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Indole-propionic acid (IPA) protects PC12M1 cells from oxidative stress induced by FeCl2 and reduces aggregation of Aβ1-42 in vitro

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Abstract

To study the potential of novel compounds for the treatment or as disease modifying agents in Alzheimer's (AD) and related CNS & PNS diseases, we used in vitro models which enable easy and fast evaluation of drugs. Compounds were assessed: a) to act as anti-lipid peroxides ligands, using the thiobarbituric acid reactive substances (TBARS) assay and b) as inhibitors of Aβ1-42 aggregation (found in plaques of AD brains) using the Thioflavin T (ThT) assay. FeCl2 exhibits lipid oxidation (TBARS) response of 2.4 and 4.1 fold increase over control (100 and 400 µM, respectively) in differentiated PC12M1 cells (have M1 muscarinic receptors) and simulate neuronal behavior. The known antioxidant ligand, indole-3-propionic acid (IPA) at 0.01 nM - 100 µM protected the cells from the lipid-peroxidation, exhibiting significant protection of 50 ±11 % at 100 nM. Also, IPA at 1 - 1000 nM inhibited the aggregation of Aβ1-42 in a concentration dependent manner. Based on these results we suggest that IPA or ligands based on the backbone of this molecule can serve as potential drugs that protect neurons from Aβ fibrils found in brains of AD patients as well as protecting neurons from oxidative stress at nanomolar concentrations

Keywords: Aβ1-42 aggregation, oxidative stress, protection from FeCl2, indole propionic acid (IPA), thiobarbituric acid reactive substances (TBARS), Thioflavin T (ThT), M1 muscarinic acetylcholine receptor (mAChR),differentiated PC12 cells, PC12M1

Introduction

It is known that fatty acid metabolism is involved in CNS disorders, especially in Alzheimer's disease (AD) [1-3] and oxidative stress is one of these processes [4-7]. In this regard, lipid peroxidation is a well-established mechanism of cellular injury and used as an indicator of oxidative stress.

Lipid peroxides are unstable and decompose to form a complex series of compounds, which include reactive carbonyl compounds, such as malondialdehyde (MDA). MDA can be quantified through a controlled reaction with thiobarbituric acid, generating 'Thiobarbituric Acid Reactive Substances' (TBARS). The method is precise, sensitive, and highly reproducible for quantitative determination [8]. Therefore, the TBARS assay provides a simple, reproducible, and standardized tool for assaying lipid peroxidation in various tissue homogenates and cell lysates.

In addition, one of the hallmark of AD is the formation of extra-cellular protein deposits in the brain that consist predominantly of aggregates of β-amyloid protein (Aβ) produced through undesired proteolytic processing of the β-amyloid precursor protein [9]. Aggregation of amyloid-plaques and tau proteins were suggested to be the major pathology found in AD brains [10-13]. The amyloid deposits result from the formation of fibrils which turn to Aβ aggregates. The fibrils are characterized by their β-sheet structures leading to neuronal cell death.

It is known that amyloid fibrils can be produced in vitro and found to simulate mechanism of β-amyloid-induced neurotoxicity [14-15]. Therefore, this in vitro model can provide a useful tool to study the mechanisms of Aβ-mediated cell toxicity and to devise strategies of protection by reducing the Aβ-load either through prevention of fibril formation or by reducing the existing deposits. One of the most common methods used to monitor protein fibril formation is the Thioflavin T (ThT) method. This method is attractive since ThT induce fluorescence only when it bound to fibrils, producing a hypochromic shift of the bound dye [16-17].

In this study we evaluated the potency of indole-3-propionic acid (IPA) to act as anti-lipid peroxided agent employing the TBARS assay. For this purpose, we used differentiated PC12 cells transfected with the M1 muscarinic acetylcholine receptor (mAChR) [PC12M1], simulating neuronal cells. In addition, we evaluated the potential of IPA to prevent the aggregation of Aβ1-42, a granulomere that exists in the core of plaques found in brains of AD patients, using the ThT method.

