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Identification of the Hydroxyl Radical and Other Reactive Oxygen Species in Human Neutrophil Granulocytes Exposed to a Fragment of the Amyloid Beta Peptide

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A fragment of the amyloid beta protein, βA(25–35), was investigated for its effect on production of reactive oxygen species (ROS) in human neutrophil granulocytes. The formation and identification of ROS were examined by using a 2′,7′-dichlorofluorescin (DCF) fluorescence assay, a luminol chemiluminescence assay, electron paramagnetic resonance (EPR) spectroscopy with DEPMPO as a spin trap, and hydroxylation of 4-hydroxybenzoate (4-HBA). The DCF assay showed that βA(25–35) stimulated formation of ROS in a concentration and time dependent manner. The inverted peptide, βA(35–25), gave no response. Also, luminol-amplified chemiluminescence was stimulated by βA(25–35). Incubation with diethyldithiocarbamate (a superoxide dimustase inhibitor) and salicylhydroxamate (SHA; a myeloperoxidase inhibitor) reduced the chemiluminescence. This indicates that hypochlorous acid (HOCl) is formed after exposure to βA(25–35). The EPR spectra indicated a concentration dependent formation of superoxide ($O_2^{\bullet -}$)- and hydroxyl ($^{\bullet}OH$)-radicals. Hydroxylation of 4-HBA to 3,4,-dihydroxybenzoate confirmed production of $^{\bullet}OH$. This response was attenuated by SHA, indicating involvement of HOCl in formation of $^{\bullet}OH$. The DCF fluorescence was inhibited with U0126 (an extracellular signal regulated protein kinase (ERK) inhibitor). Further analysis with western blot confirmed phosphorylation of ERK1/2 after exposure to βA(25–35). The phospholipase A_2 (PLA_2) inhibitor 7,7-dimethyl-(5Z,8Z)-eicosadienoic acid, and diphenyleneiodonium, which inhibits the NADPH oxidase, also led to a reduction of the DCF fluorescence. The present findings indicate that βA(25–35) stimulates the NADPH oxidase by activating the ERK pathway and PLA_2. Production of $O_2^{\bullet -}$ can lead to HOCl and further formation of $^{\bullet}OH$, which both have a cytotoxic potential.

Keywords: Alzheimer's disease; Amyloid beta protein; Human neutrophil granulocytes; Reactive oxygen species; Hydroxyl radical; Hypochlorous acid

INTRODUCTION

In Alzheimer's disease (AD), insoluble amyloid beta protein (βA) deposits as extracellular senile plaques in brain areas that are critical for learning and memory. This accumulated βA, which is an early and probably necessary stage in the development of the illness,[1] may be toxic to neurons by stimulating production of inflammatory mediators such as reactive oxygen species (ROS), cytokines and chemokines.[2,3] However, βA peptides like the (1–40) and (1–42/43) fragments can also exist as soluble forms circulating in the plasma and cerebrospinal fluid.[4,5] It has been shown that these peptides may lead directly to neurotoxic damage,[6] as well as have deleterious effects on cerebral cortex vessels.[7] In particular, soluble βA contributes to cerebral amyloid angiopathy, one of the pathological features of AD.[8] Previous studies have also shown that βA damages peripheral blood vessels in animals overexpressing the amyloid precursor protein,[9] and

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that concentrations of βA higher than 10^{-7} M result in endothelial cell death.[10,11]

Defining exactly how βA exerts its toxicity is important, but the mechanisms are still incompletely described. Many studies have focused on the production of superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) in different cells. McDonald *et al.*[12,13] have shown that exposure of microglia and THP1 monocytes to fibrillar forms of βA results in generation of $O_2^{\bullet -}$. It has also been demonstrated cellular release of $O_2^{\bullet -}$ in peritoneal macrophages,[14] and H_2O_2 in neutrophils, microglia and monocytes[15] exposed to βA. However, because of low reactivity towards biological macromolecules, these species are probably not cytotoxic in the presence of endogenous antioxidants. The toxicity may therefore be a result of conversion to more reactive species such as the hydroxyl radical ($^{\bullet}OH$) and hypochlorous acid (HOCl), which are more likely to overwhelm the defence mechanisms, leading to oxidative damage. So far formation of these molecules after exposure to βA has been scarcely investigated.

In the present paper, we have examined the effect of the amyloid beta protein fragment βA(25–35) on formation of ROS in human neutrophil granulocytes. These cells are part of the phagocytic system and possess the NADPH dependent oxidase complex, which is required for respiratory burst. Although activation of respiratory burst represents a beneficial physiological response in host defence, chronic activation may be harmful, because it leads to a sustained release of reactive molecules at the sites of inflammation.[16] The focus of our investigation has been to identify different ROS produced after exposure to βA(25–35), and to elucidate the involvement of different intracellular signaling pathways. Four methods were used: a 2′,7′-dichlorofluorescin (DCF) fluorescence assay, a luminol chemiluminescence assay, an electron paramagnetic resonance (EPR) spectroscopy assay with DEPMPO as a spin trap, and hydroxylation of 4-hydroxybenzoate (4-HBA). To identify the signaling pathways various enzyme inhibitors and western blot analysis were used.

