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Effects of oxidative stress on amyloid precursor protein processing in rat and human platelets

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Abstract

Alzheimer's disease (AD) is the most common neurodegenerative illness affecting the elderly and is characterized by beta-amyloid ($A\beta$) deposition in the brain (plaques) and in microvessels ($A\beta$ -angiopathy). The reasons for $A\beta$ deposition are not clear, but an impaired clearance of $A\beta$ at the blood-brain barrier may be implicated and oxidative stress possibly plays a major role in this process. Platelets are of particular interest, because they contain high levels of the amyloid precursor protein (APP) and in AD an abnormal expression of platelets APP fragments was found. The aim of the present study was to investigate (1) if oxidative stress induced by hydrogen peroxide (H_2O_2) affects APP expression in rat and human platelets and (2) to compare the APP changes with platelets of AD patients. In rat platelets, all three fragments of APP (130-110-106 kilo Dalton, kDa) were found. H_2O_2 (10 mM, 20 minutes) significantly reduced all three fragments in rat platelets, did not affect CD62P-staining and slightly increased the size of actin as seen in the Western blot. The effect was not seen at 1 mM H_2O_2 and was counteracted by glutathione. Immunohistochemistry for CD62P, CD61, APP and Annexin-V was used to verify the changes at the cellular level. In platelets of young volunteers (age = 33 ± 4 years), 10 mM H_2O_2 markedly reduced the smaller APP 110 and 106 kDa fragments after 20 minutes. Our data show that platelets of AD patients (age = 80 ± 1 years) had a significant reduced 130 kDa fragment compared to controls (age = 70 ± 2 years). In summary, oxidative stress may account for a dysfunctional processing of APP in rat and human control platelets and possibly in AD patients.

Keywords

Platelets; APP processing; hydrogen peroxide; oxidative stress; Alzheimer's disease

Introduction

Platelets contain the highest amyloid precursor protein (APP) levels of all peripheral tissues and APP is secreted by platelets spontaneously, as well as during their activation, aggregation or degranulation [1, 2]. Platelets express mainly APP770 and APP751 which

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contain a Kunitz-type serine protease inhibitor domain (KPI) and are also known as APP-KPI+ forms, while the APP695 form lacks this domain (APP-KPI- form) and is mainly found in neuronal tissue [3, 4]. APP 770 is the predominant form in platelets, but the role of platelets APP is still unclear. There are strong indications that soluble APP (sAPP) fragments may play a role in regulating thrombosis and hemostasis [5]. While the major population of APP is stored in alpha-granules as proteolyzed fragments, including sAPP α , approx. 10% of platelets APP is associated as a full length protein in the membrane [1, 5–9]. Activation of platelets with thrombin increases the surface expression of APP [7] and full length APP770 is cleaved by a calcium-dependent cysteine protease during platelet activation [10]. Platelets possess all the enzymatic machinery necessary for APP processing to beta-amyloid (A β) peptides [2, 11]. Platelets contain α - (ADAM10) and β -secretases (BACE1), but the α -secretase is more dominant and sAPP α levels are much higher than A β levels [2]. Interestingly, platelets are the source of 90% of peripheral β -amyloid in the blood and secrete mainly the A β ₍₁₋₄₀₎ peptide [1, 11].

In plasma of Alzheimer's disease (AD) patients a decrease of a larger 130 kilo Dalton (kDa) APP fragment, a decrease of α -secretases and an increase of β -secretases have been reported [12, 13]. Such changes may be caused by alterations in processing of the mRNA [14], altered posttranslational modifications [15–17] or might be the result of proteolytic cleavage by distinct secretases. Thus, these findings suggest an important role of platelets in AD and make them useful as a possible biomarker to diagnose AD [8, 18, 19].

There is evidence that oxidative stress is implicated in the pathomechanism of AD and neurons are particularly vulnerable [20, 21]. Age is a key risk factor for AD and it is likely that free radicals damage neurons over years. Hydrogen peroxide (H₂O₂) is one such reactive radical and commonly used to induce oxidative stress *in vivo* and *in vitro* [22–24]. There is evidence that H₂O₂ directly affects APP processing in human neuroblastoma cells where it induces apoptosis [25]. The aim of the present study was to use rat platelets as a simple and easy accessible model to study the effect of H₂O₂ on APP expression. Furthermore we compared the effects of oxidative stress in young healthy controls and AD patients. We showed that oxidative stress differentially affects APP fragments in all three used models. We hypothesize that oxidative stress may cause an aberrant APP processing and A β secretion in platelets, which may play a role in A β -angiopathy.

Methods

Collection of blood

For the animal experiments adult Sprague Dawley rats were used. The rats were anaesthetized with a high dose of thiopental (Sandoz) and blood was directly drawn from the heart by using a 21 gauge butterfly blood collection system (BD Valu- Set, BD). The blood was collected in ethylenediaminetetraacetate (EDTA) tubes (S-monovettes, Sarsted) and gently mixed. All experiments on animal tests were approved by the Austrian ethical committee. For human platelets blood was taken from young healthy volunteers from the lab (age = 33 ± 4 years, *n* = 6), collected in EDTA tubes and immediately processed. For old healthy controls (age = 70 ± 2 years, mini mental state examination (MMSE) = 27 ± 1, *n* =

15) and AD patients (age = 80 ± 1 years, MMSE = 18.0 ± 1.1 , $n = 25$) the blood was used from a previous study [26] and processed within 3 hours.

