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SHORT COMMUNICATION

Arginine deiminases: Therapeutic tools in the etiology and pathogenesis of Alzheimer's disease

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Abstract

There is, at present, no definitive pre-mortem diagnostic tool for Alzheimer's disease, (AD) which relates to a poor understanding of its etiology. Brains of AD patients contain large amounts of the toxic plaque-forming β-amyloid$_{1-42}$ fragment in addition to elevated concentrations of the amino acid L-arginine. This work proposes that lowering levels of arginine in the astrocytes surrounding amyloid plaques may serve as a therapeutic tool in this neurodegenerative disorder. Arginine deiminase (ADI), from *Pseudomonas aeruginosa*, and peptidylarginine deiminase [PAD II], from bovine brain, are inhibited by amyloid peptides that contain arginine (amyloid$_{1-42}$) and those that have no arginine (amyloid$_{12-28/22-35}$). Enhanced activity of PAD II is noted with free L-arginine.

Keywords: *Arginine deiminase, peptidyl arginine deiminase, neurodegeneration, Alzheimer's disease, L-arginine, β-amyloid plaques*

Introduction

Alzheimer's disease (AD) is a progressive, debilitating and fatal form of dementia resulting in significant neurodegeneration. While several factors have been implicated as causative agents in the onset of dementia, the pathological route of disease progression is still unclear. The hypothesis that has received most support in recent years proposes that cerebral insult (either physical or physiological) leads to the production of elevated amounts of the toxic β-amyloid$_{1-42}$ (Aβ$_{1-42}$) (Figure 1C) protein fragment. These fragments form aggregates, known as senile plaques which result in the production of neurotoxic agents that result in neurodegeneration [1]. A decrease in the efficiency of catabolism and clearance of Aβ could cause the elevated levels of Aβ that are visible in AD patients. The mechanism of this process and the factors involved in it, however, remain unknown [2].

Amyloid plaques, that form between neurons, are extracellular and insoluble [1] and are surrounded by astrocytes, which also function to store reserve arginine in brain tissue. This indicates a potential connection between brain arginine content and amyloid plaque formation *in vivo* [3]. The distribution and prevalence of the different amyloid fragments is variable: in undamaged brain tissue, β-amyloid$_{1-40}$ (Aβ$_{1-40}$) is the most abundant form while in the brain tissue of AD patients, an excess amount of the plaque-forming Aβ$_{1-42}$ fragment is found [4]. In addition to this, several other amyloid fragments exhibit neurotoxic effects. Amyloid$_{12-28}$ (Figure 1A) is known to impair post-training memory in mice, but does not participate directly in the formation of senile plaques [5]. The fragment Aβ$_{22-35}$ (Figure 1B) is not as toxic as Aβ$_{1-42}$ (Figure 1C), though it forms aggregates and fibrils *in vivo* and has a visible neurotoxic effect. The inhibition of arginine-degrading enzymes by amyloid fragments depends largely on the amino acid sequence of the enzyme, and consequently on their ability to bind to various substrates.

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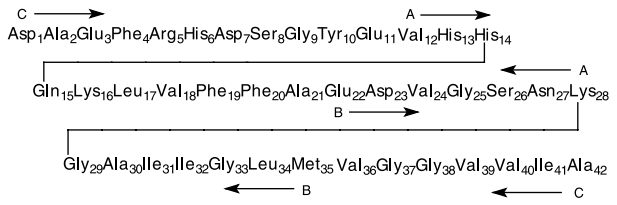


Figure 1. Amino acid sequences of amyloid peptides: [A] $A\beta_{12-28}$; [B] $A\beta_{22-35}$; [C] $A\beta_{1-42}$.

Though little is known of the mechanism of Aβ removal *in vivo*, it is thought that a reduction of Aβ catabolism is directly responsible for the accumulation of this peptide in the brain and its subsequent aggregation and plaque formation [6]. In addition, the various conditions of stress within the body (oxidative stress, trauma and/or head injury) are known to lead to the production of increased amounts of Aβ [7]. The higher the concentration of Aβ *in vivo*, the more likely it is to aggregate and form insoluble plaques. Importantly, it was shown that small amounts of Aβ can cause oxidative stress that leads to an increase of Aβ in the cell, initiating a feedback loop mechanism of enhanced oxidative damage [8].