MATERIALS AND METHODS

Materials

βA(25–35) (Lot. #521137, Lot. #536373 and Lot. #539704) and βA (35–25) (Lot. #545149) were obtained from Bachem AG (Switzerland). 2′,7′-dichlorofluorescin diacetate (DCFH-DA), phorbol 12-myristate 13-acetate (PMA), diphenyleneiodonium chloride (DPI), 7,7-dimethyl-(5Z,8Z)-eicosadienoic acid (DEDA), luminol, 4-hydroxybenzoic acid (4-HBA), 3,4-dihydroxybenzoic acid (3,4-DHBA), salicylhydroxamic acid (SHA), diethyldithiocarbamic acid (DDC), ethyl acetate and hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich AS (USA). U0126 (1,4-diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene) came from Promega (USA). 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 supplied by GibcoBRL. Oxis International Inc. (USA) supplied 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO). Mouse phospho-ERK1/2 (Tyr204) monoclonal antibody was from Santa Cruz Biotech Inc., CA (USA) and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG-antibody was from DAKO A/S (Denmark).

Separation of Granulocytes

Fresh blood samples were collected in the morning from healthy, adult males, and the neutrophil granulocytes were prepared by the standard density-gradient centrifugation method.[17]

Exposure of Granulocytes

βA(25–35) and βA(35–25) were stored at −20°C and dissolved in distilled water prior to use. In solution these peptides form fibrils spontaneously.[15] The controls contained granulocytes in buffer, and when included, granulocytes in buffer with an enzymatic inhibitor (U0126, DPI, DEDA, DDC or SHA). PMA was used as positive control.

DCF Fluorescence Assay

The effect of βA(25–35) on ROS formation was determined by using the probe 2′,7′-DCFH-DA,[18] as described by Myhre *et al.*[19]

Human neutrophil granulocytes (8×10^6 cells/ml buffer) were diluted in HEPES-buffered (20 mM) HBSS (pH 7.4) with glucose (5 mM) and DCFH-DA (2 μM), and incubated for 15 min at 37°C. Dye-loaded samples were then centrifuged for 8 min at 625*g*. After centrifugation, the extracellular medium with DCFH-DA was exchanged with fresh buffer and the pellet was resuspended gently. The reaction was started by transferring 125 μl granulocytes to a microplate (96 wells, 250 μl; final concentration 4×10^6 cells/ml buffer) containing HEPES-buffered HBSS and the compounds to be tested. The measurements of the DCF mediated fluorescence were performed every second minute for 60 min on a computerized Perkin-Elmer LS50 luminescence spectrometer, using excitation wavelength 488 nm and emission wavelength 530 nm. The incubation temperature was 37°C.

Luminol Chemiluminescence

Stimulation of ROS production was also investigated by measuring luminol amplified chemiluminescence as previously described.[20] The reaction was carried out on a microplate (96 wells) where each well contained 0.1 mM (final concentration) luminol and the compounds to be tested in HEPES-buffered (20 mM) HBSS (pH 7.4) with glucose (5 mM). The reaction was started by transferring 100 μl granulocytes to each well (250 μl; final concentration 1×10^6 cells/ml buffer). The chemiluminescence was measured every second minute for 60 min on a computerized Labsystems Luminoscan luminometer. The incubation temperature was 37°C.

EPR Spectroscopy Assay

Identification of free radicals produced after exposure of neutrophils to βA(25–35) was performed by using EPR spectroscopy in combination with the spin trap DEPMPO. The experiments were carried out at X-band frequencies using a BRUKER ESP 300E 10/12 X-band EPR spectrometer. A standard rectangular cavity (TE_{102}) and a vertically oriented flat cell (200 μl) were used.

The procedure is described by Myhre *et al.*[19] Granulocytes (final concentration 4×10^6 cells/ml buffer) were diluted in HEPES-buffered (20 mM) HBSS (pH 7.4) with glucose (5 mM) and DEPMPO (20 mM). The cells were incubated for 10 min at room temperature before exposure to increasing concentrations of βA(25–35) or PMA. Thereafter, the cells were immediately transferred to the flat cell. Spin adduct formation was measured after 30 min. The amplitude of the EPR spectrum is proportional to the amount of spin adducts formed.

For the benefit of the reader, Fig. 1 shows the benchmark EPR spectra of the superoxide adduct of DEPMPO (DEPMPO–OOH) and the hydroxyl adduct (DEPMPO–OH). In Fig. 6, two "stick diagrams" are included to show the relative positions of the partly overlapping lines from DEPMPO–OOH and DEPMPO–OH.

Aromatic Acid Hydroxylation Assay

4-HBA can be used to identify the presence of $^{\bullet}OH$ since it undergoes a specific addition reaction with $^{\bullet}OH$ producing 3,4-dihydroxybenzoate (3,4-DHBA).[21] The assay was performed as described by Myhre *et al.*[19] Granulocytes (final concentration 4×10^6 cells/ml buffer) were diluted in HEPES-buffered (20 mM) HBSS (pH 7.4) with glucose (5 mM) and 4-HBA (2 mM), and incubated for 10 min at room temperature. Thereafter, the compounds to be tested were added. The mixtures were incubated for 2 h at 37°C. After 2 h pH was adjusted to about three