Materials and Methods

Materials

ThT (purchased from Sigma #T-3516), a benzothiazole dye [increases fluorescence upon binding to amyloid fibrils] was dissolved in sterile double distilled water (DDW) in a final concentration of 1 mg/ml. The solution was centrifuged using an Eppendorf centrifuge and the upper layer was filtered using syringe filters (high efficiency micro-clean filter syringe for sterile filtration, ANOW, China). Aliquot samples were covered with aluminum foil and kept at -20ºC in the dark. Before each experiment a fresh solution of 5 µM ThT was prepared in Tris buffer (1.6 µl/1 ml), kept in dark and the unused sample was discarded.

Stock solutions of Aβ1-42 (1 mg/ml, purchased from Bachem # H-1368, Switzerland) were prepared by dissolving the content of the bottle (1 mg) in 200 µl di-methyl sulfoxide (DMSO) Then, 800 µl sterile 10 mM NaOH were added and aliquots (50 µM) were prepared and kept in -70ºC. These solutions were ready for use up to 3 weeks under freezing conditions. The aggregation process of Aβ1-42 was carried out at 37 ºC in 5% CO2 incubator for 1-8 days.

Stock solutions of IPA (100 mM, purchased from Sigma) were prepared. Before each experiment sterile solutions at various concentrations of IPA were prepared in 10 mM NaOH and 50 mM Tris buffer. Atropine sulfate (purchased from Aldrich) was dissolve in DDW to give a concentration of 10 µM. A solution of 1% thiobarbituric acid (TBA) [99% pure, Alderich] was prepared freshly for each experiment. This was completed by dissolving 400 mg of TBA in 10 ml 0.5 M NaOH, vortexing and adding 10 ml DDW and 20 ml glacial acetic acid (pure, Aldrich).

$FeCl_2$ (100 mM) was prepared at the same day of performing the experiment (since it tend to be oxidized) by dissolving 35.86 g in 2.83 ml DDW. Tetra chloro-acetic acid (TCA) (15%) was prepared by adding 567 ml DDW to 100 ml TCA (100%).

Inducing lipid oxidative stress in differentiated PC12 M1 cells

Rat pheochromocytoma (PC12) cells, stable transfected with the M1 mAChR (PC12M1) were obtained from Prof. Sokolovsy (Tel Aviv University). The cells were differentiated into neuronal characteristics to induce neurite outgrowth in presence of 50 ng/ml NGF according to Pinkas-Kramarski *et al.* [18].

Preparation of 6-well plates prior to cell seeding: 1 ml sterile poly-L-lysine (PLL) was added to each well of six-well plates (corning NY, USA) for 10 min, washed twice with DDW and left open in UV hood for 2 h for drying.

The PC12M1 cells were plated at a density of 0.8X106 cells/well in a complete RPMI1640 medium supplemented with 5% fetal calf serum, 10% heal-inactivated horse serum, 2 mM glutamine and a mixture of antibiotics and 400 µg/ml G418 (Calbiochem, USA). Two days later the medium was changed to medium containing NGF at a final concentration of 50 ng/ml in serum-free RPMI 1640 medium. Cells were washed twice with serum-free RPMI 1640 medium, containing 20 mM HEPES and 0.2 mg/ml bovine serum albumin [19]. This step was repeated on the 6th day post seeding. On the 12th day post seeding, when the cells developed axons, FeCl2 was added to give final concentrations of 100 and 400 µM (2 and 8 µl from stock solution, respectively) and incubated at 37ºC in 5% CO_2 incubator for 3h. When the protective effect of IPA (0.01 nM – 100 µM) was tested, a concentration of 100 µM $FeCl_2$ was used. A final concentration of 100 nM atropine was co-incubated with IPA and $FeCl_2$, when the involvement of the M1 mAChR was assessed.

TBARS assay

The MDA-TBA adduct is formed by the reaction of MDA and TBA under high temperature (90-100°C) and acidic conditions. This complex was followed with high sensitivity using fluorimeter at excitation of 532 nm and emission of 550 nm wavelengths [20].

