages. Casting of four conventional gels as a single large gel is done at a time with the help of two lateral and one bottom
spacers. Thus the routine use of *almost* nine spacers can be omitted. Gel casting and assembly as individual slabs and electrophoresing them as separate runs consumes more time. In multigel unit, all this is reduced to one third with respect to gel casting, assembly and man hours. The variations from batch-to-batch are absolute minimum, as all the gels are run simultaneously under same running conditions. The multigel unit facilitates multiple handling of IEF samples and allows very economical performance of the original procedure (1) with highly reproducible protein resolution pattern. This simple modification also increases the chances of getting comparable protein profile gels (publishing quality gels) rendering easy analysis of protein patterns.

5 ACKNOWLEDGEMENTS

This work is supported by the CSIR. New Delhi, project No. 9(226)86-EMR-II. We thank Prof. P.R.K. Reddy, Dean, School of Life Sciences, for the facilities.

6. REFERENCES

DNA BINDING PROTEIN CHARACTERIZATION USING-2DE

A. Raghunathan and Mohan C. Vemuri
School of Life Sciences, University of Hyderabad, Hyderabad-500
India.

1 INTRODUCTION

In brain, functional and cellular complexity is enormous [1] probably it is a consequence of brain specific gene expression which involves the mediation of DNA binding proteins (DBPs). Very DBPs have thus been characterized and to understand the basis of b specific functions, it is necessary to identify and characterize DNA binding proteins involved. The DBPs in higher mammals exist very scanty amounts and their identification is complicated [3] they follow development dependent expression. In the present study describe the identification and partial characterization of two binding proteins using 2-DE followed by ultra sensitive si staining. He suggest that the 2-DE can be potentially exploited identifying rare and scarce DNA binding proteins.

2 MATERIALS AND METHODS

Ten day old Histar strain albino rats of both sexes weighing about gms were used for the experiments. Nuclei were isolated from rat b as described earlier [4] and DNA free nuclear protein extract prepared following ammonium sulphate fractionation and polyethylene glycol precipitation as described [3]. Double strand (ds) and single strand (ss) DNA-cellulose chromatography was performed as descr: [5]. Various protein fractions were electrophoresed on SDS-1 according to Laemmli with modifications as suggested by Thomas Kornberg [6]. Two dimensional electrophoresis was done as descr by O'Farrel [7] followed by ultra sensitive silver staining [4].

3 RESULTS

Nuclear proteins were chromatographed on dsDNA-cellulose column the column was washed successively with buffers of step-1 increasing NaCl concentrations (0.1, 0.2, 0.6, 1.0 and 2.0) Several minor proteins were eluted in 0.1 and 0.2 M NaCl buffer; they were ignored as proteins bound to DNA cellulose below 200 mM; concentration are not significantly binding type [8]. The fractions of 2M NaCl eluates were analysed by SDS-PAGE (Fig.1) and proteins denoted DBP-1 and DBP-2 were identified. In order to cl whether or not DBP-1 and DBP-2 are specific for double stranded or, single stranded DNA, the 2M NaCl eluates from dsDNA cellul: chromatography were chromatographed on a ssDNA-cellulose column. proteins were found only in flow through; but not in salt elu: indicating preferential affinity of these proteins to native DNA. dimensional electrophoresis allowed further eluation <Tization of PL and DBP V. having molecular weights: 30kDa and 48kDa, with isoelect: Points (pI) 5.3 and 5.2 respectively.
The interaction of proteins with genome is usually brought about by DNA binding regulatory proteins [9]. In order to search for such regulatory proteins of cell nucleus, we chose to analyse UMA binding proteins in the cerebral cortex of rat, using a combination of methods such as DNA affinity chromatography and 2-DE followed by silver staining. He have identified two proteins DBP-1 and DBP-2 with preferential affinity to bind to DNA (Fig 2b). The isoelectric point and relative molecular mass of these proteins were derived from 2-DE. He could not observe these peptides in nuclear preparations from 1 and 2 month rnt brains (data not shown). Hence we presume DBP-1 and DBP-2 are present in very curly stages of brain development and occur in substantially low amount. Further, separation of nuclei into neuronal and glial indicated the presence of these proteins only in neurons (Fig 3). The functions of DBP-1 and DBP-2 are not clearly known at present, but as these proteins are prominent at 10 days of post-natal age, that too, only in neurons, we suspect they might be involved in neuronal differentiation as a transition from proliferating precursor cells to non-dividing terminally differentiated neurons takes place at this stage of brain development [10]. The results from UMA affinity chromatography and 2-DE suggests that DBP-1 and DBP-2 might be involved in development dependent neuronal gene expression in brain function.

5 ACKNOWLEDGEMENTS

This work was supported by CSIR, New Delhi (No. 9(226)86-EMR-II). We thank Prof. P. K. R. Reddy, Dean, School of Life Sciences for facilities.

6 REFERENCES

DECREASE OF EXCITOTOXICITY OF D-ASPARTATE REUPTAKE: ENHANCEMENT OF EXCITOTOXICITY - Wtemham R. L. and Dodd P. R. Clinical Research Centre, Harrow, Middlesex, UNITED KINGDOM.

Amyloid plaques are a pathological marker of Alzheimer's Disease (AD). Since the discovery of the amyloid protein in senile plaques, much work has focused on a relationship between the amyloid protein and neurodegeneration. Indeed, one of the main findings in AD research has been the accumulation of the amyloid protein in the extracellular compartment of the brain, particularly in the cerebral cortex and hippocampus. 

The term "amyloid plaques" refers to the characteristic deposits of an amyloid protein in the brain, which are a hallmark of AD. These plaques consist of long-fibrillar structures, often accompanied by an increase in extracellular space. The amyloid protein is composed of beta-amyloid peptide (Aβ), a small protein of 40 to 42 amino acids that is derived from the larger amyloid precursor protein (APP). 

The accumulation of Aβ in the extracellular space is thought to be a key pathological event in AD, leading to neurodegeneration and synaptic dysfunction. The Aβ peptide is a neurotoxin that can activate microglia and astrocytes, leading to the release of pro-inflammatory cytokines and matrix metalloproteinases, which can further contribute to neurodegeneration. 

Moreover, Aβ can promote the development of neurofibrillary tangles, another hallmark of AD, by aggregating with tau protein. This aggregation can lead to the formation of neurofibrillary tangles, which are bundles of hyperphosphorylated tau protein that are found in the cytoplasm of neurons.

In summary, the accumulation of Aβ in the extracellular space is a key pathological event in AD, leading to neurodegeneration and synaptic dysfunction. Understanding the mechanisms underlying Aβ accumulation and its effects on the brain is crucial for developing effective treatments for AD.