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Original Contribution

In vivo oxidative stress in brain of Alzheimer disease transgenic mice: Requirement for methionine 35 in amyloid β-peptide of APP

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ABSTRACT

Numerous studies have demonstrated oxidative damage in the central nervous system in subjects with Alzheimer disease and in animal models of this dementing disorder. In this study, we show that transgenic mice modeling Alzheimer disease—PDAPP mice with Swedish and Indiana mutations in the human amyloid precursor protein (APP)—develop oxidative damage in brain, including elevated levels of protein oxidation (indexed by protein carbonyls and 3-nitrotyrosine) and lipid peroxidation (indexed by protein-bound 4-hydroxy-2-nonenal). This oxidative damage requires the presence of a single methionine residue at position 35 of the amyloid β-peptide (Aβ), because all indices of oxidative damage in brain were completely prevented in genetically and age-matched PDAPP mice with an M631L mutation in APP. No significant differences in the levels of APP, Aβ(1–42), and Aβ(1–40) or in the ratio Aβ(1–42)/Aβ(1–40) were found, suggesting that the loss of oxidative stress in vivo in the brain of PDAPP(M631L) mice results solely from the mutation of the Met35 residue to Leu in the Aβ peptide. However, a marked reduction in Aβ-immunoreactive plaques was observed in the M631L mice, which instead displayed small punctate areas of nonplaque immunoreactivity and a microglial response. In contrast to the requirement for Met at residue 35 of the Aβ sequence (M631 of APP) for oxidative damage, indices of spatial learning and memory were not significantly improved by the M631L substitution. Furthermore, a genetically matched line with a different mutation—PDAPP(D664A)—showed the reverse: no reduction in oxidative damage but marked improvement in memory. This is the first in vivo study to demonstrate the requirement for Aβ residue Met35 for oxidative stress in the brain of a mammalian model of Alzheimer disease. However, in this specific transgenic mouse model of AD, oxidative stress is neither required nor sufficient for memory abnormalities.

Oxidative stress is elevated in the brain from subjects with Alzheimer disease (AD) and those with its arguably earliest form, amnestic mild cognitive impairment (MCI) [1–11]. Proteomic analyses of brain proteins from subjects with AD and MCI have identified oxidatively modified proteins that were generally dysfunctional and suggested to be consistent with the known biochemical, pathological, and behavioral alterations in both conditions [2,3,12–17].

Soluble amyloid β-peptide (1–42) [Aβ(1–42)] oligomers are thought to be the toxic species in brains of subjects with AD and MCI [18–21], leading to loss of learning and memory [19,22]. Aβ(1–42) added to neurons in vitro results in elevated protein oxidation and lipid peroxidation as indexed by elevated levels of protein carbonyls, 3-nitrotyrosine (3-NT), and protein-bound 4-hydroxy-2-nonenal (HNE), each of which is blocked by antioxidants [23–26]. Likewise, human Aβ(1–42) in vivo, either in *Caenorhabditis elegans* [21] or in rodent brain [27–31], leads to oxidative stress.

The 42-mer Aβ(1–42), as well as the 40-mer Aβ(1–40), contains a single methionine residue at position 35 [corresponding to residue 631 of human amyloid precursor protein (APP)]. Our laboratory has suggested that oxidative stress associated with Aβ(1–42) is dependent on the formation of a sulfur-centered, transient free radical involving this Met residue that, in turn, leads to lipid peroxidation and protein oxidation in neurons [24,32–35]. For example, substitution of norleucine (Nle) for Met (replacement of the S atom in Met by CH_2) in human Aβ(1–42) creates a peptide of the same length and same hydrophobicity as native human Aβ(1–42) that abrogates the associated oxidative stress and neurotoxic properties of Aβ(1–42) in cultured neurons, but does not prevent fibril formation [32,33,35]. In *C. elegans*, the expression of the human Aβ(1–42) peptide is asso-

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ciated with oxidative damage [21,32]; however, substitution of the codon for Met by that for Cys in DNA encoding human Aβ(1–42) prevents in vivo protein oxidation, while not affecting Aβ(1–42) deposition [32]. These results are consistent with the notion that mechanisms of oxidative stress involving Met35 of Aβ(1–42) apply in vitro and in vivo and do not involve deposition of the peptide.

In this study, we tested the hypothesis that this same Met35 residue is critical for oxidative stress and the Alzheimer-related spatial memory deficits in a well-characterized transgenic mouse model of Alzheimer disease. In this model, the PDAPP mouse model, mutations in human APP corresponding to the Swedish and Indiana familial forms of AD are expressed ($APP_{Sw,In}$) behind a platelet-derived growth factor (PDGF) β-chain promoter, resulting in Aβ accumulation, plaque formation, and memory deficits. To test this hypothesis, we generated transgenic mice matched for genetic background and $APP_{Sw,In}$ expression level, carrying an additional mutation—M631L—that substituted leucine for methionine at residue 35 of the Aβ peptides derived from APP. Leucine was chosen because it has a naturally occurring codon (whereas norleucine does not) and exhibits length and hydrophobicity characteristics similar to those of methionine. We then compared the degree of oxidative damage and memory deficits in these PDAPP Alzheimer model mice with vs without the M631L mutation. To complement these studies, we then assessed the oxidative damage in another PDAPP mutant mouse—D664A—that had been shown previously to prevent the memory deficits otherwise characteristic of Alzheimer model mice [36].

Materials and methods

All chemicals and antibodies used in Oxyblot (slot-blot) studies were purchased from Sigma–Aldrich (St. Louis, MO, USA) with exceptions noted. Dinitrophenylhydrazine (DNPH) and the primary antibody for the protein-bound 2,4-dinitrophenylhydrazone were purchased from Chemicon International (Temecula, CA, USA). The primary antibody for protein-bound HNE was purchased from Alpha Diagnostics International (San Antonio, TX, USA). Nitrocellulose membranes were purchased from Bio-Rad (Hercules, CA, USA).