Platelets isolation from rat and humans

Immediately after blood collection, anticoagulated rat or human blood was centrifuged at $250 \times g$ for 15 minutes to obtain platelet rich plasma (PRP). All centrifugation steps were performed at room temperature. PGI₂ (Prostaglandin, 500 nM, Sigma) was added to prevent platelet activation during processing. Platelets were separated from PRP by centrifugation at $2300 \times g$ for 10 minutes and washed in calcium-free Tyrode buffer (136 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.42 mM NaH₂PO₄, 1 mM MgSO₄, 5 mM glucose, pH 6.5). After further centrifugation at $2300 \times g$ for 10 minutes, platelets were finally resuspended in 1 ml Tyrode buffer (adjusted to pH 7.4) and further processed. To induce oxidative stress, platelets were exposed to freshly prepared H₂O₂ (30%, Merck) at a concentration of 1 or 10 mM for 20 minutes. In order to test protective effects, the antioxidant glutathione (1 mM, Sigma) was added 30 minutes prior and during H₂O₂ treatment. To study secretion of platelet markers, 1×10^8 platelets were incubated for 10 minutes (serotonin), 20 minutes (sAPP α and sAPP β), 3 hours (matrix-metalloproteinase-2; MMP-2) or 24 hours (A β ₄₀₊₄₂) in Tyrode buffer (pH 7.4) at 37° C, then centrifuged for 10 minutes at $2300 \times g$ and the supernatant was analyzed.

Immunohistochemistry

Immunohistochemistry was performed as described recently [26]. Platelets were pipetted onto glass slides and postfixed in 4% paraformaldehyde for 30 minutes at 4° C. Cells were washed 30 minutes with 0.1 % Triton/phosphate-buffered saline (T-PBS), pretreated with 5% methanol/1% H₂O₂/PBS. Then the cells were washed three times for 5 minutes with phosphate buffered saline, blocked with 20% horse serum/0.2% bovine serum albumin/T-PBS and further incubated with the primary antibody mouse anti-APP (Millipore; 1:2000), mouse anti-cluster of differentiation 41 (CD41, integrin alpha 2b) (Serotec, 1:2000), rabbit anti-cluster of differentiation 62P (CD62P, P-selectin) (Abcam, 1:1000) or mouse anti-cluster of differentiation 61 (CD61, integrin beta-3) (Serotec, 1:200) in 0.2% bovine serum albumin/T-PBS at room temperature overnight. Cells were washed and incubated with secondary biotinylated anti-mouse or anti-rabbit immunoglobulin G (IgG, 1:200 BA-2001 and BA-1000, Vector Laboratories) antibody for 1 hour at room temperature. After rinsing three times in PBS, slides were incubated in avidin-biotin complex solution (ABC; Elite Standard PK 6100, Vector Laboratories) for 1 hour, and then washed three times in 50 mM Tris-buffered saline (TBS). The signal was detected using 0.5 mg/ml 3,3' diaminobenzidine (DAB) in TBS with 0.0003% H₂O₂ as substrate. Reaction was stopped in TBS and slides were allowed to dry and were cover slipped.

Annexin-V-Alexa 568 staining

Treated platelets were centrifuged at $2300 \times g$ for 5 minutes, dissolved in 500 μ l Annexin-V-labeling solution (Roche, 20 μ l/ml Annexin, 10 mM 2-(4-2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethansulfonacid (HEPES), pH adjusted to 7.4, 140 mM NaCl, 5 mM CaCl),

then incubated 15 minutes at room temperature, pipetted onto glassslides and covered with Vectashield (H-1000, Vector Laboratories).

Western blot

To analyze the expression of APP fragments and platelet markers, Western blot analysis was performed as described recently [27]. Briefly, the platelet pellet was dissolved in 100 μ l ice-cold PBS with a protease inhibitor cocktail (P-8340, Sigma), homogenized by using an ultrasonic device (Branson sonifier 250, Danburry), centrifuged at 16 000 \times g for 10 minutes at 4° C and the pellet was discarded. Then platelet extracts (approximately 30–50 μ g) were loaded onto 4–12% Bis-Tris polyacrylamide gel (Invitrogen) and electrophoresed for 45 minutes at 200 V. Samples were electrotransferred to nylon PVDF Immobilon-PSQ membranes (Millipore) for 90 minutes at 30 V with 20% methanol blotting buffer (Invitrogen). For detection, the Western Breeze Chemiluminescent System (Invitrogen) was used. Blots were blocked for 30 minutes with blocking buffer, then incubated overnight at 4° C with the primary antibody mouse anti-APP (1:2000, Millipore), rabbit anti-CD62P (1:1000, Abcam), mouse anti-CD61 (1:200, Serotec), or rabbit anti-actin antibody (1:1000, Sigma-Aldrich). Blots were washed and incubated with alkaline phosphatase-conjugated anti-mouse or anti-rabbit antibodies for 30 minutes at room temperature. After being washed, blots were incubated in CDP-Star chemiluminescent substrate solution (Invitrogen) and the signal was visualized with a cooled CCD camera (SearchLight, Thermosience). The used monoclonal antibody 22C11/APP recognizes three APP fragments at 106, 110 and 130 kDa. Due to methodological difficulties to differentiate between the 106 and 110 kDa band both bands were merged for the calculations.

