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# Discussion of the Role of the Extracellular Signal-Regulated Kinase-Phospholipase $A_2$ Pathway in Production of Reactive Oxygen Species in Alzheimer's Disease*

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In this paper we show that exposure of a rat brain synaptosome fraction to the amyloid beta peptide fragment βA(25-35), but not the inverted peptide βA(35-25), stimulated production of reactive oxygen species (ROS) in a concentration- and time-dependent manner. The ROS formation was attenuated by the tyrosine kinase inhibitor genistein, the mitogen-activated protein kinase inhibitor U0126, and the phospholipase $A_2$ ($PLA_2$) inhibitor 7,7-dimethyl-(5Z,8Z)-eicosadienoic acid. This strongly suggests that βA(25-35) stimulated ROS production through an extracellular signal-regulated kinase-$PLA_2$-dependent pathway. The interaction between these enzymes and their possible involvement in free radical formation in Alzheimer's disease are discussed.

**KEY WORDS:** Amyloid; extracellular signal-regulated kinase 1 and 2; phospholipase $A_2$; reactive oxygen species; Alzheimer's disease.

## INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia in adult life. Major pathological hallmarks of the disease include neurofibrillary tangles, senile plaques, and loss of synapses in selected brain regions (1). Patients are characterized by a profound impairment of recent memory, which is due to damage in the hippocampus and associated areas in the temporal cortex (2). Different studies have shown an elevated level of oxidative stress in AD brains (3,4), and the amyloid beta peptide (βA), which is the principal constituent of plaques, has been identified as a possible source for this free radical formation (5,6). A sustained production of reactive oxygen species (ROS) will lead to oxidation of DNA, proteins and lipids, and ultimately to cell death. However, it is still controversial whether oxidative stress plays an early role in the disease, or alternatively, is secondary to deposition of plaques and formation of tangles.

The extracellular signal-regulated kinases 1 and 2 (Erk1/2), which are two mitogen-activated protein (MAP) kinases identified in the brain, need both threonine and tyrosine phosphorylation to become active (7,8). So far, most of the focus on downstream targets of Erk1/2 has been on transcription factors such as c-Fos, c-Myc, and c-Jun (9,10), but other targets may also be affected, and accumulated evidence indicates that Erk1/2 may be involved in both physiological and pathological activities in the central nervous system (11,12). The classical pathway activating Erk1/2 is through the receptor tyrosine kinase/Ras/Raf/Mek cascade. However, Erk1/2 has also been shown to be activated by protein kinase C (PKC) after stimulation of NMDA and metabotropic glutamate receptors in cultured rat hip-

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pocampal neurons (13), by nitric oxide in cultured rat cerebellar granule cells (14), by phosphatidylinositol 3-kinase in primary cultures of mouse striatal neurons (15), and by hydrogen peroxide in astrocytes (16) and oligodendrocytes (17). Erk1/2 may also be activated by calcium influx after stimulation of the NMDA receptor (18) and the AMPA/kainate receptor (19).

Activation of the Erk1/2 MAP kinase cascade is probably implicated in several glutamate receptor–dependent physiological functions, such as hippocampal long-term potentiation (20) and cerebellar long-term depression (21), which both are believed to be major mechanisms contributing to mammalian learning and memory. Phosphorylation of Erk1/2, and thereby activation, may also be of importance in the development of neurodegenerative diseases (22). Dineley et al. (23) argue that hippocampus-dependent learning and memory impairments characteristic for AD patients are due to an increased βA burden and chronic activation of the Erk1/2 MAP kinase cascade through the α7nicotinic acetylcholine receptor. Furthermore, Erk1/2 can activate cytosolic phospholipase $A_2$ (cPLA$_2$) (24). This enzyme is overexpressed in AD brains (25,26) and may be associated with oxidative stress.