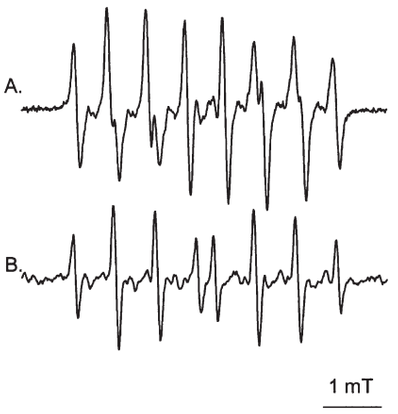

FIGURE 1 (A) EPR spectrum of the superoxide adduct of DEPMPO (DEPMPO–OOH) obtained after illuminating riboflavin (50 μM) in the presence of diethylenetriaminepentaacetic acid (DTPA) (4 mM) and DEPMPO (10 mM). The EPR spectrometer settings were as follows: modulation frequency, 100 kHz; power, 20 mW; modulation amplitude, 0.036 mT; time constant, 160 ms; size, 1024; conversion time, 160 ms. (B) EPR spectrum of the hydroxyl adduct of DEPMPO (DEPMPO–OH) obtained after X-ray irradiation (10 Gy) of DEPMPO (20 mM) in HEPES-buffered HBSS. The EPR spectrometer settings were as follows: modulation frequency, 100 kHz; power, 20 mW; modulation amplitude, 0.16 mT; time constant, 160 ms; size, 1024; conversion time, 160 ms.

(pK_a (4-HBA) = 4.48). One milliliter of the cell suspension was extracted with 2 ml 2-propanol/ethylacetate (1:100). The resulting 2-propanol/ethylacetate extract containing 3,4-DBHA was purified through a normal phase extraction column (2OH diol column Bond Elut, 100 mg, Varian, USA), and the solvents were evaporated at room temperature. The samples were stored at −80°C until the amount of 3,4-DHBA was determined by HPLC with electrochemical detection. The peak areas were calculated as volt per second and quantified by using authentic standards of 3,4-DHBA corrected for the volumes applied. Stringent tests for the experimental system to evaluate non-biological hydroxylation were made. To assure that the method actually measured production of $^{\bullet}OH$ and that adventitious iron in the buffer did not contribute to the response, cell free control experiments were done. In one experiment the buffer was irradiated with X-rays (30 Gy). In another experiment H_2O_2 alone (up to 10 mM), or H_2O_2 (2.5 mM), Fe^{2+} (0.5 mM) and ascorbic acid (0.17 mM) were added to the buffer.

Western Blotting

Human neutrophil granulocytes were isolated as described. Prior to use, the granulocytes were incubated at 37°C for 30 min to equilibrate. Thereafter the cells were exposed to βA(25–35) (25μM), βA(25–35) together with U0126 (20 μM), and βA(35–25) (25 μM) for different time-spans (1–40 min). After the indicated incubation periods, 500 μl ice-cold

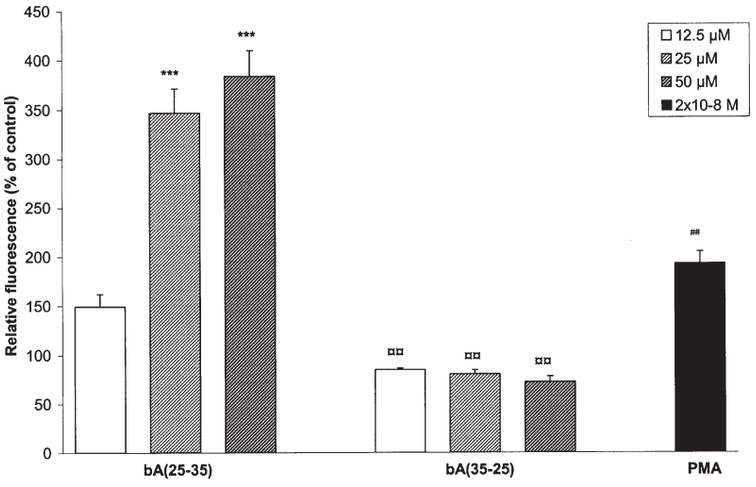

FIGURE 2 Relative DCF fluorescence as an expression for ROS formation in human neutrophil granulocytes after exposure to increasing concentrations of βA(25–35) and βA(35–25), and PMA. The control (unstimulated cells) is set to 100. Values are mean + SEM, $n = 4–6$ assayed in triplicate. *** Statistically higher than the control, $p < 0.001$. ¤¤ Statistically lower than the control, $p < 0.01$ (One-way ANOVA, Newman–Keuls; see text for details). ## Statistically higher than the control, $p < 0.01$ (Student's *t*-test).

phosphate buffered salt solution (pH 7.4) was added to the granulocytes, and the mixtures were centrifuged at 9000*g* for 5 min. The cells were lysed in sample buffer (final concentrations: 3% sodium dodecyl sulphate (SDS) and 5% glycerol in 62.5 mM Tris/HCl, pH 6.9, with bromphenol blue) and added 2-mercaptoetanol (6/100 µl). The aliquots were incubated at 95°C for 5 min and centrifuged again. Fifteen micro liters lysate was separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gel. The proteins were transferred to nitrocellulose membranes (0.45 µm). The nitrocellulose blots were incubated in blocking buffer (Tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% low-fat dry milk) for 1 h and then probed with phospho-ERK1/2 (1:200 dilution in blocking buffer) for another 1 h. The blots were washed in TBST (6 × 5 min) and then incubated with a HRP-conjugated secondary antibody (1:1000 dilution in blocking buffer) for 1 h. After washing in TBST (6 × 5 min), the blots were exposed to enhanced chemiluminescence reagent for 1 min. The signals were visualized on X-OmatBlue XB-1 film (Kodak). The transfer of proteins was confirmed by staining the nitrocellulose blots with Ponceau S. The experiments were repeated 3–4 times.