ELISA for rat $A\beta_{(1-40+42)}$, MMP-2, sAPP α and sAPP β

For the secretion studies rat or human platelets were incubated with 1 or 10 mM H_2O_2 for 10 minutes, 20 minutes (human), 3 hours, or 24 hours, centrifuged at 2300 \times g for 10 minutes to remove platelets and the supernatant was analyzed. ELISA measurements were performed as described by the manufacturers (Wako 292-64501 $A\beta_{(1-42)}$; Wako 294-64701 $A\beta_{(1-40)}$). MMP-2 ELISA was performed as described by a SearchLight Array (Searchlight, Aushon Biosystems) [28]. Soluble APP α and APP β measurements were performed by ELISA as described by the manufacturers (IBL, 27734 sAPP α and Covance, 38960 sAPP β).

Analysis of serotonin

Serotonin was measured by high-performance liquid chromatography (HPLC) and electrochemical detection, as described by us [26]. Rat platelets were incubated with 1 or 10 mM or without (controls) H_2O_2 for 10 minutes, centrifuged and the supernatant (20 μ l) was injected onto the HPLC. The samples were separated on a reversed phase C18 Nucleosil column (Bartelt) at a flow rate of 1 ml/minute using the following mobile phase: 0.05 M trichloric acid, 0.26 mM EDTA, 1.36 mM NaCl, 1.81 mM heptane sulfonic acid and 15% acetonitril in HPLC water. Detection was performed with an electrochemical detector (Antec-Leyden) at +0.55 V at 30° C. All unknown samples were correlated to external standards of serotonin (Sigma) according to the peak heights.

Quantification and statistical analysis

Measurement of optical density in Western blots was performed by computer-assisted densitometry (Photoshop). The Western blot signal has been found to be linear up to 60 μ g protein loaded ($R^2 = 0.934$). The number of platelets was counted under the microscope (Olympus, BX61) at a 40 \times magnification. Statistical analysis was performed by one way analysis of variance (ANOVA) with a subsequent Fisher LSD posthoc test and by students-*t* test; A *p*-value <0.05 was considered as statistically significant.

Results

Rat control platelets characterization

Immunohistochemistry of control platelets revealed a high number of discoid CD62P+ (Figure 1(a)) and CD61+ cells (Figure 1(d)). Control platelets were also positive for APP-like immunohistochemistry (Figure 1(g)). Annexin-V staining displayed a very low number of positive cells (360 ± 67 positive cells/mm², $n = 4$, Figure 1(j)) in controls. Western blot analysis of control platelets showed three APP fragments at 130, 110, and 106 kDa (Figure 2(a)) and a strong band of CD62P at approximately 160 kDa (Figure 2(a)). Actin was used as a control, which was detectable at approximately 40 kDa (Figure 2(a)).

Rat platelets and H₂O₂

Treatment of rat platelets with 10 mM H₂O₂ did not change the number of CD62P+ cells (Figure 1(b) and (c)), but significantly reduced the number of CD61+ platelets (Figure 1(e) and (f)). Incubation of platelets with 10 mM H₂O₂ significantly reduced the number of APP-positive cells (Figure 1(h) and (i)). H₂O₂ (10 mM, 20 minutes) markedly increased the number of Annexin-V positive cells (Figure 1(k) and (l)). Treatment of rat platelets with 10 mM H₂O₂ for 20 minutes resulted in a significant deletion of the 130, 110 and 106 kDa APP fragments (Figure 2(a) and (b)), but not CD62P, as shown in Western blot analysis. To preclude that H₂O₂ impairs the immunogeneity of the monoclonal antibody 22C11/APP the transblot was incubated with 10 mM H₂O₂ for 20 minutes before detection was performed (Figure 2(c)). The effect of H₂O₂ was dose-dependent with a marked effect at 10 mM H₂O₂, while 1 mM H₂O₂ did not affect APP-fragments (Figure 2(d)). In order to test protective effects, rat platelets were incubated with glutathione with or without H₂O₂. Glutathione (1 mM) partly counteracted the H₂O₂-induced decrease of APP fragments (Figure 2(e)). Incubation of platelets with glutathione alone did not alter APP fragments (Figure 2(e)).

Effect of H₂O₂ on secretion of serotonin, MMP-2, A β (1-40) and A β (1-42)

Serotonin secretion from rat platelets was detectable in controls and H₂O₂ did not significantly affect serotonin secretion after 10 minutes (Table I). MMP-2 secretion was not enhanced by H₂O₂ after 3 hours (Table I). Secreted A β (1-40) levels were clearly detectable in controls, but not changed after treatment with 1 or 10 mM H₂O₂ (Table I). A β (1-42) levels were very low in controls and slightly decreased after 1 and 10 mM H₂O₂ treatment.

Characterization of human platelets

Immunohistochemistry of control platelets revealed discoid CD41+ (Figure 3(a)) and APP+ cells (Figure 3(a)). Western blot analysis detected 130, 110 and 106 kDa fragments of APP in young (age = 33 ± 4 years, $n = 6$) healthy control platelets (Figure 3(b)) and in old healthy controls (age = 70 ± 2 years, $n = 15$; Figure 3(b)). Human platelets contained approximately 20–50 times more sAPP α than sAPP β (Figure 3(e)).

Effect of H₂O₂ on APP expression in human platelets

Treatment of human control platelets from young healthy volunteers with 10 mM H₂O₂ significantly decreased the lower 106 and 110 kDa APP fragments, but did not affect the 130 kDa APP fragment (Figure 3(b) and (c)). H₂O₂ treatment did not affect secretion of sAPP α and sAPP β after 20 minutes (Figure 3(e)).