We have examined the contribution of the Erk1/2-PLA$_2$ pathway concerning formation of ROS in a rat brain synaptosome fraction after exposure to the amyloid beta peptide fragment βA(25-35). The tyrosine kinase inhibitor genistein (27,28), the mitogen-activated protein kinase (Mek1/2) inhibitor U0126 (29,30), and the arachidonic acid–specific PLA$_2$ inhibitor 7,7-dimethyl-(5Z,8Z)-eicosadienoic acid (DEDA) (31) were employed to elucidate whether these enzymes participate in immediate ROS production stimulated by βA(25-35). The interaction between these enzymes and their possible role in AD are discussed.

## EXPERIMENTAL PROCEDURE

*Materials.* 2′,7′-Dichlorofluorescin diacetate (DCFH-DA), 7,7-dimethyl-(5Z,8Z)-eicosadienoic acid (DEDA), and 4′,5,7-trihydroxyisoflavone (genistein) were purchased from Sigma-Aldrich (St. Louis, MO, USA). U0126 was from Promega (Madison, WI, USA), and methyl mercury chloride was obtained from KEBOLab (Oslo, Norway). Hanks Balanced Salt Solution (HBSS) (containing 1.26 mM $CaCl_2 \times 2H_2O$, 5.37 mM KCl, 0.44 mM $KH_2PO_4$, 0.49 mM $MgCl_2 \times 6H_2O$, 0.41 mM $MgSO_4 \times 7H_2O$, 0.14 M NaCl, 4.17 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4$, 5.55 mM D-glucose) and HEPES buffer were purchased from GibcoBRL (Paisly, UK). βA(25-35) (Lot.#539704 and Lot.#551370) and βA(35-25) (Lot.#517245) came from Bachem AG (Bubendorf, Switzerland).

*Exposure to βA(25-35) and βA(35-25).* The peptides were stored at −20°C and dissolved in distilled water before use. They were further diluted in the test medium.

*Preparation of a Rat Brain Synaptosome Fraction.* A rat brain synaptosome fraction was in principle prepared by the method of Gray and Whittaker (32) as described by Mariussen and Fonnum (33). One male Wistar rat (150–200 g; Møllegaard breeding laboratories, Denmark) was decapitated in each experiment, and the cerebral cortex was quickly dissected out, placed in ice-cold 0.32 M sucrose, and homogenized (~5% w/v) gently. The homogenate was centrifuged for 10 min (1000 *g*, Sorvall SA-600 rotor). After centrifugation the supernatant was mixed (1:1) with 1.3 M sucrose to obtain a 0.8 M sucrose solution. Centrifugation for 30 min (20,000 *g*) resulted in a myelin-rich supernatant and a pellet ($P_2$) consisting of free mitochondria covered with synaptosomes. The supernatant was removed before the synaptosomes were washed out by adding 1 ml ice-cold 0.32 M sucrose. The protein content was determined by using the method of Lowry et al. (34).

*DCF Fluorescence Assay.* Formation of ROS in the rat brain synaptosome fraction was determined by using the probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) as described by Myhre and Fonnum (35). The synaptosome fraction was diluted in HEPES-buffered (20 mM) HBSS (pH 7.4) with additional glucose (10.55 mM, final concentration) (~0.3 mg protein/ml buffer), and then incubated with DCFH-DA (5 μM) for 15 min at 37°C. After centrifugation of the dye-loaded samples the extracellular buffer was exchanged with fresh buffer, and the pellet was dissolved gently. The reaction was started by transferring 200 μl of the synaptosome fraction to a microplate (96 wells, 250 μl; final concentration ~0.25 mg protein/ml buffer) containing HEPES-buffered HBSS and the compounds (peptide and/or inhibitor) to be tested. The measurements of the DCF-mediated fluorescence were performed every second minute for 40 min on a computerized Perkin-Elmer LS 50 luminescence spectrometer using excitation wavelength 488 nm and emission wavelength 530 nm. The incubation temperature was 37°C.

The mechanism involved in the βA(25-35)-induced formation of ROS was elucidated by use of different inhibitors (genistein, U0126 and DEDA). The controls contained the synaptosome fraction in buffer, and when included, the synaptosome fraction in buffer with an inhibitor.