Statistical Analysis

For the data presented in Figs. 2, 4 and 7, and in Table I statistical overall analyses were made with one-way analysis of variance (ANOVA) and group comparisons with Newman–Keuls *post-hoc* test (Sigma Stat statistical software program version 1.0). For the data

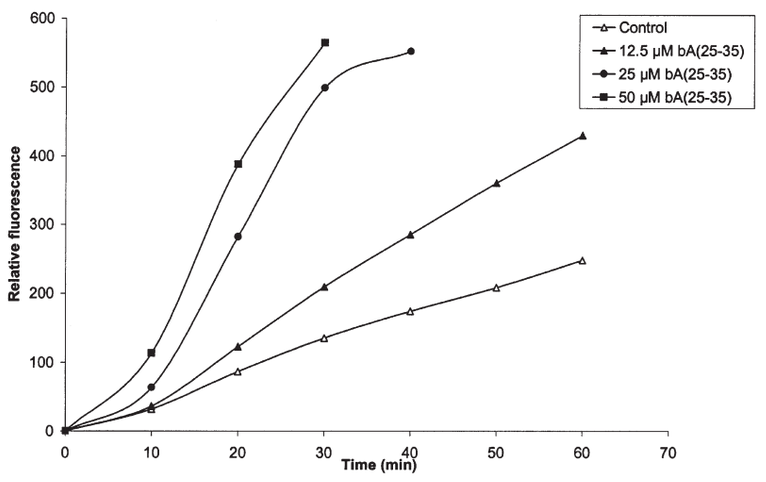

FIGURE 3 Typical response curves showing DCF fluorescence as function of time (min) in human neutrophil granulocytes after stimulation of ROS formation with increasing concentrations of βA(25–35).

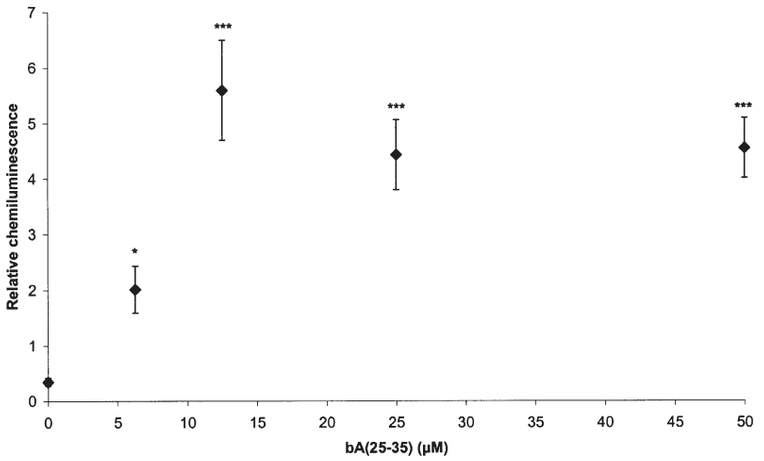

FIGURE 4 Relative chemiluminescence with luminol in human neutrophil granulocytes after stimulation of ROS formation with increasing concentrations of βA(25–35). Values are mean ± SEM, $n = 6$ assayed in duplicate. *Statistically higher than the control, $^{*}p < 0.05$, $^{***}p < 0.001$ (One-way ANOVA, Newman–Keuls; see text for details).

presented in Fig. 5 and in Table II Student's *t*-test, paired two samples of means, was performed (Excel 97 version).

RESULTS

The Effect of βA(25–35) and βA(35–25) on ROS Formation in Human Neutrophil Granulocytes

βA(25–35) increased the DCF fluorescence in a concentration dependent manner (Fig. 2). ANOVA revealed a significant difference between the concentrations tested ($F_{3,18} = 61.07$, $p < 0.0001$). *Post-hoc* comparisons with Newman–Keuls confirmed that the response obtained by 25 and 50 μM was significant different from both the control and 12.5 μM ($p < 0.001$), while 12.5 μM was not significant different compared to the control. βA(35–25) was used as negative control, and inhibited the DCF fluorescence ($F_{3,17} = 11.58$, $p < 0.0002$).

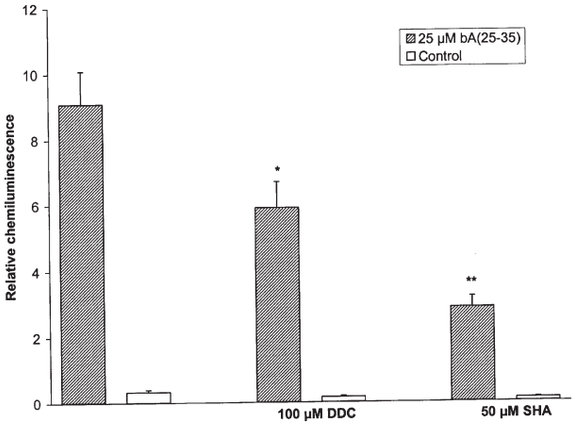

FIGURE 5 The effect of the superoxide dismutase inhibitor DDC (100 μM) and the myeloperoxidase inhibitor SHA (50 μM) on luminol amplified chemiluminescence in human neutrophil granulocytes after exposure to βA(25–35) (25 μM). Values are mean + SEM, $n = 5$–6 assayed in duplicate. *Statistically different from βA(25–35), $^{*}p < 0.05$, $^{**}p < 0.01$ (Student's *t*-test; see text for details).