Differentiated PC12M1 cells were incubated with $FeCl_2$ in presence and absence of various concentrations of IPA. At the end of the incubation period (3h), the cells were scratched (with their medium) and the content was transferred to 14 ml screwed tubes, centrifuged for 5 min at 200 g using a refrigerated centrifuge. The upper layer was discard and cold 0.4 ml PBS was added. The cells were sonicated on ice bath (3 pulse/3 sec/50% amplitude). Then, 0.4 ml of cold 15% TCA solution was added (for protein precipitation). After a vigorous mixing (by vortex), 0.6 ml of 1% TBA was added. The mixture was boiled for 30 min, and left at room temperature till reaching the ambient temperature. Centrifugation was employed at 3,000 g for 5 min at RT and 100 µl of the upper layer was transferred into 96-well plate for detection of products using NOVOstar fluorimeter (MTX Lab Systems Inc, USA). An extra tube which contained similar ingredients but with no cells was added as a blank to reduce the O.D. baseline. We found that the range for using this assay is linear between 0.7-15 pmoles/ml MDA **(Figure 1).**

ThT aggregation assay

ThT initiated after binding to fibrils of Aβ, and therefore was used to quantitatively follow the formation of fibrils (aggregated forms) and to evaluate the potential of IPA to prevent fibrils of Aβ. This method was employed after Bourhim *et al.* [21]. The reaction is initiated immediately when β-amyloid is introduced into aqueous environment, a process which is completed within 1 min.

The study was carried out in 24-well plates. Aβ1-42 (2.5 µg=275 nM and 5 µg=550 nM) were incubated with 1 ml ThT/ Tris buffer (5 µM final concentration) and with or without various concentrations of IPA (10 µl from stock solutions X100). After 1-2 & 6-7 days of incubations in 5 % CO_2 incubator at 37ºC,

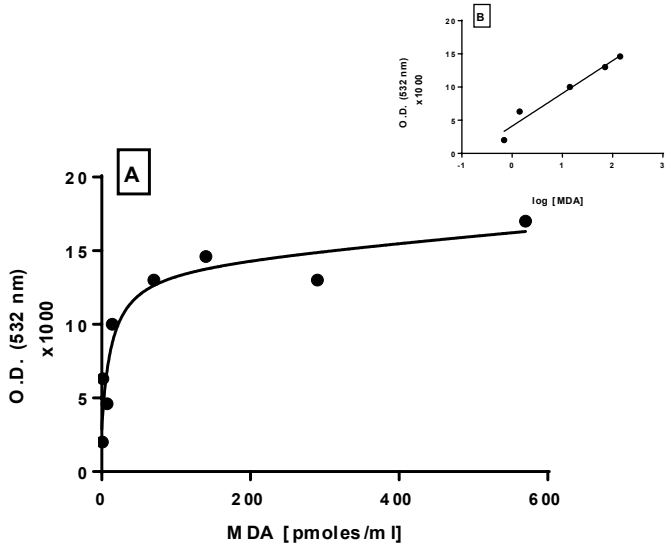

Figure 1. *TBARS assay performed in differentiated PC12M1 cells: dose response curve of MDA, displays saturation curve (0-600 pmoles/ml)* ***(A)****; linear curve of MDA* ***(B)****. This assay exhibited linearity in this model between 0.7-15 pmoles/ml MDA. For more details see section 2.3.*

200 µl from each well were transferred (in triplicates) to black 96-well plates for reading the florescence at 450 nm excitation and 480 nm emission using NOVOstar fluorimeter (MTX Lab Systems Inc., USA, excitation: 10-450 nm, emission: 10-480 nm, Gain=10% of control).

The efficacy of IPA to prevent aggregation was calculated (as percentage) by subtracting from the sample value the control value (no ThT) and dividing the result by the blank value (no Aβ1-42) and then multiple the result by 100.

Data analysis

Data are presented as means ± SEM from 5 to 8 experiments. Differences between groups was assessed by analyses of one way ANOVA followed by post-hoc Dunnet for evaluating statistical significance and a value of $p<0.05$ was accepted as statistically significant.