APP expression in AD patients

In AD patients (Figure 3(b), age = 80 ± 1 years, $n = 25$) the APP 130 kDa fragment was significantly decreased (Figure 3(b) and (d)), but the 106 and 110 kDa APP fragments were only slightly decreased (Figure 3(b) and (d); $p = 0.08$, $n = 25$).

Discussion

Our present study shows (1) that rat platelets contain all three APP fragments, (2) that H₂O₂ induces a decline of APP in rat platelets, and (3) that H₂O₂ affects APP expression in human control platelets.

Rat platelets

In order to compare isolated rat platelets with human platelets we characterized rat platelets with the well established markers CD61 and CD62P. CD61 is also known as the integrin $\beta 3$ unit and forms together with CD41 the gpIIIa/IIb complex, which is found on rat platelets and megacaryocytes mediating cell adhesion. CD61 is expressed on resting platelets and serves as a subunit of the receptor for fibrinogen [29]. Binding of fibrinogen to this receptor is required for platelet aggregation and endothelial adherence. CD62P is stored in alpha-granules of platelets, but is not expressed on the surface. This calcium-dependent protein migrates during platelet activation to the plasma membrane and mediates platelets interaction with endothelial cells or leukocytes and CD62P serves as a marker for activated platelets. In our Western blot experiments we found high CD62P levels in platelet extracts after sonication, which most likely reflects the total granular content. Thus CD62P levels may not be fully appropriate as an activation marker by using Western blot but is highly useful in immunohistochemistry where membrane bound CD62P can be seen. In rat platelets APP fragments of 130, 110 + 106 kDa were detectable using the same antibody as in human platelets, but the APP expression levels were markedly lower. When comparing the APP ratios from young, old and rat control platelets no significant difference was observed, although a tendency for a decrease in the control rat platelets ratio was seen (data not shown). We also noted that the APP content markedly varied during different preparations and partly displayed a high heterogeneity, which may be explained by cleavage of the full length APP into smaller fragments without transmembrane and cytoplasmic domains during

the preparation [1]. Indeed, we observed enhanced expression of a smaller APP form (probably sAPP as shown in the ELISA) in supernatants of control platelets. Also we cannot exclude that a part of the isolated platelets undergo rapid cell death, as we also found low actin levels in the supernatant (data not shown). Our data showed that rat platelets provide a fast, simple and easy model to study processing of APP. Moreover, rat platelets are useful to investigate effects of oxidative stress. This has the advantage to use a well controlled cell system, while in humans unknown cofactors, such as drug intake or age-dependent alterations cannot be excluded. Indeed, in agreement recently Yu et al. [30] explored the proteome of human and rat platelets suggesting the usefulness of rat platelets as a reliable alternative platelet model.

Human platelets

Human platelets were isolated from young healthy volunteers, older healthy controls, and AD patients. The isolation procedure and characterization (CD41) has been recently published by us in detail [26]. In order to test if human platelets react to oxidative stress we isolated platelets from younger volunteers from the lab and found 130, 110 + 106 APP fragments with an expression level which was not different to elderly controls. Oxidative stress mediated by 10 mM H₂O₂ (20 minutes) significantly reduced the APP 110 + 106 kDa fragments but not the APP 130 kDa fragment. This was interestingly to note because it seems that especially the smaller fragments are more sensitive to oxidative stress. In this study we used the same controls and AD patients as in a previous study [26]. We recently reported that the ratio of APP 130/110-106 was slightly reduced in AD patients [26] and show that the APP 130 kDa cleavage product was significantly reduced and the APP 110 + 106 kDa fragments were only slightly reduced. This is in full line with several previous reports showing altered APP metabolism in AD patients [8, 12, 13, 18, 19, 26].

H₂O₂ mediated oxidative stress in platelets

Oxidative stress may play a role in AD neuropathology [20, 31]. Hydrogen peroxide is commonly used to induce oxidative stress *in vivo* as well as *in vitro* [22–24]. Under physiological conditions the mean hydrogen peroxide level is <0.7 μM and does not induce oxidative stress. In blood, neutrophils produce sufficient amounts of H₂O₂ leading to high local endogenous peroxide levels *in vivo* [32, 33]. Furthermore, hyperoxic conditions after stroke or heart attack may give rise to high H₂O₂ concentrations in the brain [34] and generation of H₂O₂ may also be induced by different drugs, such as sulfonamide [35]. In general, exogenously applied H₂O₂ to cells normally results in approximately 7 to 10-fold less intracellular levels [36] and thus, in our experiments oxidative stress was induced by 10 mM H₂O₂. The lack of comparability between the *in vivo* H₂O₂ concentration and the dose of H₂O₂ that was used *in vitro* is a limitation of this study. However, there are some good reasons for using H₂O₂ levels in the mM range. (1) Hydrogen peroxide is a very unstable reactive molecule and rapidly reacts with other molecules. (2) We used a relative high density of platelets and it is known that platelets contain H₂O₂-degrading enzymes [32, 37], which may limit the effects of H₂O₂ treatment at low concentrations. (3) The number of morphologically CD62P+ platelets was not affected by this H₂O₂ treatment. It cannot be excluded that CD62P is already activated during the isolation or culturing conditions. (4) We could not see any effects on APP expression with 1 mM H₂O₂ and the most prominent