*Statistical Analysis.* One-way analysis of variance (ANOVA) and group comparisons with Newman-Keuls post-hoc test (Sigma Stat statistical software program version 1.0), or Student's t test, paired two samples of means (Excel 97 version), was performed to determine statistical significance. A *P*-value less than .05 was considered statistically significant.

## RESULTS

βA(25-35) stimulated production of ROS in the rat brain synaptosome fraction in a concentration- (Fig. 1) and time-dependent (Fig. 2) manner. ANOVA revealed a significant difference between the concentrations tested ($F_{4,27} = 30.9$, $P < .0001$). Post-hoc comparisons with the Newman-Keuls method confirmed that all the concentrations, except 6.25 μM, were significantly different from both the cell control and each other ($P < .05$). The inverted peptide βA(35-25) was used as a negative control and did not elevate the DCF fluorescence, showing that the effect of βA(25-35) was not due to a nonspecific

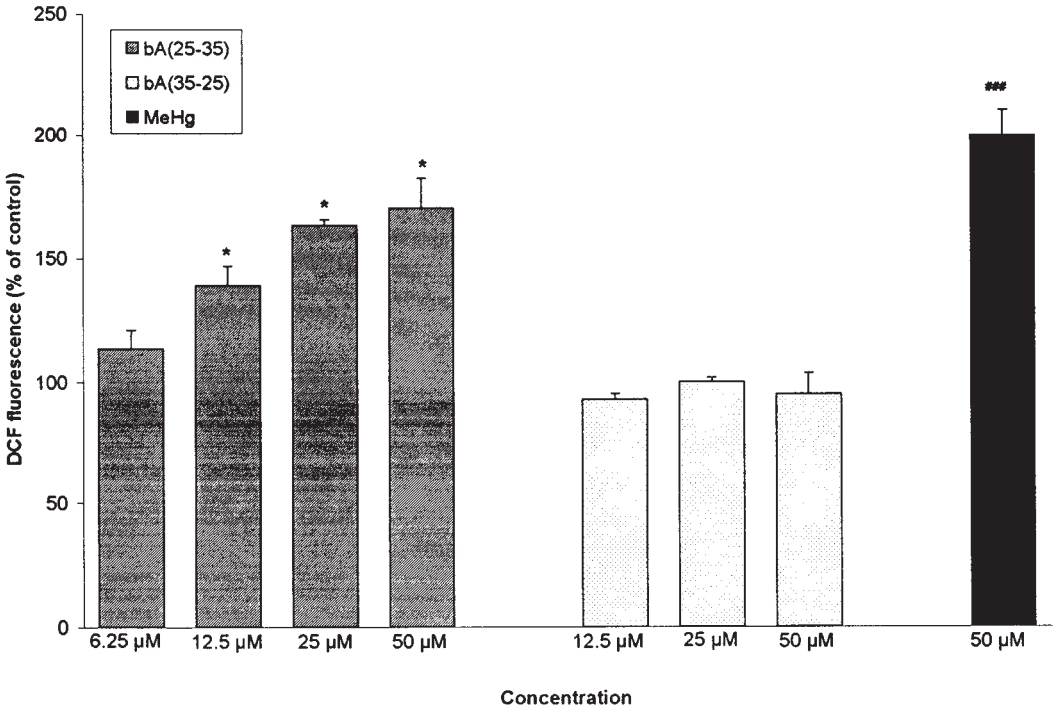


**Fig. 1.** Increased DCF fluorescence as an expression for ROS formation in a rat brain synaptosome fraction after exposure to increasing concentrations of βA(25-35) and βA(35-25), and MeHg (50 µM). The control (nonstimulated cells) is set to 100. Values are mean + SEM, n = 4–11 assayed in triplicate. *Statistically higher than the control, $P < .05$ (One-way ANOVA, Newman-Keuls; see text for details). ###Statistically higher than the control, $P < .0001$ (Student's t test).