Results

$FeCl_2$ induced lipid-oxidative stress in differentiated PC12M1 cells

The actual range which fit the TBARS response in the differentiated PC12M1 cell model was established. We found that the linear range for calculating MDA in this model was 0.7-15 pmoles/ml **(Figure 1).**

In order to measure the potency and the extent of $FeCl_2$ to increase TBRAS response in this model, we used the concentrations of 100 and 400 µM. We found that Fe^{2+} significantly increased TBRAS response by 2.4 ± 0.22 and 4.7 ± 0.89 fold over control, respectively (data not shown). For further studies, the concentration of 100 µM $FeCl_2$ was chosen to evaluate the potential of IPA to protect differentiated PC12M1 cells from $FeCl_2$-induced oxidative stress.

IPA protected differentiated PC12M1 cells from lipid-oxidative stress induced by $FeCl_2$

IPA displayed a significant protection against $FeCl_2$-induced oxidative stress **(Figure 2).** This effect was observed at very low concentrations of IPA, showing 29.0 ± 1.0 % protection at 0.01 nM and 49.5 ± 11.3 % protection at 100 nM **(Figure 2).** Thus, IPA displayed a significant protection at 0.01nM - 100 µM concentrations with a maximal significant protection of 58.0 ± 6.5 % at 100 µM **(Figure 2).**

When the protection effect of IPA (0.01nM - 100 µM) was tested in differentiated PC12 cells (with no M1 receptors), the protection effect, whatsoever was not seen (data not shown). Moreover, we found that 100 nM atropine (a muscarinic antagonist) did not block the protecting effect of IPA (data not shown). These results indicate that although the M1 muscarinic receptors are somehow involved in the process of protecting the cells from oxidative stress, they are not functioning via the traditional (orthosteric) site.

In summary, IPA at nM concentrations, exhibited significant protections against lipid-oxidative stress induced by 100 µM FeCl2 in differentiated PC12M1 cells.

Aggregated Aβ1-42 formation, concentration- and time-dependent calibration; using ThT method

Aggregation of Aβ1-42 was observed after incubation with ThT/Tris buffer in 5% CO_2 incubator at 37ºC for 1 day. We found that the fluoresce intensity of 2.5 µg (=275 nM) and 5 µg (=550 nM) Aβ1-42 were increased after the incubation by 3.6 and 6.9 over control **(Figure 3A)**. Thus, the fluoresce intensity (which represent the intense of the aggregation) of 5 µg was about twice higher than that of 2.5 µg Aβ1-42.

Similar results were obtained after 2, 6 and 7 days incubations of Aβ1-42 under the same conditions **(Figure 3B),** displaying

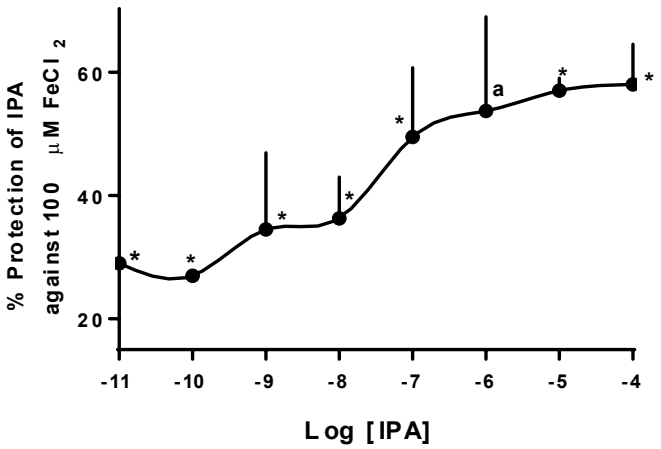

Figure 2. *Protection of differentiated PC12M1 cells from 100 µM $FeCl_2$-induced peroxidative stress by various concentrations of IPA, as measured with the TBARS assay. Statistical analysis was based on one way ANOVA repetitive measure followed by post-hoc Dunnet. Significant differences were found in all the concentrations tested (0.01 nM-100 µM), as indicated for each concentration, [a]$p<0.05$; *$p<0.001$.*