Animals

Only male animals were used in the studies reported here. All animal studies were approved by the IACUC of the Buck Institute for Age Research and were carried out in the Institute's AAALAC-accredited vivarium. Derivation of transgenic mice has been described previously [36]. The PDAPP mice, J20 line, were kindly provided by Professor Lennart Mucke and kept in their original C57BL/J6 background. The PDAPP(D664A) mice have been described and characterized previously [36]. The PDAPP(M631L) mice were derived directly in the same genetic background as the other lines, i.e., C57BL/J6, using the same approach described previously for the PDAPP(D664A) mice [36]. A mutation was introduced in the PDGF β-chain promoter-driven human APP minigene carrying the Swedish and Indiana mutations that mutated Met631 (APP_{695} numbering) to Leu to generate PDAPP(M631L) transgenic mice by direct injection into C57BL/6J embryos. Mice from the PDAPP(D664A) B21 transgenic line were described previously [36]. PDAPP(M631L) transgenic mice express the PDAPP(Swe,Ind,M631L) transgene to levels 12.5% higher than those of the PDAPP(Swe,Ind) transgene in the PDAPP(J20) line. Groups of male nontransgenic (non-Tg) littermates from each transgenic line were used as controls. All transgenic lines were maintained by heterozygous crosses with C57BL/6J breeders (The Jackson Laboratory, Bar Harbor, ME, USA). All transgenic animals were heterozygous with respect to the transgene. Animals showing no motivation to swim (percentage of time floating >60%, i.e., "floaters") were not found in the studies reported here. Thus, no animals were excluded (see Behavioral testing and the supplementary material). Experimental groups of animals were Tg PDAPP, $n=11$; Tg PDAPP(M631L), $n=10$; non-Tg PDAPP, $n=4$; non-Tg PDAPP(M631L), $n=4$. Nontransgenic littermates were used as controls in all studies.

Oxyblots

Sample preparation

One-half of the brain was homogenized using a Wheaton glass homogenizer (~100 passes) in 1 ml of Media I buffer (0.32 M sucrose, 0.10 mM Tris–HCl, pH 8.0, 0.10 mM $MgCl_2$, 0.08 mM EDTA, 10 μg/ml leupeptin, 0.5 μg/ml pepstatin, and 11.5 μg/ml aprotinin; pH 8.0). Homogenates were vortexed, aliquotted into Eppendorf tubes, and sonicated with a Fisher 550 Sonic Dismembrator (Pittsburgh, PA, USA) for 10 s at 20% power. Protein concentrations were determined according to the Pierce BCA method (Pierce, Rockford, IL, USA).

Protein carbonyls

Protein carbonyls are a marker of protein oxidation and were determined as previously described [37]. Samples (5 μl) were derivatized at room temperature for 20 min in 10 mM DNPH and 5 μl of 12% sodium dodecyl sulfate (SDS). Samples were neutralized with 7.5 μl of neutralization solution (2 M Tris in 30% glycerol). Derivatized samples (250 ng) were then blotted onto a nitrocellulose membrane under vacuum pressure using a slot-blot apparatus (Bio-Rad). Membranes were blocked with 3% bovine serum albumin in Wash Blot [a PBS solution containing 0.04% (v/v) Tween 20 and 0.10 M NaCl] for 1.5 h and incubated with a 1:100 dilution of rabbit polyclonal anti-DNP primary antibody in Wash Blot for 2 h at room temperature on a rocker. Blots were rinsed three times for 5 min each in Wash Blot and subsequently incubated with a 1:8000 dilution of anti-rabbit IgG alkaline phosphatase secondary antibody in Wash Blot for 1 h at room temperature on a rocker. The membrane was washed three times for 5 min each in Wash Blot and developed using a solution of nitrotetrazolium blue chloride (0.2 mM) and 5-bromo-4-chloro-3-indolyl phosphate dipotassium (0.4 mM) in ALP buffer (0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$; pH 9.5). Dried blots were quantified using Scion Image software (PC version of Macintosh-compatible NIH software). Controls, using samples with no primary antibody or samples pretreated with $NaBH_4$ to reduce protein carbonyls, resulted in no staining (see Results).

HNE

Levels of protein-bound HNE are used as a marker of lipid peroxidation and were determined as previously described [38]. Samples (5 μl) were incubated at room temperature for 20 min in 5 μl of 12% SDS and 10 μl of Laemmli buffer [0.125 M Tryzma base, pH 6.8, 4% (v/v) SDS, and 20% (v/v) glycerol] and diluted with 7.5 μl PBS. Samples (250 ng) were then blotted onto a nitrocellulose membrane under vacuum pressure using a slot-blot apparatus. A 1:200 dilution of rabbit polyclonal anti-HNE primary antibody was used. Blots were developed and quantified as described above for protein carbonyls. Controls in which the primary antibody was preabsorbed with HNE gave no staining (data not shown).

3-NT

3-NT levels are used as another marker of oxidative damage [5,37]. Samples (5 μl) were incubated at room temperature for 20 min in 5 μl of 12% SDS and 10 μl of Laemmli buffer and diluted with 7.5 μl PBS. Samples (250 ng) were blotted onto a nitrocellulose membrane (as described above) and a 1:2000 dilution of rabbit polyclonal anti-3-NT primary antibody was added. Blots were developed and quantified as described above for protein carbonyls. Controls in which samples were treated with dithionite to reduce 3-NT residues to amines resulted in no staining (data not shown).

ELISA measurements of Aβ(1–40) and Aβ(1–42)

The unused half of the brains of the mice was used for measurements of Aβ(1–40) and Aβ(1–42) levels by ELISA. Aβ40 and 42 were measured in 5 M guanidine brain homogenates following the manufacturer's protocol (Invitrogen). Briefly, mouse hemibrains were weighed frozen and dissolved in 50 mM Tris buffer, pH 8, containing 5 M guanidine to achieve 100 mg brain/ml buffer. Brains were kept on ice and sonicated several times until homogeneous (buffer was added in stages). Homogenates were rotated at room temperature for 3–5 h and stored at −20 °C. Aβ(1–40) and Aβ(1–42) levels were then measured by ELISA (Invitrogen). Samples were diluted 1:50 in the provided standard diluent buffer and that same amount of guanidine was also included in the standard curve.