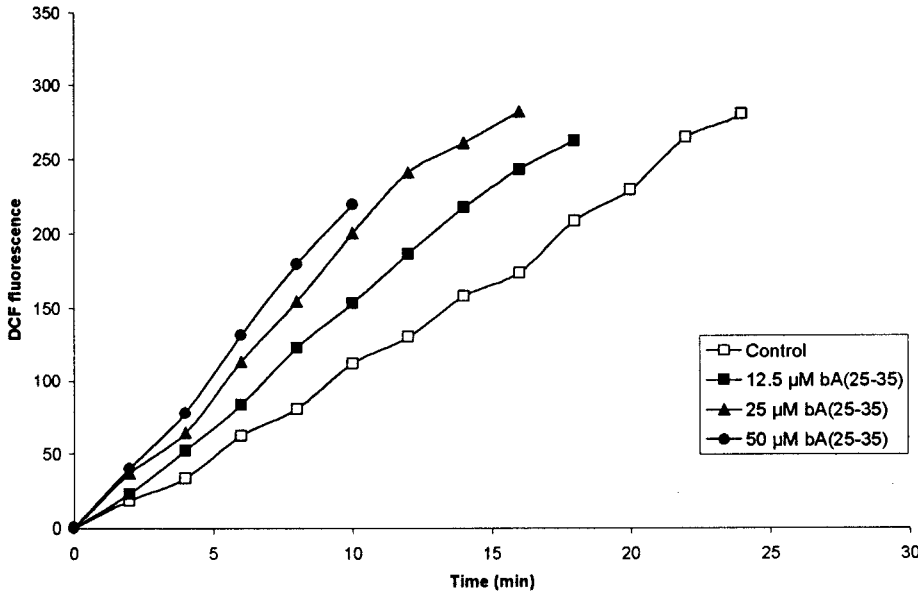


**Fig. 2.** Typical response curves showing DCF fluorescence as a function of time (min) in a rat brain synaptosome fraction after stimulation of ROS formation with increasing concentrations of βA(25-35).

effect of the peptide length. Methyl mercury (MeHg; 50 μM), which was used as a positive control, stimulated the DCF fluorescence significantly, measured by Student's t test, compared to the cell control ($P < .0001$).

Different inhibitors were used to elucidate involvement of the intracellular signaling pathway (Fig. 3). Treatment of the synaptosome fraction with the protein tyrosine kinase inhibitor genistein (100 μM) (27,28) reduced the fluorescence by 78%. One-way ANOVA revealed a reliable overall effect ($F_{2,18} = 69.6$, $P < .0001$), and the Newman-Keuls post-hoc test showed that genistein reduced the βA(25-35)-mediated response significantly ($P < .05$). ANOVA also uncovered a reliable effect of the noncompetitive Mek1/2 inhibitor U0126 (10 μM) (29,30) ($F_{2,24} = 19.5$, $P < .0001$). The βA(25-35) response was reduced by 75% ($P < .05$). Incubation of the synaptosome fraction with DEDA (5 μM), a competitive arachidonic acid–specific $PLA_2$ inhibitor (31) lowered the fluorescence by 67% ($F_{2,24} = 97.7$, $P < .0001$). Group comparisons showed that this concentration of DEDA had a significant effect on the βA(25-35)-stimulated response ($P < .05$).

The inhibitor controls of U0126 and DEDA were significant lower than the cell controls ($P < .05$, Student's t test).

## DISCUSSION

Our results show that exposure of a rat brain synaptosome fraction to βA(25-35) (6.25–50 μM), but not the inverted peptide βA(35-25), stimulated production of ROS in a concentration-dependent manner (see Fig. 1). The response started immediately and increased during the experiment (see Fig. 2). MeHg has been used to show formation of ROS in several papers, and was used as a positive control in the present experiments (see Fig. 1) (For discussion see [35]). Both clinical and epidemiological studies have indicated that βA may be associated with oxidative stress and cell death (3,4), but the exact mechanism(s) for this toxicity is still uncertain. An interesting and, as far as we know, new finding from our study is that βA(25-35) may stimulate formation of ROS through an Erk1/2-$PLA_2$-dependent pathway (see Fig. 3). The Mek1/2 inhibitor U0126 (29,30), the tyrosine kinase inhibitor genistein (27,28), and the $PLA_2$ inhibitor DEDA (31) all reduced the formation of ROS. In the following discussion we concentrate on the interaction between these affected enzymes and their possible involvement in free radical formation in AD.