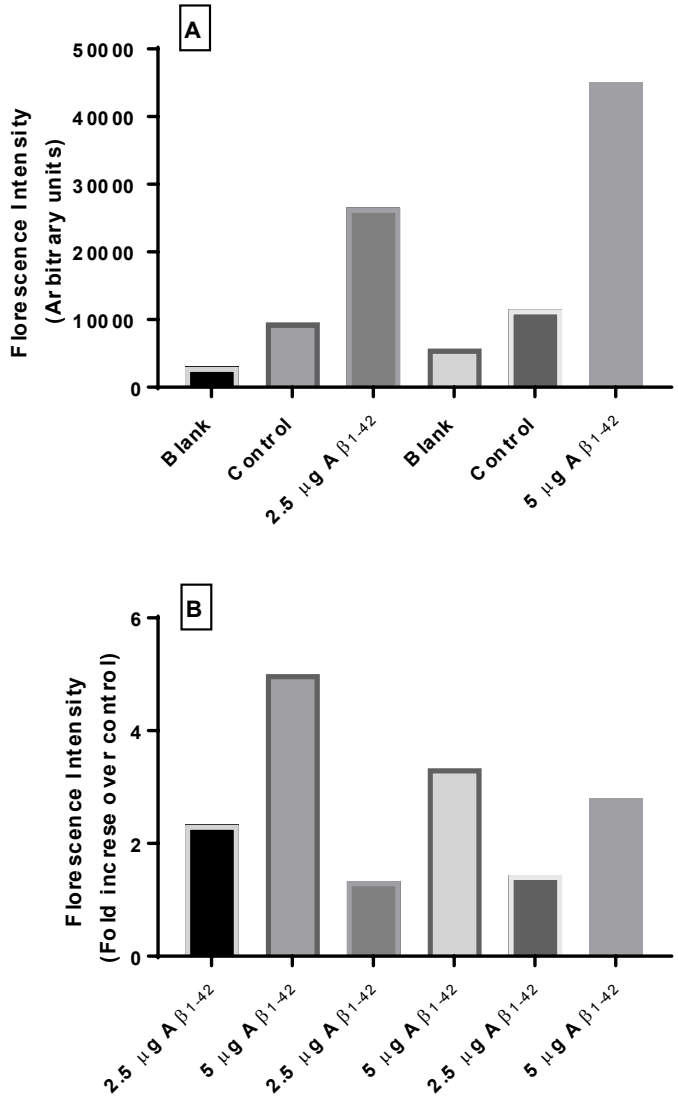

Figure 3. *Representative experiment showing the aggregation of Aβ1-42 as measured in vitro by the ThT assay. Concentration effect is seen with 2.5 and 5 μM after 1 day of incubation at 37ºC* ***(A)****. Effect of concentration response is seen after 2, 6 and 7 days of incubation at 37ºC* ***(B)****. For more details see 2.4.*

a 2.0 fold increase when 5 μg was used compared to 2.5 μg **(Figure 3B).** For further evaluation of the potency of IPA to inhibit this Aβ aggregation, we used 5 μg (=550 nM) Aβ1-42.

Inhibition of Aβ1-42aggregation by IPA

Aggregation of 550 nM Aβ1-42 was measured after short and long periods of incubations (1-2 and 6-7 days at 37ºC, respectively) in the absence or presence of IPA.

We found that IPA (1-1000 nM) inhibited the aggregation of Aβ1-42 in a concentration-dependent manner. Thus, 8-10% inhibition was noticed at 1 nM and 33-35% at 1 μM (significant at $p<0.01$ & $p<0.001$ after short and long incubation periods, respectively). IPA significantly inhibited the beta-amyloid aggregation already at 10 nM (by 22 ± 1% after 6-7 days). Similar results were obtained when short or long aggregation periods were employed displaying maximal significant inhibition of 33 ± 7.1 and 35 ± 3.3 %, at 1 μM IPA, respectively **(Figure 4).**