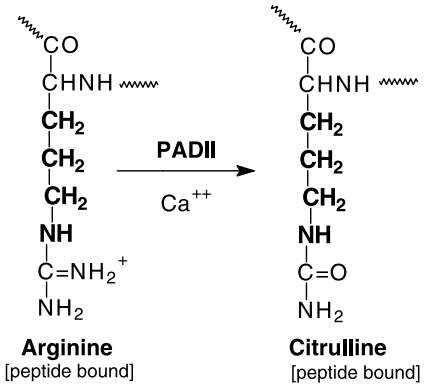
L-Arginine is an amino acid that maintains a positive charge at physiological pH and plays an important role in various physiological processes including cellular regeneration, immune function and polypeptide anabolism and catabolism [9]. Elevated levels of arginine have been observed in the cerebral tissue of persons suffering from AD [3] though it is not known whether this is a cause or a result of the disorder. In addition to its potential role in neurodegeneration, an increase in arginine levels have been implicated in the etiology of various types of cancer [10]. In order to lessen these effects attempts have been made to utilize the arginine-lowering ability of the enzyme arginine deiminase (ADI). It is therefore reasonable to assume that ADI, in a similar manner, could lower the levels of arginine in Alzheimer's patients, thereby acting as a therapeutic tool [11]. ADI converts arginine into citrulline and ammonia and while it lowers levels of arginine *in vivo*, its efficacy and safety as part of a treatment regimen are questioned since the enzyme is not endogenous to mammals [12].

The current therapeutic focus in AD is limited predominantly to treatment with acetylcholinesterase inhibitors. A lack of insight into the precise etiology of AD is hampering the development of efficient therapeutics and the use of enzymes implicated in neurodegeneration, either as drug targets or therapeutic agents, may offer a beneficial alternative to conventional drug use [13]. For an agent to be useful in the treatment of Alzheimer's disease, it must have a low toxicity to the patient; have the ability to cross the blood-brain barrier and be resistant to modification by any naturally occurring substance in the brain.

While the therapeutic potential of ADI in AD pathogenesis has not been verified, it makes the exploration of arginine degrading enzymes as potential therapeutic agents viable. This is significant in the etiology of Alzheimer's disease as the levels of β-amyloid present in affected brain tissue could inhibit the efficiency with which ADI would function if administered to patients suffering from AD. The use of an arginine-degrading enzyme that is not inhibited by characteristic changes in AD brain tissue (such as elevated levels of β-Amyloid) could also have implications in the etiology and pathogenesis of the disorder.

Though current literature suggests the mammalian enzyme peptidylarginine deiminase (PAD II) does not use free arginine as a substrate [14,15] the enzyme is responsible for the conversion of peptide-bound positive arginine to neutral citrulline by means of a calcium (Ca^{2+}) induced deimination reaction [15,16][Scheme 1]. This deimination affects the behaviour of proteins in the cellular environment since it induces proteins to unfold, which could subsequently act as a catalyst for the aggregation of susceptible proteins [17]. The reaction catalyzed by PAD II is localized to peptides in the astrocytes in the cerebral tissue that, as mentioned above, are reservoirs of arginine storage within the brain [18,19]. Since citrullinated proteins occur exclusively in brain tissue of patients affected by AD, they may serve as novel markers for neurodegeneration suggesting that an understanding of the function and action of PAD II may facilitate an understanding of neurodegeneration in general, and AD in particular [14,20]. The activity of PAD II may be a catalyst for amyloid aggregation and the subsequent formation of senile plaques in brain tissue of patients affected by AD.

It is the purpose of this paper to determine whether ADI and/or PAD II activity are inhibited by the amyloid fragments in order to ascertain the potential efficacy of these enzymes as markers/therapeutic agents in the etiology of Alzheimer's disease.



Scheme 1. Conversion of peptide-bound arginine to citrulline by PAD II.

Materials

1-Piperazineethane sulphonic acid, 4-(2-hydroxyethyl)-monosodium salt (HEPES), L-arginine, DEAE cellulose, dithiothreitol (DTT), β-mercaptoethanol, diacetyl monoxime, N-α-benzoyl-L-arginine ethyl ester (BAEE), thiosemicarbazide, phenyl-methylsulphonyl fluoride and amyloid peptides 12–28; 22–35 and 1–42 were obtained from Sigma. Bovine brain was obtained from a local abattoir.