Immunohistochemistry

Amyloid plaques/Aβ and microglia were labeled, and plaques were counted, in six PDAPP and six Tg PDAPP(M631L) mice at 8 months of age. Mice were anesthetized and perfused with saline, and a sagittal hemibrain from each mouse was submersion-fixed in 2% paraformaldehyde. The fixed brains were processed into paraffin and 10-μm coronal sections cut from the area where the hippocampus is readily apparent (bregma −2.18) and mounted on charged glass slides (Platinum line; Mercedes Medical, Sarasota, FL, USA). Two slides were used for each antibody label for each individual mouse, two sections per slide. Both the anti-Aβ (6E10) and the anti-ionized calcium binding protein 1 (Iba-1) antibodies require that antigen retrieval be performed in 10 mM sodium citrate, pH 6, for 5 min at 98 °C. For the mouse monoclonal 6E10, endogenous mouse IgG was blocked with chicken anti-mouse (Vector) at 1:200 for 1 h in TBST (20 mM Tris base, 137 mM NaCl, pH 7.8, 0.05% Tween 20). Blocking of nonspecific antibody binding and all antibody incubations were done in 5% normal donkey serum in TBST; and all washes were done in TBST. Both primary antibodies were used at 1:500 overnight at 4 °C. The secondary antibodies were anti-mouse and anti-rabbit AlexaFluor 488 raised in donkey (Invitrogen).The labeled sections were viewed and imaged at 20 and 40× on a Nikon Eclipse E800 fluorescence microscope using the FITC filter and Nikon Act 1 software. Plaques were counted only from the molecular layer of the hippocampus within the granular cell layer of the CA regions and above the dentate gyrus.

Behavioral testing

The Morris water maze [39] was used to test spatial memory. All animals had normal motor and visual skills as determined by sensorimotor tasks performed before testing. All groups were assessed for swimming ability with a straight water alley (15 by 200 cm) containing a submerged (1 cm) 12×12-cm platform 2 days before testing. The procedure described by Morris [39] was followed. Details are included in the supplementary material.

Statistical analyses for behavioral testing

Statistical analyses are described in the supplementary material.

Results

Many lines of PDAPP(M631L) were produced (>10) to derive two with high-level APP expression, which is needed both for the phenotype and for accurate comparison to the PDAPP(J20) line (which has similar APP expression levels). The two highest-expressing lines, A1058 and A1059, are shown for measures of Aβ(1–40 and Aβ(1–42). However, the higher of these two lines, A1058, is quite similar in APP expression to the PDAPP(J20) line, and therefore it was used for the studies reported in this article.

Oxidative stress

Compared to non-Tg PDAPP littermate mice, brains isolated from mice with the Swedish and Indiana mutations in human APP [Tg PDAPP(J20)] displayed elevated indices of oxidative stress (Figs. 1a–c). Levels of protein oxidation [indexed by protein carbonyls ($P<0.003$, Fig. 1a) and 3-NT ($P<0.002$, Fig. 1b)] and lipid peroxidation [indexed by protein-bound HNE ($P<0.02$, Fig. 1c)] were elevated in brains from Tg PDAPP(J20) mice. To determine the specificity of the marker for protein carbonyls, J20 samples were pretreated with $NaBH_4$, which reduces carbonyls to alcohols, thereby preventing covalent attachment of DNPH. Fig. 2 shows that $NaBH_4$ treatment prevents staining by anti-DNP-protein antibody, in marked contrast to that of the J20 samples, demonstrating specificity of the antibody for protein carbonyls. This result confirms our previous reports of this same finding [16,40].

In contrast to the oxidative stress present in brains of PDAPP(J20) mice, replacement of methionine 35 of Aβ by Leu [corresponding to residue 631 of APP; Tg PDAPP(M631L)], which does not alter hydrophobicity or side-chain length, completely prevented the elevation in these oxidative stress parameters in brain [protein carbonyls, $P<0.35$; 3-NT, $P<0.76$; protein-bound HNE, $P<0.95$ (Figs. 1a–c)]. To ensure that these results were not due to a difference in expression of APP or accumulation of Aβ(1–42) or Aβ(1–40), levels of APP expression were compared by Western blotting, and levels of both peptides were determined by ELISA. The ratio of Aβ(1–42)/Aβ(1–40) was also determined. No significant differences in expression of Aβ(1–40) and Aβ(1–42) were found in the brains of Tg PDAPP(M631L) mice compared to those of Tg PDAPP(J20) mice (Fig. 3). If anything, the ratio of Aβ(1–42) to Aβ(1–40) was elevated in the brains of the Tg PDAPP(M631L) mice. Plaque load was determined by 6E10 immunohistochemistry. There was a marked reduction in Aβ-immunoreactive plaques in the M631L mice (Fig. 4), and the M631L plaques were smaller than the plaques in the J20 mice. Accompanying this plaque reduction was a clear increase in punctate 6E10 labeling, along with a microglial response (Fig. 4); the latter demonstrated by Iba-1 staining and the observation of microglial processes characteristic of activation (Fig. 4).

To complement these studies, we assessed the oxidative damage in another PDAPP mutant mouse—D664A—which had been shown previously to escape the memory deficits otherwise characteristic of PDAPP mice [36]. Unlike the Tg PDAPP(M631L) mice, the D664A mutation did not result in any reduction in protein carbonyls, 3-nitrotyrosine, or protein-bound 4-hydroxy-2-nonenal (Supplementary Fig. 1).

Behavioral measures

Before behavioral analyses, mice were evaluated to ensure that their visual and somatic motor systems were intact and that they were capable of swimming. None of the mice evaluated in this study displayed any problems completing these preliminary tests.