effect on APP was only observed at 10 mM H₂O₂. As mentioned before, immunodetection of CD62P was not influenced by H₂O₂ treatment, as seen in Western blot analysis or in immunohistochemistry, clearly showing that H₂O₂ did not cause morphological discrimination of the platelets. CD61 immunodetection was affected and the sensitivity of the gpIIb/IIIa complex (CD 61) to H₂O₂ may also be indicated by the finding that platelets aggregation is modified by hydrogen peroxide [38, 39]. In addition, our experiments showed a slight increase in molecular weight of actin, indicating actin polymerization during H₂O₂ treatment. This alteration in molecular weight may advise to an early apoptotic process in response to H₂O₂, which is well established [26]. Interestingly, we found low levels of actin also in the supernatant of control and H₂O₂-treated rat platelets (data not shown), which possibly points to rapid cell death of a few platelets during the isolation procedure. In addition, we showed enhanced Annexin-V-staining in rat platelets after 20 minutes of 10 mM H₂O₂ treatment, but not in control platelets. It is well known that the translocation of phosphatidylserine from the inner part of the plasma membrane to the outer layer binding site is seen during platelet activation. Taken together, our findings may indicate activation in rat and human platelets *in vitro* due to oxidative stress.

Serotonin and MMP-2 in platelets during oxidative stress

To proof functionality of our platelets preparation during oxidative stress, we measured the serotonin and MMP-2 secretion. Platelets contain high levels of serotonin, which directly reflect the blood serotonin levels. Platelets do not synthesize serotonin and this biogenic amine is taken up from the plasma, stored in dense granules and secreted upon platelets activation. Serotonin secretion was detectable in controls, but not changed by 1 and 10 mM H₂O₂ treatment, which again indicated a moderate peroxide effect. However, at 100 mM H₂O₂ (data not shown) a significant serotonin secretion has been seen, being in agreement with others [32]. Interestingly, serotonin is also implicated in APP processing [40, 41] and may thus contribute to any APP metabolic processes during isolation. MMPs are zinc-dependent endopeptidases, which are capable of degrading extracellular matrix proteins [42]. MMP-2 is expressed and secretion by platelets and is associated in blood-brain barrier impairment and axonal regeneration [43, 44]. It is interesting to note that MMP-2 is capable to degrade A β -peptides [45, 46], possibly indicating a role in A β -clearance. In AD a reduction in MMP-2 levels in the cerebrospinal fluid [47] and decreased levels in platelets have been found [26], pointing to an important role of MMP-2 in AD pathology. In the present study the secretion of MMP-2 was not altered after treatment with 1 and 10 mM H₂O₂, suggesting that MMP-2 is not directly involved in the H₂O₂-induced APP processing.

APP and A β in platelets during oxidative stress

A β -peptides are derived from amyloid precursor protein after processing by different secretases mainly leading to A β ₍₁₋₄₀₎ and A β ₍₁₋₄₂₎ peptides, which accumulate in the brain (plaques) and vessels (angiopathy) of AD patients. Platelets contain high levels of APP [1, 2, 11] and share some metabolic similarities with neurons, as they store and release neurotransmitters, such as e.g. serotonin. The processing of APP is altered in patients suffering from AD [4] and a reduced ratio between upper (130 kDa) and lower (106-110 kDa) APP fragments has been reported in AD compared to controls [12, 13], suggesting that APP ratios in platelets could be a biomarker for AD [10, 20]. The different sizes of APP

detected by Western blot analysis do not necessarily reflect different APP fragments [15] but rather APP cleavage products of a different size. Reasons for differences in molecular weight may also be due to alternative splicing or posttranslational modifications, such as glycosylation, phosphorylation and sulfatation [15–17]. In the present study, using Western blot analysis, we could detect three immunoreactive bands at 106, 110, and 130 kDa size in rat and human platelets. The monoclonal antibody 22C11 detects mature and immature APP, which may differ in the degree of glycosylation, as well as secreted APP fragments, at the N-terminal cysteine containing region [48]. Since the 106, 110 kDa fragments were hardly to distinguish, both bands were combined for measurements in the study. H₂O₂ markedly decreased the APP 106 110 kDa bands and less the 130 kDa band indicating cleavage and processing of full length APP and different metabolic APP-fragments. Interestingly, after H₂O₂ treatment of neuroblastoma cells a 5.5 kDa peptide was detected, possibly containing the A β -sequence [25]. Indeed, we were able to detect a smaller APP product at approximately 80 kDa in platelet supernatants, maybe indicating a secreted form of APP and indicating platelet activation and APP processing during platelets isolation. sAPP α is generated by the α -secretase and may have neuroprotective effects [49] sAPP α levels are much higher than A β levels due to the fact that the non-amyloidogenic pathway is predominant in platelets [2]. This is in line with our study where higher sAPP α than sAPP β levels were measured in human platelets. Thus, higher levels of sAPP α point to a predominant non-amyloidogenic pathway with α -secretase cleavage. Interestingly, treatment with H₂O₂ did not affect the secretion of sAPP α and sAPP β in platelets.