Methods

Preparation of cell-free extract from pseudomonas aeruginosa

This was a modification of the published method [21]. Cultures of *P. aeruginosa* were prepared in nutrient broth (1.6%) and incubated at 30°C overnight. After 28 h, cells were harvested by centrifugation (5000 × g, 20 min, 4°C) on a Beckman centrifuge J2-21 equipped with a JA-14 rotor. The pellet was washed with NaCl (0.85%) [10 ml/l original culture medium] and finally suspended in phosphate buffer (0.01 M, pH 7.0, 10 ml/g wet weight cells). Lysis of the cells was effected by sonication using a Vibra Cell Sonicator (Sonics and Material, Inc.) (30 s bursts, 30 s intervals, 3 min). The cell debris was removed by centrifugation (46000 × g, 45 min, 4°C) with a Beckman centrifuge J2-21 equipped with a JD-20 rotor and the supernatant was stored at −80°C until required.

Purification of ADI from pseudomonas aeruginosa

This was a modification of the method already described [21]. Thawed cell-free extract (5 ml) was treated with protamine sulphate (10%), centrifuged (38000 × g, 40 min, 4°C) and the supernatant loaded on to a Sephacryl S-400 column (2 × 30 cm) previously equilibrated in 0.01 M phosphate buffer, pH 7.0. The column was then eluted with the same buffer and fractions (4.0 ml) collected and assayed for protein and arginine deiminase activity. All active fractions were pooled, dialysed against double distilled H_2O and stored at −20°C.

Purification of PAD II from bovine brain

The method outlined [15] was used in an attempt to purify PAD II. Briefly, bovine brain was homogenized in three volumes of buffer A (50 mM HEPES, pH 7.6, 1.0 mM EDTA, 0.5 mM DTT and 0.43 mM PMSF) prior to centrifugation (10 000 × g, 30 min) using a JA-14 rotor (Beckman). The supernatant (5.0 ml) was applied to a DEAE cellulose column (2 × 30 cm) and eluted stepwise with a 0–0.5 M sodium chloride gradient in buffer A. Active fractions were pooled and dialyzed overnight against buffer B (50 mM HEPES, pH 7.6, 0.1 M NaCl, 0.5 mM DTT, 10% glycerol, 10 mM β-mercaptoethanol and 10 mM $CaCl_2$).

Assay of PAD II

PAD II activity was determined by means of citrulline detection using a modification of the method described [22]. Samples (0.1 ml) were incubated in assay buffer (50 mM Tris-HCl, pH 7.6, 5 mM $CaCl_2$, 2 mM DTT and 5 mM BAEE) in a total volume of 3.0 ml for 30 min at 50°C prior to termination of the reaction by the addition of 5 M perchloric acid (1.0 ml). Chromogenic reagent (18 mM thiosemicarbazide in diacetyl monoxime solution added to a 1:1 sulphuric: ortho-phosphoric acid, 3.0 ml) was then added to the terminated reaction and the reaction mixture boiled for 5 min. The absorbance at 530 nm was compared to a citrulline standard curve. One unit of PAD II enzyme activity is defined as the amount of enzyme that deiminated 1 μmol of BAEE per hour at 50°C.

Assay of ADI

ADI activity was assayed, in a total volume of 320 μl by measuring the rate of NADH disappearance coupled with ammonia production from glutamate dehydrogenase (25 U, 16 μl), α-keto-glutarate (3.2 μl, 780 mM in TES Buffer, 50 mM, pH 7.2), NADH (3.2 μl, 40 mM), triton X-100 (160 μl, 1%), TES buffer (114.4 μl, 50 mM, pH 7.2) and sample (20 μl).The addition of arginine (3.2 μl, 600 mM) initiated the reaction.

SDS page

SDS-PAGE [23] was used to estimate enzyme purity and molecular mass. Samples (15 μl) and molecular mass markers (10 μl) ranging from 14–250 kDa were applied to a 12% SDS-PAGE at 200 v. The gels were stained using Coomassie brilliant blue R-250 followed by destaining in methanol-acetic acid-water (1:1:8 v/v/v). The distance moved by the enzyme was measured and its corresponding molecular size calculated from the calibration curve of log molecular weight versus distance migrated. Proteins used for calibration were lysozyme, 14.6 kDa; soybean trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; ovalbumin, 45 kDa; bovine serum albumin, 66.2 kDa; phosphorylase b, 97.4 kDa; β-galactosidase, 116.3 kDa; myosin, 205 kDa.

Determination of the effect of Aβ on enzyme activity

PAD II. Bovine brain was homogenized as described above and centrifuged (10000 × g, 30 min). The supernatant was incubated with amyloid fragments,

$A\beta_{12-28}$, $A\beta_{22-35}$ and $A\beta_{1-42}$ (0–150 nM) prior to assaying for remaining PAD II activity.

ADI. The effect of $A\beta_{22-35}$ (0–100 μM) on arginine deiminase activity was investigated by adding the β-amyloid to the enzyme assay mixture and measuring activity as described above.

Determination of the effect of free arginine on PAD II

The effect of varying concentrations of free arginine on PAD II activity was determined by incubating L-arginine (0–37.5 μM) with bovine brain tissue extract that was prepared by homogenization in buffer (50 mM Tris-HCl, pH 7.6, 10 mM β-mercaptoethanol and 10 mM EDTA) and centrifuged (10000 × g, 30 min) prior to the incubation. PAD II activity was assayed as described previously.

Results and discussion

Arginine deiminase from *Pseudomonas aeruginosa* was purified in 3.3% yield and 29.3 fold purity by column chromatography on Sephacryl S-400 according to a modified procedure [21]. The final dialysed fraction had a specific activity of 7656.6 Umg^{-1}, with two subunits of molecular mass of 66 and 39.5 kDa and was regarded as sufficiently pure to investigate its interaction with the amyloid peptides.

The specific activity of PAD II in the crude brain homogenate was 0.21 Umg^{-1}, and all attempts to purify the enzyme failed. PAD II was reported [24] to be unstable at 4°C and lost an appreciable amount of activity when stored at this temperature. Furthermore the enzyme, from *Halophilic solinarum*, is rapidly denatured in low salt concentrations (< 2 M) [25] and despite reports that the enzyme can be purified on DEAE cellulose with a 0–0.5 M NaCl gradient [15] all attempts, in our laboratory, led to rapid inactivation. The crude active enzyme (0.21 Umg^{-1}; 2.27 Ug^{-1} tissue) obtained for PAD II from bovine brain was similar to the value obtained for cerebral tissue (2.77 Ug^{-1}) [26] but far less than that of ADI isolated from *Pseudomonas aeruginosa* with specific activity of 7656.6 Umg^{-1}.

A preliminary study was conducted to determine the effect of arginine free $A\beta_{22-35}$ on arginine deiminase. A steady decrease in activity was noted with increasing concentrations of the peptide (Figure 2A) resulting in 97% inhibition at 100 μM indicating an interaction between the amyloid peptide and ADI. Since this latter enzyme is not endogenous to mammals the mammalian counterpart (PAD II) was also studied to determine if similar interactions with amyloid peptides would be observed.

The interaction between various amyloid fragments and PAD II was conducted on crude bovine brain enzyme homogenate with specific activity of 0.21 Umg^{-1}. The use of an amyloid blank compensated for the potential interfering ability of amyloid fragments with the citrulline assay [22]. The results showed a decrease in enzyme activity (Figure 2B, 2C and 2D).

In terms of etiological studies on Alzheimer's disease, it is important to note that the amyloid fragments investigated above were free in solution, and that the aggregates found in AD brain tissue may have a very different effect on PAD II activity. Aggregation would drive the hydrophilic portions of the $amyloid_{1-42}$ fragment away from the surrounding solvent. In the aqueous environment at pH 7.4 the

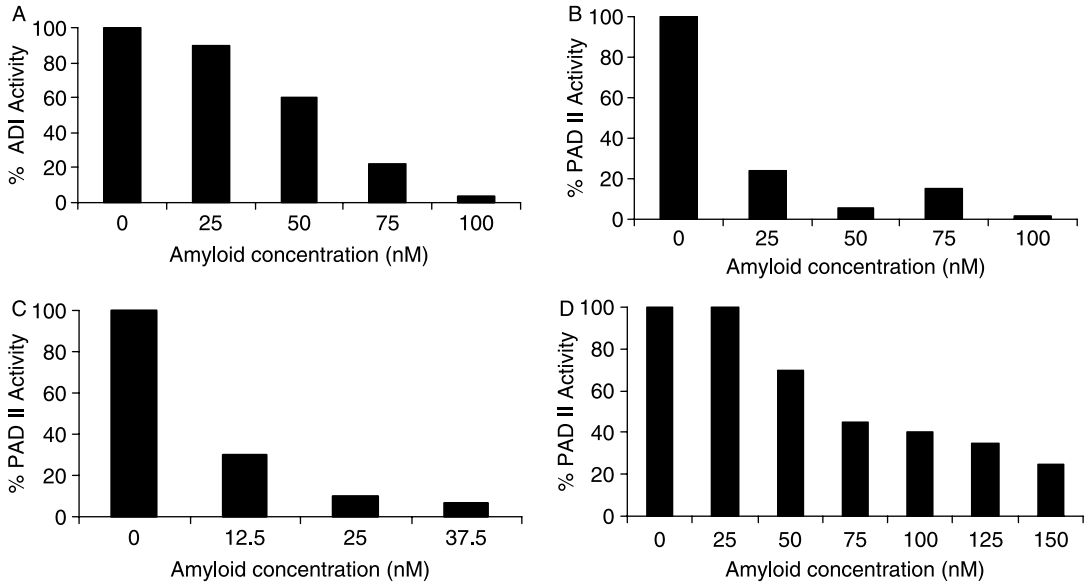


Figure 2. Effect of different concentrations of amyloid peptides (1–42; 12–28; 22–35) on ADI and PAD II activity; 100% = 0.2 Umg^{-1}. [A] 22–35 on ADI [B] 12–28 on PAD II [C] 22–35 on PAD II [D] 1–42 on PAD II. Data points are averages of duplicate readings.

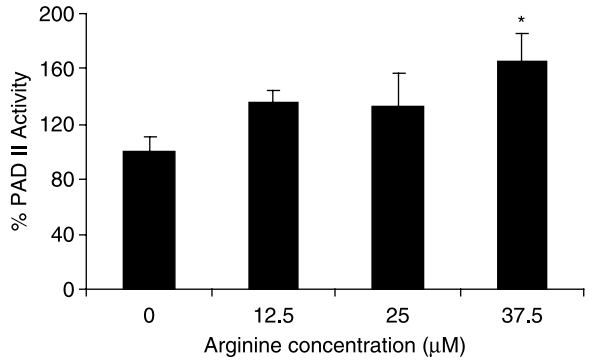


Figure 3. Effect of L-arginine on PAD II activity from crude bovine brain; 100% = 0.2 Umg^{-1}. Data points are the mean of triplicate readings. $p < 0.05$ vs control; * $p < 0.01$ vs control.

arginine is protonated and would therefore occur in the outer region of a senile plaque comprised of $A\beta_{1-42}$. It may be that aggregated forms of amyloid (in the form of senile plaques) do not interact with PAD II, which could explain the elevated levels of enzyme activity during the progression of AD. The fact that PAD II levels are elevated in AD patients suggests that deimination may be directly involved in AD pathology, potentially deiminating proteins that are important in the amyloid cascade reaction.

Free L-arginine has been reported to not have an appreciable effect on PAD II *in vitro* [15,27] yet results from our current study suggest that this may be incorrect. Addition of free arginine to the enzymatic reaction increased the activity of PAD II in the crude homogenate (Figure 3) raising the possibility that free arginine binds to the enzyme and that PAD II is able to lower arginine levels in AD brain tissue providing a potential therapeutic agent. The ability of PAD II to interact with free arginine is significant as the enzyme occurs in astrocytes, which are storage sites for reserve arginine in cerebral tissue. AD brain tissue is characterized by an increase in levels of free arginine and this finding could potentially explain the concomitant increase of PAD II activity in AD patients.

Acknowledgements

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