In the analyses of behavior employing the Morris water maze, two indices were examined. The latency index, which reflects the time necessary to find a hidden escape platform, is a measure of visuospatial learning and is characteristically abnormal in Alzheimer model transgenic mice, as well as other mice with hippocampal dysfunction. The M631L mutation led to no improvement over the PDAPP mice in latency index: performance of Tg PDAPP and Tg PDAPP(M631L) mice was indistinguishable and in both cases significantly impaired with respect to that of the non-Tg PDAPP group [$P<0.001$ and $P<0.001$, respectively, Bonferroni's posttest applied to a significant effect of genotype $F(3,78)=7.05$, $P=0.0013$, repeated-measures two-way ANOVA] (Fig. 5a). Transgenic PDAPP(M631L) mice also showed significantly increased floating behavior with respect to all other groups, which may reflect helplessness associated with memory

dysfunction [$P<0.001$, $P<0.01$, and $P<0.001$ with respect to non-Tg PDAPP, non-Tg PDAPP(M631L), and Tg PDAPP, respectively, Bonferroni's posttest applied to a significant effect of genotype, $F(3,78)=9.22$, $P=0.0003$, repeated-measures two-way ANOVA], which were not significantly different from each other (Fig. 5b).

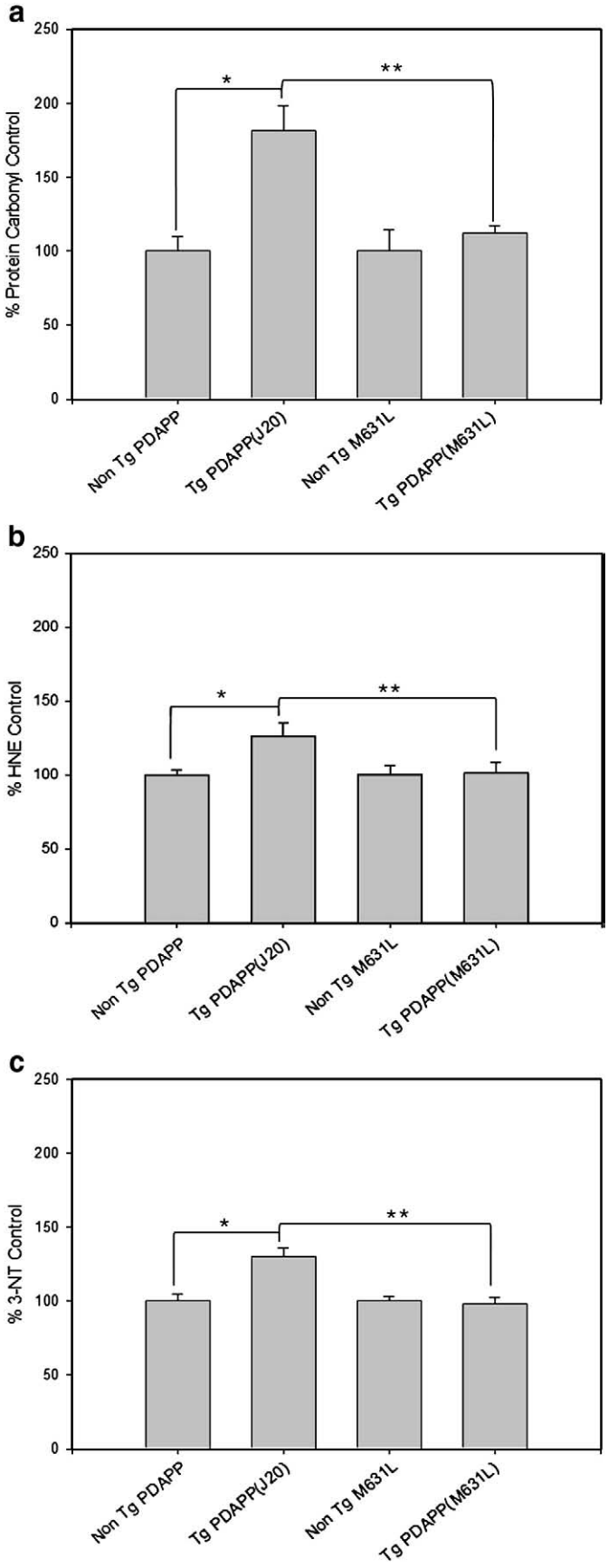

Fig. 1. In vivo oxidative modification of brain in transgenic and nontransgenic mice: role of Met35 of Aβ. (a) Oxidative stress as indexed by protein carbonylation. Results show a significant increase in the levels of protein carbonyls in Tg PDAPP(J20) mice ($n=10$) compared to age-matched non-Tg PDAPP controls ($n=5$;*$P<0.003$). Tg PDAPP (M631L) mice ($n=10$) did not show a significant increase in protein carbonyls compared to their respective non-Tg PDAPP(M631L) controls ($n=5$; $P<0.35$), but did, however, have significantly reduced protein carbonyl levels compared to age-matched Tg PDAPP(J20) mice (**$P<0.0004$). Nontransgenic control values were set to 100%, to which transgenic values were compared. These data are presented as means ± SEM. Statistical analyses employed Student's two-tailed t test. (b) Lipid peroxidation as indexed by protein-bound HNE. A significant increase in the levels of protein-bound HNE in Tg PDAPP(J20) mice ($n=10$) compared to age-matched non-Tg PDAPP(J20) controls ($n=5$; *$P<0.02$) was found; however, Tg PDAPP(M631L) mice ($n=10$) did not show a significant change in protein-bound HNE levels compared to non-Tg PDAPP (M631L) controls ($n=5$; $P<0.95$). Moreover, there were significantly reduced protein-bound HNE levels in Tg PDAPP(M631L) mice compared to age-matched Tg PDAPP(J20) mice (**$P<0.04$). Nontransgenic control values were set to 100%, to which transgenic values were compared. These data are presented as means ± SEM. Statistical analyses employed Student's two-tailed t test. (c) Protein-resident 3-NT in brain. In agreement with both protein carbonyl and protein-bound HNE parameters, a significant increase in Tg PDAPP(J20) mouse ($n=10$) 3-NT levels was found compared to age-matched non-Tg PDAPP(J20) controls ($n=5$; *$P<0.002$). In contrast, Tg PDAPP(M631L) mice ($n=10$) did not show a significant change in 3-NT levels compared to non-Tg PDAPP (M631L) controls ($n=5$; $P<0.76$), whereas a significant reduction in Tg PDAPP (M631L) 3-NT levels compared to age-matched Tg PDAPP(J20) mice was observed (**$P<0.0001$). Nontransgenic control values were set to 100%, to which transgenic values were compared. These data are presented as means ± SEM. Statistical analyses employed Student's two-tailed t test.