Indeed, we observed a higher variability in APP fragments during different platelet isolations and the H₂O₂ treatment did not exactly mimic the AD platelets phenotype. In fact, AD has a multifaceted pathogenesis and oxidative stress may only partly account for the changes seen in this disease. One limitation of measuring APP ratios is the strong variability of APP isoforms. Several studies suggested the usefulness of APP as a biomarker for AD [8, 18, 19], but these studies also reported that a high number of subjects was necessary for verification due to a high variance or instability during isolation of APP isoforms. This could be a reason for the observation that the changes seen in young treated platelets after oxidative stress did not exactly mimic the AD platelet profile. Furthermore, APP isoform expression in platelets may be influenced by many other factors, such as age, gender, and obesity. It is important to note that we used a calcium-free buffer to prevent calcium-dependent APP processing [8]. To eliminate the possibility that H₂O₂ may directly destroy the APP molecule we pretreated the Western blot membrane with 10 mM H₂O₂ just before detection of APP. Our data clearly show that all 3 APP bands were detectable pointing to a direct effect of H₂O₂ on APP processing and not just an artificial modification of the APP-binding site. In neuroblastoma cells H₂O₂-induced cleavage of APP into A β ₍₁₋₄₂₎ was observed, accompanied by a downregulation of the α -secretase, and up-regulation of β - and γ -secretase enzymes [50]. Others reported diminished levels of full length APP and a modification of APP-processing mediated by H₂O₂ in human neuroblastoma cells [25]. The predominant isoform of A β in platelets is the 40 amino acids long peptide A β ₍₁₋₄₀₎ and in our study, A β ₍₁₋₄₀₎ was clearly detectable in controls and after treatment with H₂O₂. It has been shown that A β ₍₁₋₄₀₎ accumulates in apoptotic platelets [51], indicating declined A β ₍₁₋₄₀₎ secretion. The longer A β ₍₁₋₄₂₎ form is the peptide which more likely aggregates and

contributes to plaque formation. It has been reported that H₂O₂ can increase A β production in human neuroblastoma cells [25], but A β ₍₁₋₄₂₎ can also mediate H₂O₂-induced cytotoxicity [52]. Our data show slightly decreased A β ₍₁₋₄₂₎ secretion after treatment with H₂O₂, indicating an accumulation of A β ₍₁₋₄₂₎ in platelets, which could affect the A β clearance at the blood-brain barrier and thus, contribute to plaque development in a long term.

Counteracting H₂O₂-mediated APP processing

In order to investigate if anti-oxidants counteract the aberrant APP processing upon H₂O₂ treatment, we tested the well known anti-oxidant glutathione. Glutathione prevents damage caused by reactive oxygen species and is generated in many cells [53]. A ratio between reduced and oxidized form of glutathione may indicate the intensity of oxidative stress [54]. It is well established, that H₂O₂-induced cytotoxicity in hippocampal brain slice cultures is attenuated by pretreatment with the anti-oxidant glutathione [55]. In our present study, glutathione partly counteracted the altered APP processing in H₂O₂-treated rat platelets indicating that indeed anti-oxidative molecules may be useful in AD therapy.

Conclusion

In conclusion our data show altered APP processing in rat platelets exposed to oxidative stress, which was partly prevented by glutathione, similar to the aberrant APP processing in AD. Oxidative stress similarly declined APP processing in young healthy volunteers and a decline of APP fragments has been seen in platelets of AD patients. Thus, chronic mild longlasting oxidative stress may influence the peripheral APP processing in platelets. In combination with a dysfunctional A β clearance these processes may play an important role in the development of AD and β -amyloid angiopathy.

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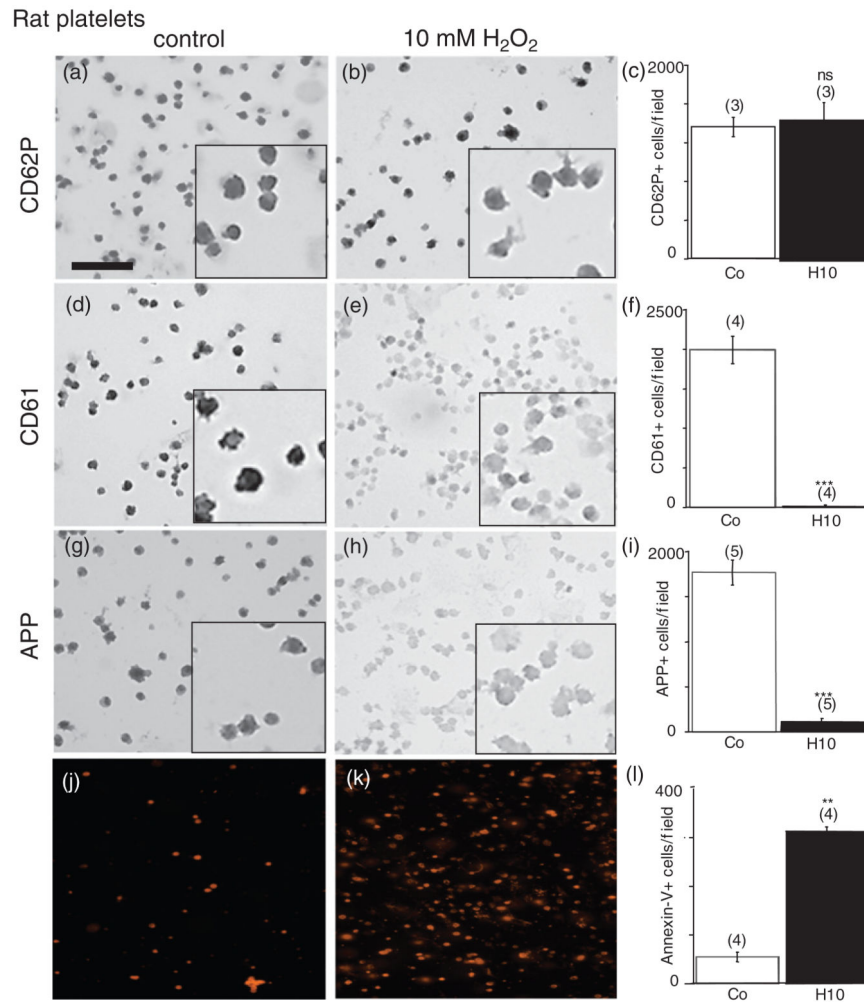


Figure 1.