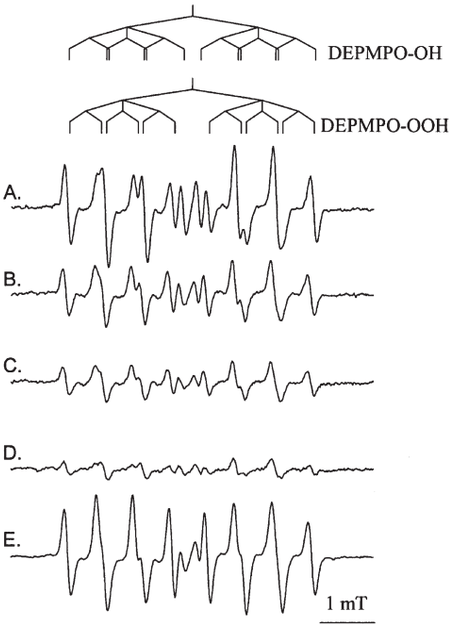

FIGURE 6 Typical EPR spectra obtained after stimulation of free radical formation in human neutrophil granulocytes. (A) 100 μM βA(25–35), (B) 50 μM βA(25–35), (C) 25 μM βA(25–35), (D) Control, (E) 2×10^{-8} M PMA (× 0.5). The spectra were recorded 30 min after start of exposure of the granulocytes, and show both DEPMPO–OOH and DEPMPO–OH, as illustrated by the stick diagrams. For comparison see Fig. 1. The EPR spectrometer settings were as follows: modulation frequency, 100 kHz; power, 12.6 mW; modulation amplitude, 0.185 mT; time constant, 640 ms; size, 1024; conversion time, 64 ms. Hyperfine splitting constants used in stick diagrams: (DEPMPO–OOH) $a_P = 5.01$ mT, $a_N = 1.32$ mT, $a_H^\beta = 1.13$ mT; (DEPMPO–OH) $a_P = 4.73$ mT, $a_N = 1.35$ mT, $a_H^\beta = 1.43$ mT.

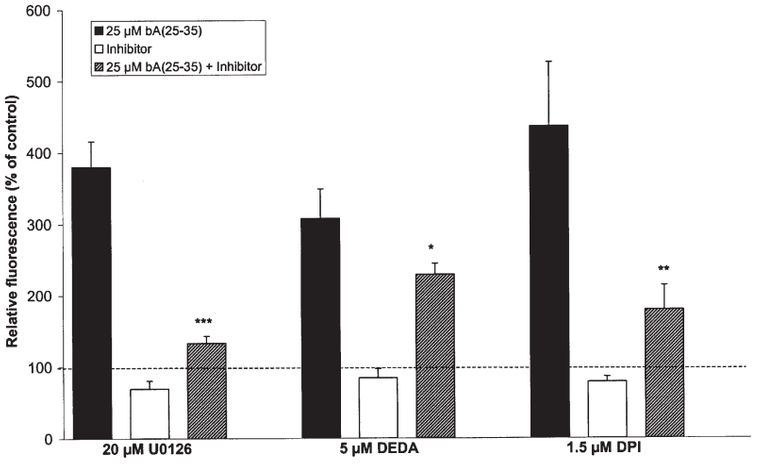

FIGURE 7 The effect of the MEK 1/2 inhibitor U0126 (20 µM), the PLA_2 inhibitor DEDA (5 µM), and the NADPH oxidase inhibitor DPI (1.5 µM) on ROS formation in human neutrophil granulocytes after exposure to βA(25–35) (25 µM). Values are mean + SEM, $n = 5–7$ assayed in triplicate. *Statistically different from 25 µM βA(25–35); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (One-way ANOVA, Newman–Keuls; see text for details).

Newman–Keuls showed that the response was significant lower than the control ($p < 0.01$), and that there were no differences between the three concentrations of βA(35–25) tested. PMA elevated the DCF fluorescence significantly, measured by Student's *t*-test, compared to the control ($p < 0.01$).

Elevation of the DCF fluorescence was time dependent (Fig. 3). The response triggered by βA(25–35) started after a lag time of about 10 min. βA(25–35) by itself did not oxidize DCFH (data not shown).

Identification of ROS After Exposure to βA(25–35)

The luminol experiments confirmed production of ROS after exposure to βA(25–35) (Fig. 4). The response was fast with peaks after 8–16 min (data not shown). ANOVA revealed a significant difference between the concentrations tested ($F_{4,25} = 15.41$, $p < 0.0001$). Newman–Keuls confirmed that the response obtained by all the concentrations was significant different from the control (6.25 µM: $p < 0.05$; 12.5, 25 and 50 µM: $p < 0.01$). 25 and 50 µM did not elevate the response compared to 12.5 µM. Student's *t*-test showed that 100 µM of the superoxide dismutase inhibitor DDC,[22,23] and 50 µM of the myeloperoxidase inhibitor SHA,[24,25] attenuated the luminol response mediated by 25 µM βA(25–35) significantly by 29% ($p < 0.05$) and 66% ($p < 0.01$), respectively (Fig. 5). This indicates that at least a part of the ROS response is due to formation of HOCl. In the absence of any stimulus, chemiluminescence was negligible.