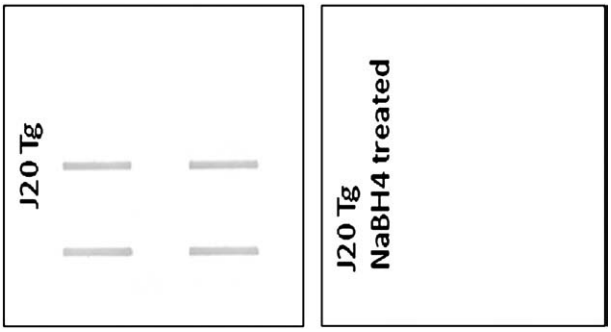

Fig. 2. Specificity of the assay for protein carbonyls. Duplicate brain samples from PDAPP(J20) mice ($n=2$) were subjected to analysis of protein carbonyls (left) without and (right) with prior treatment with the strong reducing agent $NaBH_4$, as described previously [16,40], which reduces carbonyls to alcohols. As in our earlier studies, prior treatment of brain samples with $NaBH_4$ abrogates staining with anti-DNP-protein antibodies, demonstrating the specificity of the assay for protein carbonyls.

The second behavioral index assessed was the number of passes the mice made over the original position of the platform after acquisition training was completed and the platform was subsequently removed; this index measures memory retention. As described previously [36,41,42], transgenic PDAPP mice showed a significant reduction in crosses over the former platform position (target) compared to non-Tg littermates [$P<0.05$ as a result of Dunnet's multiple comparison test applied to a significant effect of genotype ($P=0.03$) in one-way ANOVA]. The PDAPP(M631L) mice showed, just as for the latency index, no significant difference between the PDAPP and the PDAPP(M631L) mice ($P=0.63$, two-tailed unpaired t test), suggesting that the M631L mutation did not improve retention (Fig. 5c).

Discussion

To our knowledge, this is the first study to demonstrate the in vivo requirement for the single Met residue at position 35 of human Aβ in the production of Alzheimer-associated oxidative stress in mammalian brain. Substitution of Met by Leu at residue 631 of

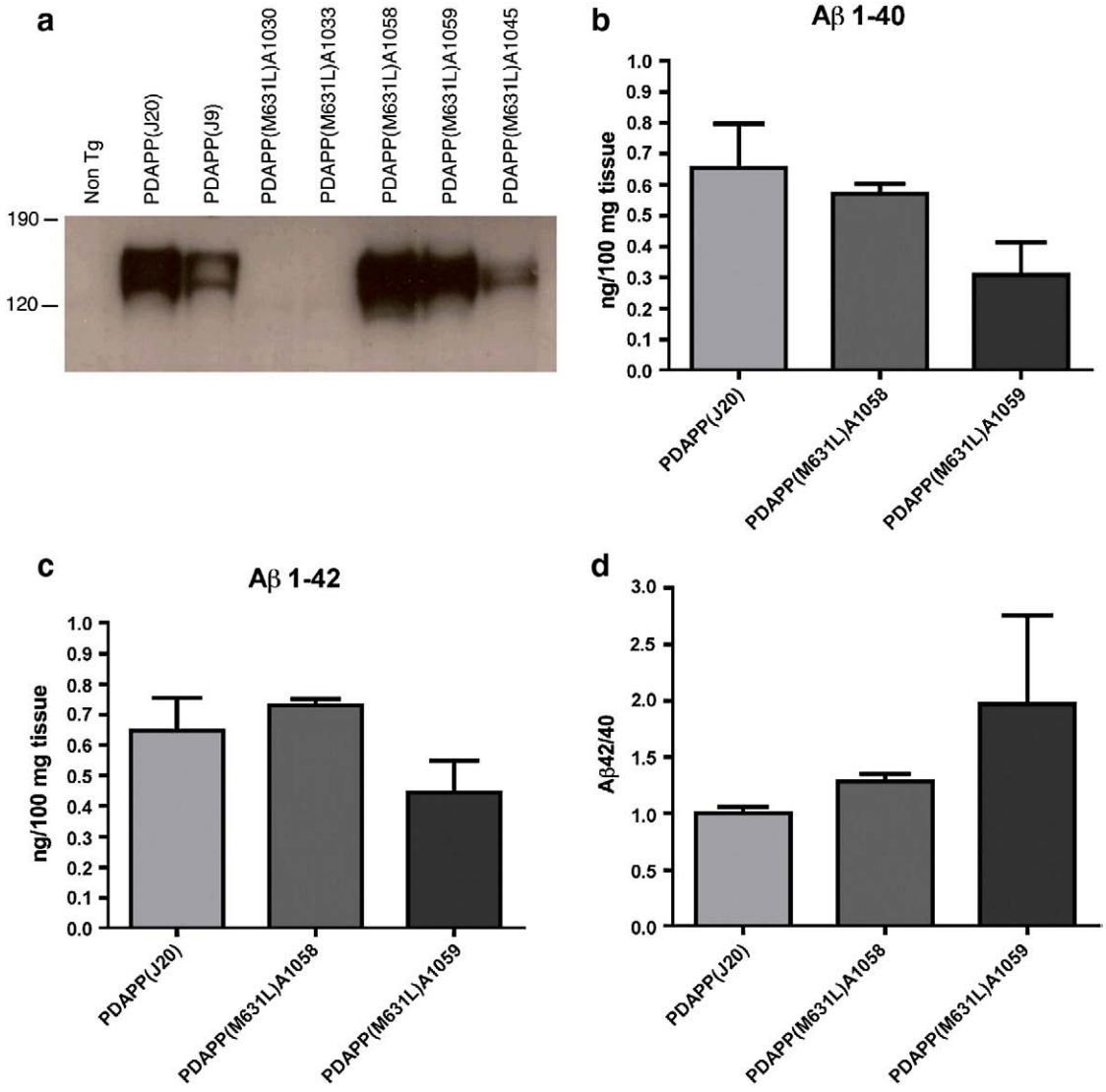

Fig. 3. PDAPP(M631L) mice express APP, and produce Aβ(1–40) and Aβ(1–42), at levels similar to those of the PDAPP mice. APP expression and Aβ quantitation were performed as previously described [36]. No significant differences were found in APP expression, Aβ(1–40) production, or Aβ(1–42) production between the PDAPP(J20) line and the PDAPP (M631L) A1058 line, which were the lines used in this study.