Immunohistochemical staining of rat platelets. Rat platelets were treated with 10mM H₂O₂ for 20 minutes (b, e, h, k) or without (a, d, g, j) and spotted onto glassslides. Immunohistochemistry against CD62P (a, b) or CD61 (d, e) or amyloid precursor protein (APP) (g, h) or Annexin-V-staining (j, k) was performed. H₂O₂ markedly reduced the number of CD61 (e, f) and APP-positive platelets (h, i), but not of CD62P (b, c) and enhanced Annexin-V-positive cells (k, l). Platelets were counted under the microscope at 40 × magnification in a 336 × 448 μm field. Values are given as mean ± SEM. Statistical analysis was performed by Student's *t*-test. Values in parenthesis give the number of experiments. *** *p* < 0.001; ***p* < 0.01; ns, not significant; Scale bar in (a) = 24 μm (large image) and 9 μm (small inserts), and 40 μm (j, k).

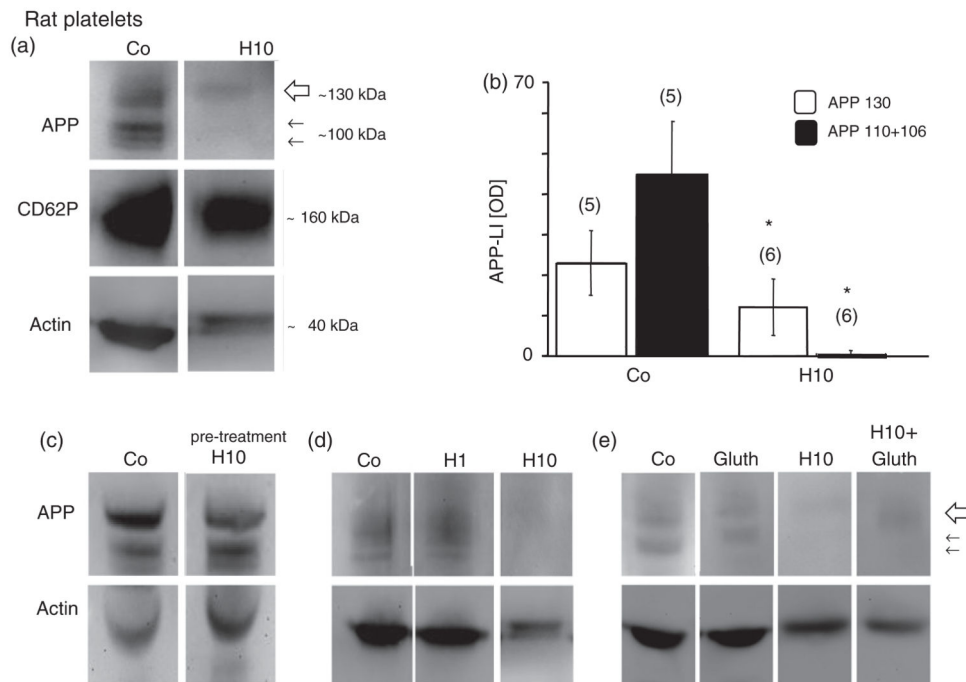
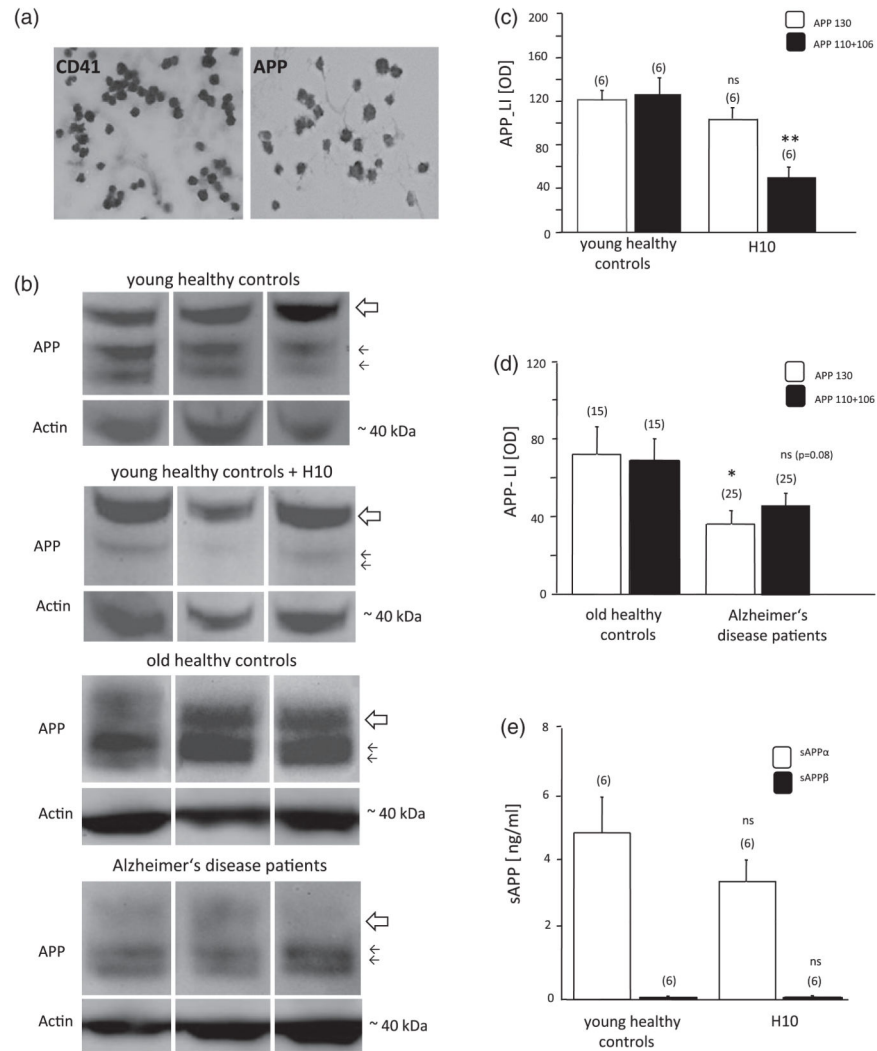