TABLE I Hydroxylation of 4-HBA to 3,4-DHBA as an expression of $^{\bullet}OH$ formation in human neutrophil granulocytes after exposure to βA(25–35) (50 µM) alone, and in combination with the myeloperoxidase inhibitor SHA

Treatment	3,4-DHBA detected after 120 min (nmol)
Control	0.031 ± 0.019
50 µM βA(25–35)	0.097 ± 0.010*
50 µM SHA	0.014 ± 0.004
50 µM βA(25–35) + 50 µM SHA	0.052 ± 0.010†
100 µM SHA	0.011 ± 0.004
50 µM βA(25–35) + 100 µM SHA	0.035 ± 0.007†

Values are mean ± SEM, $n = 4$. *Statistically different from control (unstimulated cells), $p < 0.01$. †Statistically different from 50 µM βA(25–35), $p < 0.01$ (One-way ANOVA, Newman–Keuls; see text for details).

The EPR experiments revealed that both $O_2^{\bullet -}$ and $^{\bullet}OH$ were produced during the respiratory burst triggered by βA(25–35) (Fig. 6). The spectra reached their highest amplitude after 30 min, and showed a concentration dependent formation of DEPMPO–OOH and DEPMPO–OH. The positive control, PMA (2×10^{-8} M), gave a strong EPR spectrum, which shows mainly DEPMPO–OOH but also small amounts of DEPMPO–OH. The control spectra showed only minor amounts of the adducts.

Formation of 3,4-DHBA confirmed an increase in production of $^{\bullet}OH$ after exposure of granulocytes to 50 µM βA(25–35). The myeloperoxidase inhibitor SHA reduced this response (Table I). ANOVA revealed a significant difference between the groups ($F_{5,18} = 9.46$, $p < 0.0001$). Multiple comparisons, using the Newman–Keuls *post-hoc* test, showed that 50 µM βA(25–35) was significant different from the control ($p < 0.01$), and that both 50 and 100 µM SHA reduced this response significantly ($p < 0.01$). This indicates that HOCl is involved in formation of $^{\bullet}OH$ in granulocytes. PMA (1×10^{-7} M) was the strongest stimulator (control: 0.169 ± 0.027, PMA: 0.792 ± 0.055, $p < 0.001$, Student's *t*-test).

TABLE II Cell free control experiments showing formation of •OH after irradiation with X-rays (30 Gy), and incubation with Fe^{2+} (0.5 mM), H_2O_2 (2.5 mM) and ascorbic acid (0.17 mM) (Fenton chemistry; reaction time 120 min)

Treatment (cell free system)	3,4-DHBA formed (nmol)
Control	0.020 ± 0.009
X-ray irradiation (30 Gy)	0.095 ± 0.024*
Control	0.019 ± 0.003
Fenton chemistry	1.24 ± 0.212*

Values are mean ± SEM, $n = 3–4$. *Statistically different from control (buffer), $p < 0.05$ (Student's *t*-test). Only H_2O_2 added to the buffer gave no response (data not shown).

Cell free control experiments were done to assure that the aromatic acid hydroxylation assay measured formation of •OH. Both X-ray irradiation (30 Gy), which produces •OH directly, and addition of H_2O_2 (2.5 mM), Fe^{2+} (0.5 mM) and ascorbic acid (0.17 mM) to the system, elevated the formation of 3,4-DHBA ($p < 0.05$, Student's *t*-test; Table II). Addition of H_2O_2 (up to 10 mM) alone gave no significant response (control: 0.024 ± 0.0046, H_2O_2: 0.038 ± 0.004, measured in nmol after 120 min; $p > 0.05$), showing that trace amounts of iron in the buffer were not responsible for formation of •OH.

The Role of the ERK Pathway, PLA_2 and the NADPH Oxidase

Enzymatic inhibitors were used to elucidate involvement of different intracellular signaling pathways. The concentrations of the inhibitors used in Fig. 7 were chosen from previous dose response experiments with U0126 (5–40 μM), DEDA (1–20 μM) and DPI (1.5–14 μM) (data not shown). Treatment of the granulocytes with the non-competitive ERK inhibitor U0126 (20 μM)[26,27] reduced the fluorescence by 77%. One-way ANOVA revealed a reliable overall effect ($F_{3,20} = 53.75$, $p < 0.0001$), and Newman–Keuls *post-hoc* test showed that U0126 reduced the βA mediated response significantly ($p < 0.001$). Incubation of the granulocytes with DEDA (5 μM), a competitive arachidonic acid-specific PLA_2 inhibitor,[28] lowered the fluorescence by 30% ($F_{3,24} = 21.88$, $p < 0.0001$). Group comparisons showed that this concentration of DEDA had a significant effect on the βA mediated response ($p < 0.05$). ANOVA also uncovered a reliable effect of 1.5 μM of the NADPH oxidase inhibitor DPI[29] ($F_{3,16} = 9.757$, $p < 0.0007$). The βA response was reduced by 70% ($p < 0.01$).

Neither of the inhibitor controls were significant different from the cell controls (One-way ANOVA, Newman–Keuls, $p > 0.05$).

To confirm the ability of βA(25–35) to phosphorylate ERK1/2, a phospho-specific antibody that recognizes phosphorylated Tyr204 of ERK1/2 was employed (Fig. 8). The results show that βA(25–35) (25 μM) induced phosphorylation of ERK 1 and 2 in a time dependent manner (1, 5, 10, 20 and 40 min) demonstrated by an increase in the relative intensities of the two immunodetectable bands, 44 and 42 kDa, respectively, compared to the basal level (control). Incubation of the granulocytes with βA(25–35) (25 μM) for 20 min in combination with U0126 (20 μM) eliminated this response. The inverted peptide βA(35–25), which does not stimulate formation of ROS, did not lead to phosphorylation of ERK1/2 after incubation for 20 min.