Swedish and Indiana human mutant APP (and therefore position 35 of the derivative Aβ peptides) completely abolished the oxidative stress observed when Met was present (Figs. 1a–c). Differential expression of APP, Aβ(1–42), and Aβ(1–40) was shown not to be the cause of this lack of oxidative stress in brains of Tg PDAPP (M631L) mice (Fig. 3). However, the M35L mutation in Aβ peptides did influence the plaque formation, because the M631L mice displayed a reduction in immunoreactive plaques; instead, they showed nonplaque punctate staining accompanied by a microglial response. These findings suggest that Met35 may be involved in Aβ plaque formation. Moreover, the results are consistent with the notion that any nonspecific or compensatory changes secondary to the presence of a human transgene are unlikely to be responsible for the lack of oxidative stress in brain isolated from the Tg PDAPP (M631L) mouse.

Methionine has important cellular functions, such as shielding the active sites of enzymes against oxidation [43], promotion of helical secondary structure of proteins [44], aiding activity of certain repair or chaperone proteins [45], and participation in maintenance of the redox state of cells [46]. In addition, within the Aβ peptide, the current studies indicate that Met35 plays a critical role in the oxidative damage associated with Alzheimer disease.

We previously demonstrated that oxidative stress associated with Aβ(1–42) in neurons is via catalytic processes [35]. This previous study demonstrated two important aspects of Aβ-mediated oxidative stress in neurons: (a) A small amount of Met-centered free radical on Aβ(1–42) can be greatly amplified. The oxidative damage to neurons induced by Aβ(1–42) results in part from the chain reaction produced in the lipid phase of the membrane initiated by abstraction of allylic H atoms from unsaturated acyl chains on lipids by the radical cation on the S atom of Met, i.e., the sulfuranyl radical [35]. This chain reaction continues as long as there are such allylic H atom sites available and products of lipid peroxidation, such as HNE, are produced. These, in turn, react with Cys, His, and Lys residues of proteins to cause massive changes in structure [47] and function [38,48,49]. (b) When the sulfuranyl free radical formed on the single Met residue of Aβ(1–42) abstracts the allylic H atom from an unsaturated acyl chain of phospholipids, it forms an acid whose pK_a is −5. Hence, any base, including water, can remove the H^+ from this acid, forming again Met itself, which can undergo a one-electron oxidation again to the sulfuranyl free radical. That is, the process is catalytic. Of course, HNE can be formed only by a radical located *within* the bilayer, because the reactivity of the radicals is too high for diffusion from outside the cell into the bilayer to find an allylic H