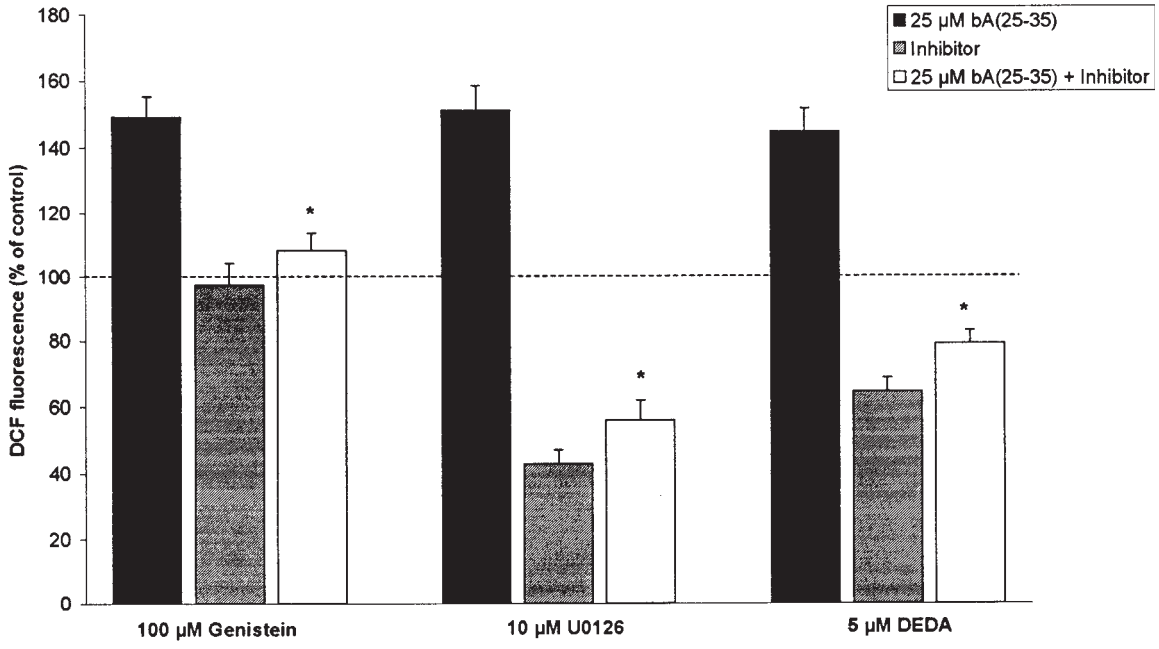


**Fig. 3.** The effect of the tyrosine kinase inhibitor genistein (100 μM), the Mek1/2 inhibitor U0126 (10 μM), and the arachidonic acid–specific $PLA_2$ inhibitor DEDA (5 μM) on ROS formation in a rat brain synaptosome fraction after exposure to 25 μM βA(25-35). Values are mean + SEM, n = 7–9 assayed in triplicate. *Statistically different from 25 μM βA(25-35), $P < .05$ (One-way ANOVA, Newman-Keuls; see text for details).

In mammals, three major groups of MAP kinase pathways have been identified, namely Erk1/2, JNK/SAPK, and p38 (36,37). The Erk1/2 MAP kinases play a pivotal role in mediation of cellular responses to a variety of signaling molecules (38–40), and activation by growth factors, such as NGF and EGF, is well documented (41–43). Also, numerous neurotransmitters and hormones stimulate Erk1/2 through either G-protein–coupled receptors or ligand-gated ion channel–coupled receptors (44). In addition to physiological roles, accumulating evidence suggest that Erk1/2 MAP kinases may be involved in neuronal death caused by oxidative stress in vitro (45,46) and in glutamate-induced toxicity in primary cortical neuron cultures and the hippocampal cell line HT22 (47). In the rat brain in vivo Erk MAP kinase is stimulated following electroconvulsive shock (48), generalized seizure (49), and transient ischemia (50).

MAP kinase activation has also been documented in AD, where neurons immunoreactive for Mek1 and Erk1/2 are found in the direct vicinity of neuritic plaques or even within plaques (22). By use of immunocytochemistry, Zhu et al. (12) provided evidence of a sequential activation of Erk1/2, JNK/SAPK, and p38 in susceptible neurons in individuals with this disease. In mild and severe cases (Braak stages III to VI), all three MAP kinase cascades are activated in the same neurons, while in predemented cases with limited pathology (Braak stages I and II) (2), Erk1/2 and JNK/SAPK are activated, but not p38. Given that the predemented cases have a high tendency to develop AD (51), it is likely that activation of Erk1/2 and JNK/SAPK represents one of the earliest stages in the pathogenesis. Because activation of Erk1/2 is generally thought of as a response to mitotic signaling, whereas activation of JNK/SAPK and p38 is associated with a response to oxidative stress, the authors argue that abnormalities in either of these events can serve to initiate the disease. Microglia that are in contact with senile plaques also exhibit high levels of tyrosine-phosphorylated proteins (52). McDonald et al. (53,54) and Combs et al. (55) have shown that exposure of microglia and THP1 monocytes to fibrillar forms of βA results in activation of two parallel intracellular signaling pathways, involving Erk1/2 and p38, and generation of superoxide ($O_2^{\bullet -}$) radicals.

Taken together, these experiments indicate that βA, which is an important component in plaques, may stimulate production of ROS through activation of specific MAP kinases. It is interesting that MAP kinases also can phosphorylate the cytoskeleton-associated protein tau in vitro (56) and in vivo (57). Hyperphosphorylated tau gives paired helical filaments, which is a pathological hallmark in the brains of AD patients (2).

We found that both the tyrosine kinase inhibitor genistein, the Mek1/2 inhibitor U0126, and the arachidonic acid–specific $PLA_2$ inhibitor DEDA reduced the βA-induced formation of ROS. The question then is whether there is a connection between the Erk1/2 pathway and $PLA_2$. Several laboratories have suggested that Erk1/2 and p38 may play an important role in regulation of the $PLA_2$ activity (58–60). Erk MAP kinase increases the enzymatic activity of $cPLA_2$ by phosphorylating Ser-505 (24). In addition to phosphorylation, full activation of $cPLA_2$ requires increased cytosolic $Ca^{2+}$ (61,62). Upon stimulation, $cPLA_2$ is rapidly translocated from the cytosol to the membrane, where it initiates degradation of phospholipids at the *sn*-2 position, liberating free fatty acids and lysophospholipids (63). Induction of $PLA_2$ and release of arachidonic acid and other free fatty acids have been reported in various neuronal disorders, including ischemia (64), schizophrenia (65), and AD (25). An uncontrolled arachidonic acid cascade may lead to formation of $O_2^{\bullet -}$ and set the stage for lipid peroxidation and oxidative damage to membrane proteins. Physiological roles of arachidonic acid itself may involve control of gene expression, modulation of ion channels, and regulation of the activity of enzymes such as protein kinase A, PKC, and the NADPH oxidase. Free fatty acids and lysophospholipids can uncouple oxidative phosphorylation and change the membrane permeability (63). Hence, activation of $PLA_2$ may have widespread effects.

In a previous work we have shown that free radical formation in human neutrophil granulocytes after exposure to βA(25-35) involves activation of the Erk1/2 MAP kinase pathway, arachidonic acid–specific $PLA_2$, and the NADPH oxidase complex (66). Traditionally, the NADPH oxidase, which produces $O_2^{\bullet -}$ in inflammatory cells, has been assumed to be restricted to nonneuronal cells, but recently it has been shown that this enzyme complex may contribute directly to oxidative stress and apoptosis in neurons (67). We cannot completely rule out a contribution of the NADPH oxidase complex in our system, because the arachidonic acid cascade activated by arachidonic acid–specific $PLA_2$ forms free fatty acids that can activate the NADPH oxidase both directly (68) and via PKC (69). It is also known that the Erk1/2 pathway has the ability to stimulate the NADPH oxidase by phosphorylating $p47^{phox}$ (70), which is present in neurons (67).