DISCUSSION

The present paper shows elevated production of $O_2^{\bullet -}$, HOCl and •OH in human neutrophil granulocytes

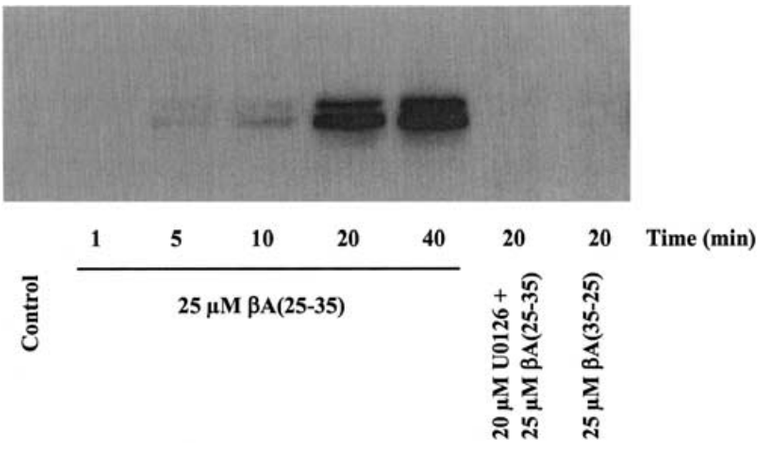

FIGURE 8 Phosphorylation of ERK1/2 in human neutrophil granulocytes exposed to βA(25–35) (25 μM) is time dependent (1, 5, 10, 20 and 40 min). Incubation of βA(25–35) in combination with U0126 (20 μM) for 20 min eliminated this response. The inverted peptide βA(35–25) (25 μM) did not lead to phosphorylation of ERK1/2 after incubation for 20 min. The experiments were repeated 3–4 times.

after exposure to βA(25–35), measured with DCF fluorescence, luminol chemiluminescence, EPR spectroscopy in combination with the spin trap DEPMPO, and aromatic acid hydroxylation. To avoid artifacts it is important to combine different methods. The DCF assay is rather non-specific for detection of ROS, but is an attractive and sensitive method as an overall index for oxidative stress in biological systems. It is reported to detect several kinds of reactive molecules such as H_2O_2, $OONO^-$, HOCl and NO.[18,30,31] Of the concentrations tested, only 25 and 50 μM βA(25–35) elevated the DCF response significantly compared to the control (Fig. 2). Control experiments with βA(35–25) did not elevate the DCF response, showing that the ROS inducing capacity of βA(25–35) was not a non-specific action due to the peptide length.

The identity of the ROS was characterized. βA(25–35) elevated the luminol chemiluminescence significantly (Fig. 4). In activated phagocytes luminol largely detects production of HOCl.[32] This molecule has attracted much attention because of its high reactivity. If myeloperoxidase and Cl^- are present, HOCl can be produced from H_2O_2.[33] Neutrophils are known to have a large amount of myeloperoxidase, and the myeloperoxidase inhibitor SHA reduced the chemiluminescence (Fig. 5). This confirms production of HOCl after stimulation with βA(25–35). Also incubation with DDC abolished the luminol chemiluminescence. This observation is in accordance with earlier findings showing that superoxide dismutase inhibition in isolated neutrophils and cerebellar neurons attenuated H_2O_2 production,[34,35] which will consequently reduce the level of HOCl. The inhibition by SHA was stronger than the inhibition by DDC. The reason for this can be that although DDC inhibits Cu–Zn superoxide dismutase, $O_2^{\bullet -}$ can also dismutate spontaneously, to form dioxygen and H_2O_2.[36,37]

The EPR spectra in Fig. 6 show concentration dependent formation of both the superoxide adduct of DEPMPO (DEPMPO–OOH) and the hydroxyl adduct (DEPMPO–OH) after stimulation with βA(25–35). The formation of $^{\bullet}OH$ was confirmed by hydroxylation of 4-HBA to 3,4-DHBA (Table I), which is a sensitive and reliable technique used to detect $^{\bullet}OH$ down to picomol concentrations[38] (Table II). As far as we know, we are the first to demonstrate production of HOCl and $^{\bullet}OH$ in phagocytes after exposure to βA(25–35) (Figs. 4–6, and Table I). In biological systems these molecules are highly reactive and will attack cell membranes, initiate lipid peroxidation, and injure DNA and sensitive proteins,[32] resulting in cellular dysfunction. The formation $^{\bullet}OH$ can occur through different mechanisms. In phagocytes, an important source of $^{\bullet}OH$ is from HOCl reacting with $O_2^{\bullet -}$.[39] Depending on the stimuli, HOCl may account for 20–70% of the liberated H_2O_2 by neutrophils (for review see Ref. [40]). When our granulocytes were incubated with βA(25–35) together with the myeloperoxidase inhibitor SHA, both the chemiluminescence and the level of 3,4-DHBA were reduced significantly (Fig. 5 and Table I), confirming that production of HOCl via myeloperoxidase is important for formation of $^{\bullet}OH$ in these cells. The reaction between $O_2^{\bullet -}$ and HOCl leading to $^{\bullet}OH$ is rapid even in the absence of metal ions,[39] and has a rate constant of $7.5 \times 10^6\ M^{-1}\ s^{-1}$, which is greater than that for the reaction of Fe^{2+} with H_2O_2. This reaction also represents an alternative pathway for the toxicity of HOCl, in addition to the well-established formation of chloramines and inactivation of antiprotease.[40,41] Lastly, it is a mechanism by which neutrophils can transform the less reactive $O_2^{\bullet -}$ into the highly reactive $^{\bullet}OH$. However, SHA did not fully eliminate the βA mediated response. It may therefore be that other sources, such as the Fenton reaction, are involved. It has been shown that iron facilitates βA injury.[42] Normally, organisms take great care in handling of this specie to minimize the amount of free iron within cells and in extracellular fluids.[43] However, an excess production of $O_2^{\bullet -}$ can lead to release of iron from e.g. ferritin.[44]