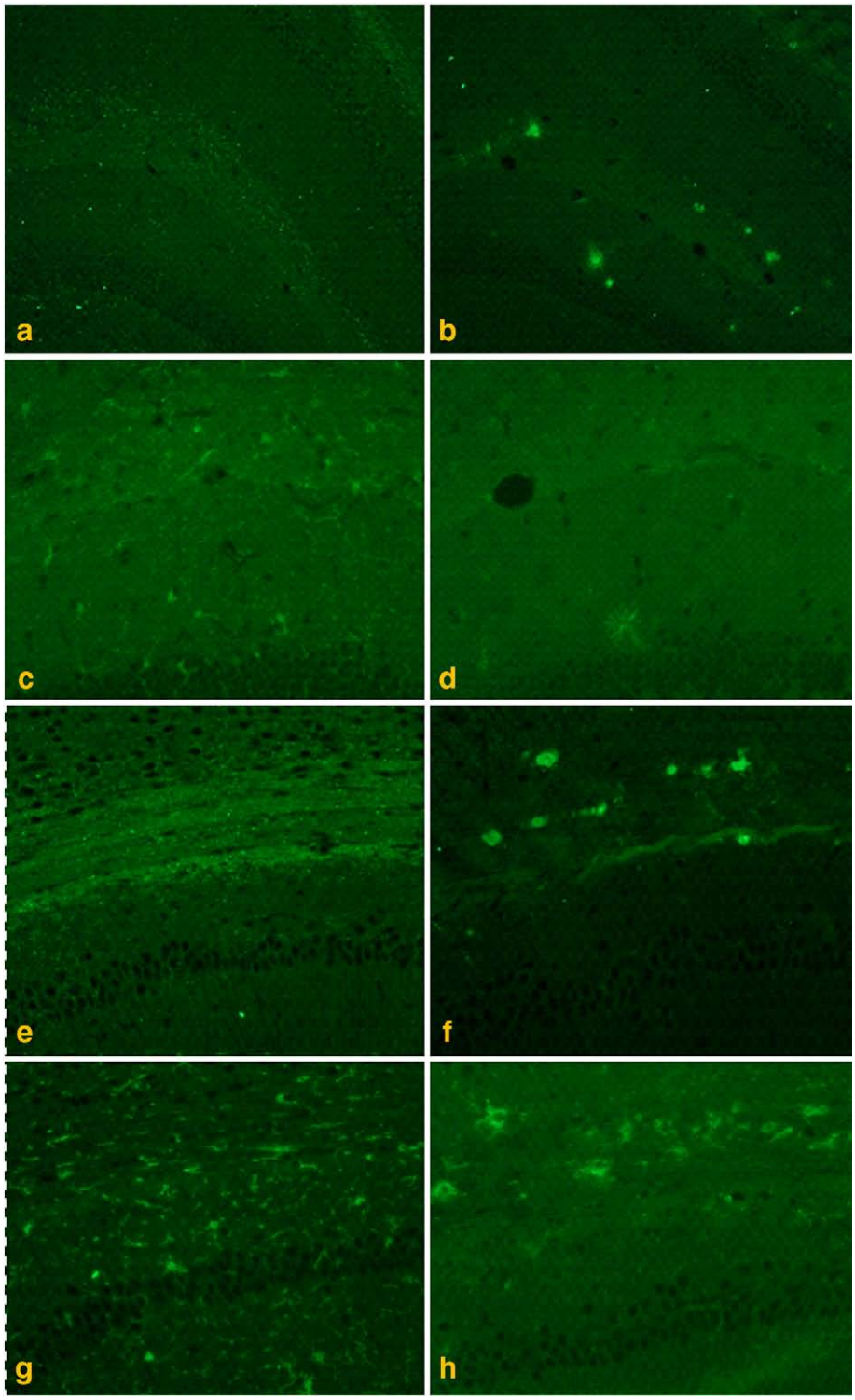

Fig. 4. Immunohistochemical staining of amyloid plaques and microglia. Coronal paraformaldehyde-fixed paraffin-embedded sections were labeled with antibodies against Aβ (6E10) and Iba-1, a marker for microglia. (a) In the molecular layer of the hippocampus just superior to the dentate gyrus in the M631L mice there were few plaques but distinctive punctate staining; (b) in the J20, however, greater plaque formation was seen in the same area (6E10 images at 10× original magnification), but no punctate staining. (c) The punctate pattern of Aβ distribution was associated with microglial activation in the M631L, which was not observed (d) in the J20, which instead showed amoeboid phagocytic microglia surrounding plaques (Iba-1 images at 20× original magnification). (e) The punctate labeling in the M631L mice was even more marked in the corpus callosum and (g) the white matter tracts lateral to the hippocampus and surrounding the thalamus, associated with a proportionately greater microglial response; again, (f) the J20 did not demonstrate punctate staining, but instead formed plaques in the corpus callosum, surrounded (h) by phagocytic microglia (all 20× original magnification).

atom on unsaturated chains of lipids. Yet, Aβ does indeed lead to HNE formation [38,50].

The notion proposed by the Butterfield laboratory, that a one-electron oxidation of Met35 of Aβ(1–42) forms a sulfuranyl free radical that in turn leads to lipid peroxidation and protein oxidation in neurons in the brain of subjects with AD and MCI [33–35], has been supported by other laboratories. For example, pulse radiolysis studies revealed a sulfur-centered free radical on Aβ(1–40), but not on the peptide with the nontoxic reverse sequence, Aβ(40–1) [51]. Our studies showing a lack of neurotoxicity, protein oxidation, and lipid

peroxidation in brain cells exposed to Aβ(1–42) when the single Met residue of this peptide was substituted by Nle [32,35] or Cys [32] have been confirmed by others [52–54]. The idea of a one-electron oxidation of Met35 within Aβ(1–42) [32–34] has also been supported by the experimental and theoretical research of others [55,56]. That study suggested that Cu^{2+} was bound weakly to Met in Aβ(1–42). This is consistent with our earlier hypothesis [33] and with the results of studies with the divalent cation chelator clioquinol [57,58]. Previously, researchers had shown that this agent could dissolve plaques from transgenic mice containing human mutant APP [57], even though the K_D of Cu^{2+} for clioquinol reportedly was 9 orders of magnitude greater than that for Aβ(1–42) [58]. Hence, we suggested [33] that a weakly bound Cu^{2+} on Met could account for two observations: (a) an electron transfer reaction involving a one-electron oxidation of Met to form the sulfuranyl radical, while Cu^{2+} is simultaneously reduced to Cu^{+}, the latter capable of undergoing Fenton-type chemistry to produce highly reactive hydroxyl free radicals; and (b) the clioquinol data, i.e., a weakly bound Cu^{2+} could easily be removed from Met by clioquinol, whereas it is unlikely that clioquinol could remove Cu^{2+} bound to the three His residues of Aβ(1–42) (positions 6, 13, and 14) with a K_D in the attomolar range.

Oxidative stress has been demonstrated in the brain of another transgenic mouse model of AD [59], as well as a knock-in mouse model of AD [27]. Although some have questioned the relevance of oxidative stress associated with Aβ(1–42) in vivo [60], it is clear from the present study that oxidative stress does not occur in brain in vivo if the single Met residue of Aβ(1–42) is not present. The importance of this study is that the results establish unequivocally the critical nature of the Met35 residue of Aβ for oxidative stress in brain in vivo in a mammalian species. We posit that a similar importance of Met35 in Aβ(1–42) exists in the brains of subjects with MCI and AD. Clearly, genetic manipulation of Met in Aβ(1–42) in humans is currently not possible, so our results point to clinical translational possibilities for therapy to potentially treat, slow the progression of, or prevent MCI and AD. Specifically, blocking the oxidative damage dependent on the Met residue of Aβ(1–42) in the brains of subjects with MCI and AD offers a highly focused therapeutic approach. Studies to test this notion are in progress.

In contrast to the clear role of Met at residue 35 of Aβ(1–40 and/or 1–42) in oxidative damage, spatial learning and memory in mice with the Tg PDAPP(Swe,Ind) double-mutant APP did not show a dependence on Met35 of Aβ. In Morris water maze studies, neither the latency period nor the number of times the Tg PDAPP (M631L) mice swam over the platform site differed significantly from that of the Tg PDAPP mice. Hence, in this mouse model, Met35 and its associated oxidative damage are not required for the spatial memory loss that is characteristic of the model (and which also characterizes human Alzheimer disease). Furthermore, a different mutation in the same model—PDAPP(D664A)—prevented spatial memory abnormalities, yet failed to show any effect on oxidative damage indices, demonstrating that such oxidative damage is not sufficient for the behavioral phenotype, either. However, it is important to note that it is still possible that we have, to date, missed a behavioral effect of the M631L mutation: mice were analyzed for markers of oxidative stress at 9 months of age, but for behavior necessarily at an earlier age (6 months), so earlier or later differences in behavior, if present, would have been missed by the studies reported here. The possibility that later deficits might be mollified by the M631L mutation is compatible with recently published studies showing that the PDAPP(D664A) mice do show