Sustained production of free radicals in nerve tissue may damage central cellular constituents and ultimately lead to cell death, which is one of the pathological

hallmarks in AD. From our results and the discussion above it seems likely that at least part of the oxidative damage observed in AD brain neurons may be due to activation of the Erk1/2-$PLA_2$ pathway because βA has the capability to both stimulate Erk1/2 (55) and elevate the intracellular concentration of $Ca^{2+}$ in different cell systems (71,72). Neurons express a basal level of phosphorylated Erk (73). The increased DCF fluorescence in the nonstimulated control (see Fig. 2) may therefore be a result of basal ROS formation due to continuous Erk activity.

In Fig. 4 we have predicted a working hypothesis for βA-mediated ROS production in neuronal tissue. Genistein, which inhibits Erk1/2 by inhibiting tyrosine kinases, and U0126, which inhibits Erk1/2 via Mek1/2, both prevent activation of arachidonic acid–specific $PLA_2$, and thereby the arachidonic acid cascade. The result is reduced production of ROS in the cell. Erk1/2 may also stimulate the NADPH oxidase directly, but the observed reduction in ROS formation after incubation with the arachidonic acid–specific $PLA_2$ inhibitor DEDA makes the Erk1/2-$PLA_2$ pathway more likely. McDonald et al. (54) have suggested that the cascade may be activated by an interaction between the tyrosine kinase receptor and βA(25-35).

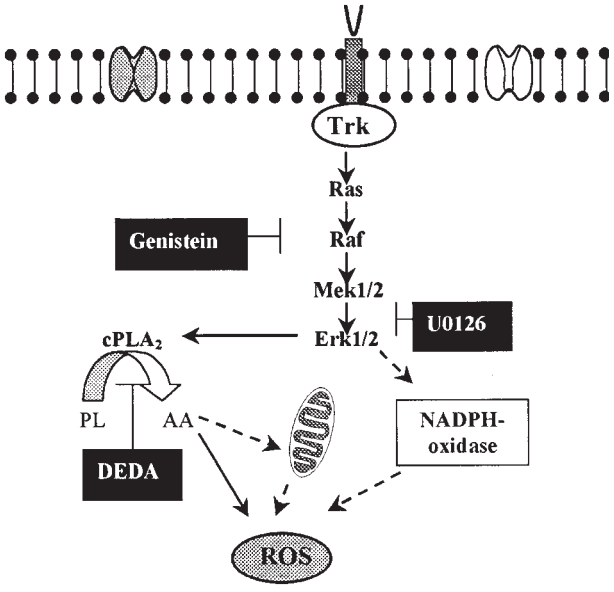


**Fig. 4.** A possible mechanism for Erk1/2-induced oxidative stress in neuronal tissue after exposure to βA(25-35) may be through activation of $cPLA_2$, and thereby uncontrolled arachidonic acid (AA) release and formation of ROS. AA may also lead to uncoupling of the mitochondrial respiratory chain, and thereby increased production of $O_2^{\bullet -}$. Alternatively, Erk1/2 may activate the neuronal NADPH oxidase directly. The sites of action of the tyrosine kinase inhibitor genistein, the Mek1/2 inhibitor U0126, and the AA-specific $PLA_2$ inhibitor DEDA used in our experiments are denoted. For further details, see text. PL, phospholipids; AA, arachidonic acid; Trk, receptor tyrosine kinase.

The inhibitors we used are supposed to be specific, but may, at least hypothetically, have other effects than the proposed. Further studies using other inhibitors and/or strategies to block the pathways should therefore be performed to verify the working hypothesis. Antioxidant activity of the blockers cannot be excluded, but it seems unlikely because antioxidants like spin traps only scavenge free radicals when present in high concentrations (10 mM) (46).

In summary, we suggest that neuronal formation of cytotoxic ROS in the vicinity of senile plaques may be due to βA-stimulated $PLA_2$ activity via Erk1/2. This observation should be further investigated in the future.

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