Figure 2.

Western blot analysis revealed three bands of amyloid precursor protein (APP) fragments at 130 (large arrow) and 110, 106 (small arrows) kilo Dalton (kD) in rat platelets (a). CD62P was used as a well established marker for control platelets and revealed a size of approximately 160 kDa (a). Actin was used for quantitative correlation and revealed a size of approximately 40 kDa (a) in control rat platelets. Incubation of rat platelets with 10mM H_2O_2 (H10) dramatically decreased the APP immunoreactivity of all 3 bands after 20 minutes (a), but did not affect CD62P (a). The size of actin was slightly increased after H_2O_2 treatment (a). H_2O_2 markedly reduced the APP 130, 110, and 106 kDa fragments, as shown in quantitative analysis (b). Pre-treatment of blots with 10mM H_2O_2 did not affect immunogenicity of the monoclonal 22C11/APP antibody (c). The effect of H_2O_2 was dose-dependent (d) and seen at 10mM H_2O_2 (H10), but not at 1mM H_2O_2 (H1). Glutathione (Gluth) partly counteracted the H_2O_2 -induced (H10) decline of APP expression (e). Each lane represents an individual subject. Values are given as mean \pm SEM optical density (OD). Statistical analysis was performed by students *t*-test. Values in parenthesis give the number of subjects; **p* < 0.05.

Human platelets

**Figure 3.**

Immunohistochemical staining of human platelets for CD41 and APP was performed (a). Western blot analysis of amyloid precursor protein (APP) in human platelets extracts revealed three fragments of APP at 130 (large arrow) and 110, 106 (small arrows) kilo Dalton (kDa) in young healthy controls (age = 33 ± 4 years; b) and old healthy controls (70 ± 2 years; b). Actin (40 kDa) was used for quantitative correlation. Each lane represents an individual subject. In platelets of Alzheimer disease (AD) patients the larger 130 kDa APP fragment was significantly decreased (b), while the smaller 110 and 106 fragments were only slightly reduced ($p = 0.08$), as shown by quantitative analysis (d). Treatment of platelets of young volunteers with 10mM H_2O_2 (H10) significantly reduced the smaller 110 and 106 kDa APP fragments (b, c), but did not affect the large 130 kDa form. Soluble APP α (sAPP α) levels were higher than soluble APP β (sAPP β) levels, however not changed after H_2O_2 treatment (e). Values are given as mean \pm SEM optical density (OD; c, d) or ng/ml (e).

Statistical analysis was performed by students *t*-test. Values in parenthesis give the number of subjects, **p* < 0.05; ns, not significant.

Table ISecretion of serotonin, matrix-metalloproteinase-2 (MMP-2), beta-amyloid (A β).

	Controls	1 mM H ₂ O ₂	10 mM H ₂ O ₂
Serotonin	27 ± 3 (8) -	45.5 ± 29 (6) ns	30.5 ± 13 (3) ns
MMP-2	115 ± 24 (5) -	207 ± 99 (3) ns	131 ± 19 (3) ns
A β ₍₁₋₄₀₎	17.1 ± 0.6 (6) -	13.6 ± 2.4 (4) ns	19.9 ± 2.4 (6) ns
A β ₍₁₋₄₂₎	3.2 ± 1 (5) -	1.2 ± 0.4 (4) *	1.0 ± 0.4 (6) *

Rat platelets (1×10^8) were incubated without (controls) or with 1 or 10mM hydrogen peroxide (H₂O₂) for 10 minutes (serotonin), 3 hours (MMP-2), or 24 hours (A β), then centrifuged and supernatant was evaluated by HPLC-EC (serotonin) or ELISA (MMP-2, A β). Values are given as mean ± SEM ng/mlx10 minutes (serotonin), pg/mlx3 hours (MMP-2), or pM/24 hours (A β). Statistical analysis was performed by one way analysis of variance with a subsequent Fisher LSD posthoc test. Values in parenthesis give the number of experiments.

ns, not significant.

*
 $p < 0.05$;