Discussion

In the present study, we found that IPA significantly protected differentiated PC12M1 cells from oxidative stress induced by 100 μM $FeCl_2$. The protection effect of IPA was shown at very low concentrations, starting at 0.01 nM with 29% protection and reaching 50% protection at 100 nM. We also found that IPA reduces the aggregation of Aβ1-42 in vitro, when it was co-incubated with the β-amyloid for short or long periods (1-2 and 6-7 days, respectively). Thus, 1 μM IPA inhibited significantly the aggregation by 33-35%. These results indicate that IPA may delay neuronal death caused by oxidative stress or by the β-amyloid toxin aggregates. To our knowledge, this is the first report to show that IPA retain neuroprotective effects against oxidative stress induced by $FeCl_2$ at nanomolar range in neuronal cells and as an inhibitor of Aβ1-42 aggregation in vitro. Schuster *et al.* [22] showed a protective effect of colostrinin on the survival of neuroblastoma cells after reduction of beta-amyloid aggregation. The protection effect of IPA was reported in primary neurons, neuroblastoma cells, and rat brain against oxidative damage induced by β-amyloid protein [23]. IPA was reported as a potent neuro-protectant against lipid peroxidation in the thyroid gland and rat kidney [24-25]. Furthermore, IPA has been shown to be a potent scavenger of hydroxyl radicals [26] and was shown to act synergistically with the anti-oxidant, glutathione [26]. IPA significantly decreased the activation of astrocytes and microglia as well as GFAP and Iba-1 protein levels in the hippocampus region of ischemic Mongolian gerbils [27].

IPA acts as an electron donor, detoxifying reactive radical since it does not possess a hydroxyl group [28]. In vivo studies, using IPA, show that IPA protects neurons from ischemia-induced neuronal damage by reducing DNA damage and lipid peroxidation in the hippocampus of Mongolian gerbils [27]. In this regard, it was reported that IPA suppresses indoxyl sulfate-induced expression of fibrotic and various inflammatory genes in proximal tubular cells [29].

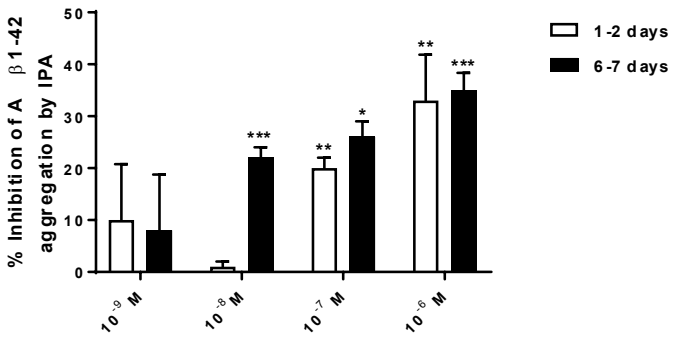

Figure 4. *IPA (1 nM-1 μM) inhibited the aggregation of Aβ1-42 (5 μM = 550 nM) after short (1-2 days) or long (6-7 days) incubations at 37ºC. Statistical analysis was based on one way ANOVA repetitive measure followed by post-hoc Dunnet. Significant differences as indicated: *p<0.05, **p<0.01.; ***p<0.001.*

We found that IPA was effective at very low concentration (nM range) in inhibiting the iron-induced oxidative damage. This data is novel and important since in the literature it was reported that only at high concentrations (mM range) IPA protects against iron-induced oxidative damage in hepatic microsomal membranes [30]. These authors also reported that at mM concentrations IPA influenced the membrane fluidity, and was proposed as potential drug against carcinogenesis. In addition, IPA inhibited basal lipid peroxidation levels in homogenates of hamster testis to reduce testicular cancer only when used in the highest concentrations of 2.5 and 5 mM [31].

In this work we found that the M1 mAChR is somehow involved in the protection of IPA against $FeCl_2$-induced oxidative stress in differentiated PC12M1 cells. This is based on results showing that the protective effect of IPA cannot be observed in differentiated PC12 cells which do not possess the M1 mAChR. However, we may assume that IPA is not acting via the traditional orthosteric M1 receptor, but rather via an allosteric/ectopic mAChR binding site [32-35], since the muscarinic antagonist, atropine did not block the protective effect of IPA in differentiated PC12M1 cells. The involvement of M1 mAChR in oxidative stress was reported in mice with chronic liver injury [36-37]. It appears that a non-traditional binding site in the M1 mAChR is involved in the mechanism of protecting cells from oxidative peroxidation. Thus, we suggest that IPA affect the M1 mAChR via a modulatory/ectopic site [38] rather than via the traditional binding locus. However, more experiments are needed in order to establish this theory.

In summary, we suggest that drugs based on IPA backbone may be used as beneficial drugs for diseases were oxidative stress is involved such as AD and other PNS and CNS disorders.

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