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RESEARCH ARTICLE

4-Hydroxynonenal Immunoreactivity is Increased in Human Hippocampus After Global Ischemia

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Oxidative stress and lipid peroxidation may contribute to the pathology of neurodegenerative disorders such as Alzheimer's disease (AD) and cerebral ischemia. 4-Hydroxynonenal (4-HNE) is a toxic by-product of lipid peroxidation, and immunoreactivity to 4-HNE has been used to examine lipid peroxidation in the pathogenesis of AD and ischemia. This study sought to determine *1)* if there are cellular alterations in 4-HNE immunoreactivity in the human hippocampus after global ischemia, and *2)* whether possession of an apolipoprotein E (*APOE)* ϵ4 allele influenced the extent of 4-HNE immunoreactivity. 4-HNE immunoreactivity was assessed semi-quantitatively in the temporal lobe of a group of controls (n = 44) and in a group of patients who had an episode of global ischemia as a result of a cardiorespiratory arrest and subsequently died (n = 56, survival ranged from 1hr to 42days). There was minimal cellular 4-HNE immunoreactivity in the control group. However, compared to controls, 4-HNE immunoreactivity was significantly increased in neurons ($p < 0.0002$) and glia ($p < 0.0001$) in the hippocampal formation after global ischemia. Possession of an *APOE* ϵ4 allele did not influence the extent of neuronal or glial 4-HNE immunostaining in the control or global ischemia group. There was a significant negative correlation between the extent of neuronal 4-HNE immunoreactivity with survival period after global ischemia ($r^2 = 0.0801$; $p < 0.036$) and a significant positive correlation between the extent of glial 4-HNE immunoreactivity and survival after global ischemia ($r^2 = 0.2958$; $p < 0.0001$). The data indicate a marked increase in neuronal and glial 4-HNE. This substantiates a role for lipid peroxidation in the pathogenesis of cerebral ischemia. There was no indication that *APOE* genotype influenced the extent of 4-HNE immunoreactivity.

Introduction

Lipid peroxidation is a self-propagating process that damages lipid membranes and as part of this process, releases a cytotoxic aldehyde, 4-hydroxynonenal (4-HNE) from polyunsaturated fatty acid side chains (4, 5). 4-HNE can diffuse readily from the site of origin inducing both intra and extracellular damage. 4-HNE is an electrophilic species that can bind to cytoskeletal proteins, such as neurofilaments, microtubule associated proteins and glial fibrillary acidic protein (8, 20, 23, 27). Modification of these proteins leads to neuronal, axonal and glial cell damage (3, 21, 36). 4-HNE also covalently binds to ionic transporters (35); mitochondrial sites (31) and glutamate uptake transporters (2, 13, 36). These actions could lead to increased production of free radicals (12, 13, 14, 19), increased levels of intracellular calcium, or induce excitotoxicity (2, 13, 36); all of which contribute to cell death. 4-HNE has also been proposed to play a role in modifying an inflammatory response and interact with signalling transduction pathways that are involved in neurodegenerative disorders (32).

Increased immunoreactivity of 4-HNE, a marker of lipid peroxidation (6), has been demonstrated after experimental traumatic brain injury (45), and in a model of cerebral ischemia in rats (21, 42). 4-HNE modified proteins are also present in post-mortem Alzheimer's disease (AD) tissue compared to controls (17, 18, 24, 25). In AD, 4-HNE modified proteins are associated with the neuropathological hallmarks: neurofibrillary tangles (24) and amyloid β-protein plaques (1). There is also some evidence that detection of 4-HNE in AD, is apolipoprotein E genotype dependent (24, 26). These data suggest that lipid peroxidation and 4-HNE may be involved in the pathogenesis of both acute and chronic neurodegenerative conditions.

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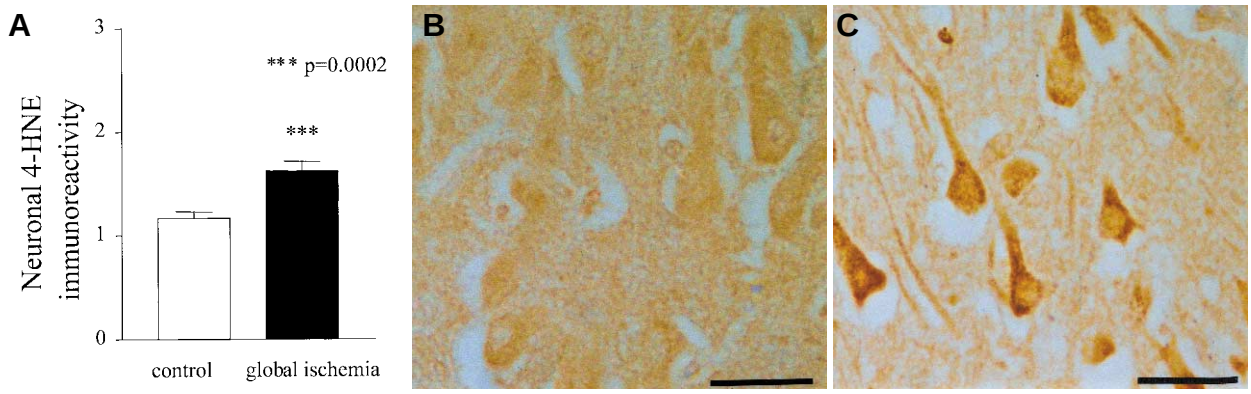

Figure 1. *Neuronal 4-HNE immunoreactivity in the control and global ischemia group.* The semi-quantitative assessment of the extent of neuronal 4-HNE immunoreactivity in the control and global ischemia group in the hippocampal formation A). