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Estrogen receptor α promotes non-amyloidogenic processing of platelet amyloid precursor protein via the MAPK/ERK pathway

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ABSTRACT

Deposition of amyloid β peptide (Aβ), a proteolytic product of amyloid precursor protein (APP), in senile plaques and in the walls of cerebral blood vessels is a hallmark of Alzheimer's disease (AD). Platelets contain high levels of APP and Aβ and may contribute to amyloid deposits seen in AD. However, the biochemical mechanism(s) involved in the regulation of platelet APP metabolism are largely unknown. The estrogen receptor α (ERα) is found to be expressed in platelets. It has not been elucidated whether ERα-mediated non-genomic signaling intervenes with platelet APP processing. Using ERα knock-out (α-ERKO) mice and wild type (WT) littermates, the present study demonstrated that ERα-specific agonist propylpyrazole triol (PPT) promoted non-amyloidogenic processing of platelet APP via the mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) pathway. The underlying basis involves direct association of activated ERK with a disintegrin and metalloprotease domain 17 (ADAM17, an α-secretase candidate) and ERK-dependent threonine phosphorylation of ADAM17. These results suggest that selective modulation of ERα in peripheral target tissues may serve as an anti-amyloidogenic strategy for AD and other amyloidogenic diseases.

1. Introduction

Aβ is a 36–43 amino acids peptide derived proteolytically from APP. APP is commonly processed by two competing pathways; one involves cleavage by α-secretase and then by γ-secretase, whereas the other involves processing by β-secretase and then by γ-secretase [1–3]. The former pathway produces sAPPα and p3 and the latter pathway produces sAPPβ and Aβ [1–3]. It was previously demonstrated that the two pathways exert antagonistic effects on the generation of Aβ [4]. β-secretase-mediated APP metabolism produces Aβ, whereas α-secretase cleavage of APP within the Aβ domain precludes the generation of Aβ [4].

Deposition of Aβ in senile plaques and in the walls of cortical and leptomeningeal blood vessels is a hallmark of AD [5,6]. APP is expressed in a variety of cell types in the brain and elsewhere, but the origins of Aβ deposits in AD brain and deposits in the cerebral vessels are uncertain [6]. Intracerebral deposition of Aβ is most likely to have been produced locally by neurons [6]. However, investigators have shown that circulating Aβ can be internalized and accumulated in vascular smooth muscle cells [7,8], and Aβ – apolipoprotein E and Aβ – apolipoprotein J complexes can cross the blood brain barrier (BBB) [8,9]; thus, circulating Aβ may also contribute to the development of Aβ deposits in AD brain and deposits in the cerebral vessels.

Platelets are considered a major source of APP and Aβ in circulation. Platelets contain high levels of APP and proteases involved in APP processing (α-, β-, and γ-secretases) [10]. Platelet APP and APP proteolytic products (sAPPα, sAPPβ and Aβ) are stored in a-granules, and are readily released by agents that induce platelet degranulation, such as collagen [10]. To date, however, the biochemical mechanism(s) involved in the regulation of platelet APP metabolism are largely unknown.

Recent studies suggest that ERα-mediated signaling pathways such as the MAPK/ERK pathway are involved in the regulation of APP metabolism by some phytoestrogens (e.g., ginsenosides and epigallocatechin gallate) in cell lines of neuronal origin and in the brains of animal models of AD [11–14]. The ERα is found to be expressed in circulating platelets [15]. ERα-mediated non-genomic signaling may occur in these anucleated cells. However, the

Abbreviations: Aβ, amyloid β peptide; APP, amyloid precursor protein; AD, Alzheimer's disease; ADAM, a disintegrin and metalloprotease domain; BBB, blood brain barrier; ERα, estrogen receptor α; α-ERKO, estrogen receptor α knock-out; ERK, extracellular-signal-regulated kinase; EDTA, ethylene dinitrilotetra-acetic acid; EGTA, ethylene glycol tetraacetic acid; MAPK, mitogen-activated protein kinase; PPT, propylpyrazole triol; PGI2, prostacyclin; PRP, platelet-rich plasma; PMSF, phenylmethylsulfonyl; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WT, wild type.

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mechanism(s) of ERα regulation of platelet APP metabolism have not been elucidated. Using α-ERKO mice and their WT littermates, this study assesses whether and how ERα modulates platelet APP metabolism. Knowledge of this mechanism could provide a rational basis for selective modulation of ERα in peripheral target tissues as an anti-amyloidogenic strategy for AD and other amyloidogenic diseases.

2. Methods

2.1. Reagents

Antibodies to Shc ERα (H184), phosphospecific ERK1/2 (Thr202/Tyr204) and total ERK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to APP, Aβ, ADAM17, ADAM10 and phosphothreonine were purchased from Abcam (Cambridge, MA, USA). Antibody to sAPPα was purchased from Covance-Signet (Princeton, NJ, USA). IRDye 800CW Donkey Anti-Rabbit IgG was purchased from LI-COR Biosciences (Lincoln, NE, USA). Unless stated otherwise, all chemicals were purchased from Sigma (St. Louis, MO, USA). Stock solutions (1000×) of ERα-specific agonist PPT and MAPK inhibitor PD98059 were prepared in ethanol and added to the platelet suspensions at the indicated concentrations. Stock solutions of collagen (5 mg/ml, human placenta collagen type I) were prepared in water (the pH was adjusted to 3.0 with acetic acid). In all experiments, an equivalent volume of ethanol (0.1%) was added to the control group.

2.2. Mice

Seventy-four 8–12 weeks old C57BL/6J mice (half male and half female) and 49 8–12 weeks old α-ERKO mice (half male and half female) were purchased from Jackson Laboratory. The α-ERKO mice (B6.129P2-Esr1tm1Ksk/J) have been maintained on a C57BL/6J background for 10 generations. Prior to the experiments, these mice were housed under standard conditions, 25 °C with a 12 h light/dark cycle, and allowed free access to water and a standard diet. The care and use of animals were in accordance with the code of (Laboratory Animal-Requirements of Environment and Housing Facilities) (GB 14925 – 2001) and (Beijing Administration Rule of Laboratory Animal).

2.3. Blood collection and platelet preparation

Mice were euthanatized by CO_2 inhalation. Blood was collected from the right ventricle and then mixed with sodium citrate to a final concentration of 0.38%. The citrated blood was incubated with 10 ng/ml prostacyclin (PGI_2) for 5 min. Platelet-rich plasma (PRP) was prepared by centrifugation of the blood at 200 g for 15 min. Platelets were pelleted from PRP by centrifugation at 1500 g for 10 min. The resultant platelet pellets were then washed twice with platelet wash buffer (10 mM sodium citrate, 150 mM NaCl, 1 mM ethylene dinitrilotetra-acetic acid (EDTA), 1% dextrose, pH 7.4). The washed platelet was gently resuspended in modified Tyrode's buffer (150 mM NaCl, 5 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, 0.55 mM NaH_2PO_4, 7 mM $NaHCO_3$, 2.7 mM KCl, 0.5 mM $MgCl_2$, 5.6 mM glucose, pH 7.4) The final platelet counts were adjusted to 1×10^8/ml. Platelets were prepared from animals immediately before a given assay.

2.4. Pharmacological treatments of platelets

To determine the role of ERα in platelet APP metabolism, platelets suspensions were incubated with 0.2–100 nM ERα agonist PPT under gentle agitation at 37 °C for 30 min [16]. To determine whether ERα regulation of platelet APP metabolism involves the MAPK/ERK signaling, platelets were incubated with PPT in the presence or absence of MAPK inhibitor PD98059 (25 μM). After drug treatment, APP proteolytic products Aβ and sAPPα released from inactivated and activated platelets were examined. Other parameters were examined when platelets were at rest. To make platelets at rest, platelets were treated with PPT in the presence of 3 μg/mL apyrase, 10 ng/ml PGI_2, and 1 mM ethylene glycol tetraacetic acid (EGTA). To activate platelets, after PPT treatment, platelets were incubated with collagen (human placenta collagen type I, 10 mg/ml) for additional 10 min [17].

2.5. Measurement of sAPPα levels

Platelet secretion of sAPPα was measured using a mouse/rat sAPPα (highly sensitive) ELISA kit (IBL International, Hamburg, Germany). Following platelet treatment as described above, the medium was collected and sAPPα levels were measured according to the manufacturer's protocol. Spectrophotometric data were collected using the Victor-2 Multilabel counter (PerkinElmer/Wallace, Akron, OH, USA) at the wavelength of 450 nm.

2.6. Measurement of Aβ levels

Platelet secretion of Aβ was measured by chemiluminescence using a BetaMark total Aβ ELISA kit (Covance, Dedham, MA). Following platelet treatment as described above, the medium was collected and Aβ levels were measured according to the manufacturer's protocol. The chemiluminescentic data were collected using the Victor-2 Multilabel counter.

2.7. Measurement of α-secretase activity

In this study, platelet α-secretase activities were measured using an α-secretase activity kit (R&D Systems, Minneapolis, MN, USA). Following treatment, platelets were collected by centrifugation of the platelet suspension at 1500 g for 10 min. Platelet pellets thus obtained were lysed in the extraction buffer provided in the kit. Platelet α-secretase activities were then measured by fluorescent spectroscopy according to the manufacturer's protocol. The cleavage-dependent release of fluorescence signal was quantified using the Victor-2 Multilabel counter at an excitation wavelength of 340 nm and an emission wavelength of 495 nm.

Total protein concentration of platelet pellets was determined with a Lowry-based detergent compatible protein assay kit (Bio-Rad Hercules, CA, USA). Briefly, platelets were lysed with RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 1 mM EGTA, 10 mM NaF, 1 mM Na3VO4, 1% TritonX-100, 0.5% sodium deoxycholate, plus 1 mM phenylmethylsulfonyl (PMSF), 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin). Total protein concentration in platelet lysates was then measured according to the manufacturer's protocol. Spectrophotometric data were obtained using the Victor-2 Multilabel counter at a wavelength of 650 nm. The enzyme activities were corrected for protein content.

2.8. Western blot analysis of sAPPα, Aβ and p-ERK

Following drug treatment, the platelets were collected and lysed with RIPA buffer. Platelet lysates were boiled in two-fold loading buffer (100 mM Tris–HCl, pH 6.8, 4% sodium dodecyl sulfate, 200 mM dithiothreitol, 0.2% bromophenol blue, and 20% glycerol) for 5 min. The proteins (50 μg per well) were subjected to a 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 3 h and then transferred onto nitrocellulose membranes using a semi-dry transfer apparatus (Bio-Rad Hercules, CA, USA). The transfer of proteins was performed for 1 h at 10 V. The blots thus