In control experiments, without granulocytes, H_2O_2 alone was not able to increase the formation of 3,4-DHBA significantly. Adventitious iron in the buffer seems therefore not to be involved in the observed production of $^{\bullet}OH$. To get $^{\bullet}OH$ in the cell free system iron and ascorbic acid had to be added together with H_2O_2 (Table II).

Dewas *et al.*[45] have demonstrated that the ERK pathway participates in phosphorylation of the NADPH oxidase component $p47^{phox}$. As shown in Fig. 7, the potential anti-inflammatory compound U0126, which inhibits MEK1/2[26,27] had a strong inhibitory effect on the βA(25–35) stimulated DCF fluorescence. Phosphorylation of ERK1/2 after exposure to βA(25–35) was confirmed by using a specific monoclonal antibody against phospho-ERK1/2 and western blot analysis (Fig. 8). The results show that the peptide phosphorylated ERK1/2 time dependently up to 40 min. Incubation with U0126 eliminated this response. This indicates that the ERK pathway, which has been shown to be involved in βA mediated ROS response in microglia an THP1 monocytes[12,13,46] also is important in human neutrophil granulocytes. Interestingly, both ERK1/2, MEK1,[47] and the NADPH oxidase[48] are found to be activated in AD brains. The NADPH oxidase inhibitor DPI did not reduce the fluorescence completely (Fig. 7), indicating that other sources may be involved. Since elevated cytosolic PLA_2 activity is associated with the inflammation and oxidative stress seen in AD,[49,50] the cells were incubated with the PLA_2 inhibitor DEDA. This inhibitor reduced

the fluorescence significantly, suggesting that PLA_2 plays a role in the observed response. The arachidonic acid cascade, which is activated by this enzyme, leads both to production of $O_2^{\bullet -}$, and forms free fatty acids that can stimulate the NADPH oxidase directly[51] or via PKC.[52] PLA_2 itself can be activated by the ERK/MAP kinase pathway.[53] Consequently, inhibition of MEK1/2 by U0126 not only suppresses a possible direct activation of the NADPH oxidase but also the βA induced activation of PLA_2. The inhibitors we used are supposed to be specific, but may, at least hypothetically, have additional effects than the proposed. Studies using other inhibitors and/or strategies to block the pathways should therefore be performed in the future to verify the results.

Clinical and epidemiological studies have indicated that βA may be associated with oxidative stress and cell death.[54,55] Consistent with this, it has been claimed that use of anti-inflammatory drugs and antioxidants slow progression of the illness.[56,57] However, the mechanisms for βA mediated toxicity are still uncertain. The peptide is known to stimulate inflammatory activation of microglia,[2,46] and examination of AD-afflicted brain tissue has shown a correlation between these cells, senile plaque and oxidative damage.[58,59] In general, microglia produce $O_2^{\bullet -}$ and H_2O_2,[12,13,15] but these species react poorly with biomolecules and must therefore be converted to more reactive species, such as $^{\bullet}OH$ and HOCl, to give cell toxicity.

In addition to direct neurodegeneration induced by amyloid plaque, recent findings propose a prominent role for vascular βA in the pathology of AD.[60,61] The level of soluble βA has been shown to be elevated in both brain and plasma of AD patients,[5,6] and several experiments indicate that this βA may mediate production of ROS resulting in vasoconstriction and endothelial destruction.[60,61,62]

Our *in vitro* results, showing formation of HOCl and $^{\bullet}OH$, may be of relevance for AD. HOCl is the main oxidant produced by neutrophils, and may account for a major part of injury induced by these cells, resulting in vascular dysfunction. Normally, myeloperoxidase is not present in microglia and brain-specific macrophages, but in AD patients this enzyme has been shown to colocalize with βA in senile plaques in cerebral cortex.[63] This suggests a role for HOCl also in the neuronal damage. Concerning the extremely reactive $^{\bullet}OH$ radical, the level of this molecule is elevated in the blood of AD patients compared to controls.[64] It has also been shown that mitochondria from AD brains have damages consistent with $^{\bullet}OH$ injury. Generation of $^{\bullet}OH$ may therefore occur against the background of intra- and extracellular antioxidant defences in these patients. In agreement with this a significant deficiency in the endogenous $^{\bullet}OH$ scavenger melatonin is common in AD.[65]

The present paper, identifying production of cytotoxic ROS in human neutrophil granulocytes after exposure to βA(25–35), supports the oxidative stress hypothesis for the pathogenesis in AD.[66] Our results show that βA(25–35) can stimulate the NADPH oxidase by activating the ERK/MAP kinase pathway and PLA_2. Activation of the NADPH oxidase produces $O_2^{\bullet -}$, which is converted to H_2O_2. This molecule can lead to HOCl and further formation of $^{\bullet}OH$. Continuous production of HOCl and $^{\bullet}OH$ can be deleterious to vital cellular functions and may be involved in the pathology seen in AD.

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