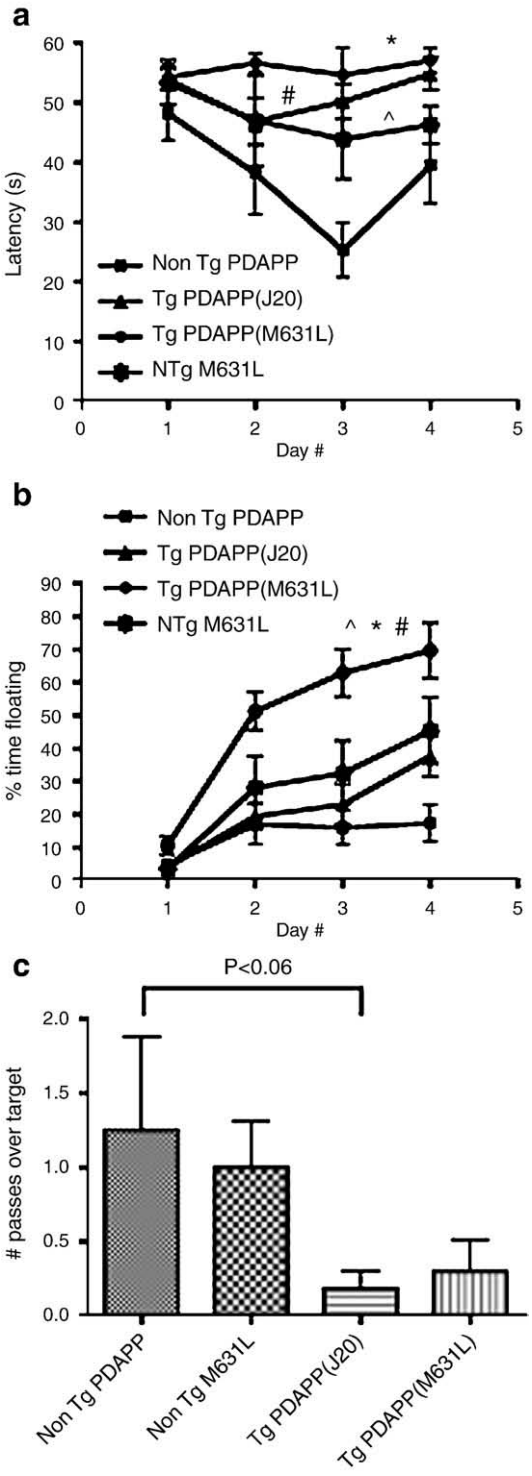

Fig. 5. Effect of the M631L mutation on behavior as assessed with the Morris water maze in PDAPP mice. (a) Spatial training. Mean latencies to reach a hidden platform were significantly different for the Tg PDAPP, the Tg PDAPP(M631L), and the non-Tg PDAPP(M631L) groups with respect to non-Tg PDAPP littermates [$^{\#}P<0.01$, $^{*}P<0.01$, and $^{\wedge}P<0.05$, respectively; Bonferroni's posttest applied to a significant effect of genotype $F(3,78)=7.05$, $P=0.0013$, repeated-measures two-way ANOVA]. (b) Floating. Percentage time spent floating increased significantly as a function of day number during training [$F(3,78)=40.14$, $P<0.0001$, two-way ANOVA]. Tg PDAPP (M631L) mice spent a significantly larger percentage of trial time floating than all other groups [$^{*}P<0.001$, $^{\#}P<0.01$, and $^{\wedge}P<0.001$ with respect to non-Tg PDAPP, non-Tg PDAPP(M631L), and Tg PDAPP, respectively; Bonferroni's posttest applied to a significant effect of genotype $F(3,78)=9.22$, $P=0.0003$, repeated-measures two-way ANOVA]. "Floaters" were excluded from all other analyses. (c) Probe trial. Retention of the former platform site was impaired in Tg PDAPP mice with respect to the non-Tg group [$P<0.05$; Dunnet's multiple comparison test applied to a significant effect of genotype ($P=0.03$) in one-way ANOVA]. No significant difference was observed for the comparison between Tg PDAPP and Tg PDAPP(M631L) ($P=0.63$). Data are means ± SEM.

a trend toward Morris water maze abnormalities at 13 months of age that they do not show at earlier ages [41]; and although it is not clear that this trend is the result of oxidative damage, the PDAPP (D664A) mice do indeed show such damage. Therefore, subsequent studies will assess transgenic mice with both the D664A and the M631L mutations, to determine whether they resist the trend toward late deterioration in Morris water maze performance.

Finally, immunoreactive Aβ deposits in the brains of PDAPP (M631L) mice clearly revealed marked differences from the frank plaque deposits of PDAPP(J20) mice. Moreover, there is evidence of microglial activation in the M631L mice. Hence, it is conceivable that the latter influenced behavior in the PDAPP(M631L) mice, potentially contributing to the lack of behavioral improvement relative to that of PDAPP(J20) mice.

In contrast to the lack of effect of the M631L mutation on memory in Alzheimer model mice, in the *C. elegans* AD model, in which human Aβ(1–42) was expressed in muscle (as opposed to full-length APP in neurons), substitution of the Met of Aβ(1–42) by Cys abrogated the paralytic phenotype of the worm without changing the deposition of the modified peptide [32]. Also in potential contrast to the present results, aged beagle dogs (whose Aβ sequence is identical to that of humans) fed a high antioxidant diet and given a program of behavioral enrichment showed decreased oxidative stress in brain, improved learning and memory, and decreased levels of Aβ(1–42) [61]. Although the fundamental differences of these previous studies make direct comparisons to the current studies difficult—for example, comparison of mutant APP expression in mouse brain to Aβ expression in nematode muscle may be a nonproductive exercise—such comparisons may nonetheless offer some clues to pathogenesis and strategies for future studies: first, the mouse model employed full-length APP with familial AD-associated mutations, whereas the nematode model expressed only the Aβ peptide. Thus, the lack of improvement in the mouse model may have been due to the other, non-Aβ peptides derived from APP—sAPPβ, Jcasp, and C31—all of which have been implicated in aspects of the AD phenotype [36,62–64]. Second, the mouse, dog, and nematode data are all compatible with the notion that multiple mechanisms are involved in AD pathogenesis, such that the threshold mechanism is likely to differ from model to model. If this proves to be the case, then the results from these different systems argue that it will be critical to test potential new therapeutics in more than one system, and ultimately it will be crucial to determine which model or combination of models provides the greatest predictive value for human Alzheimer disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.freeradbiomed.2009.10.035.

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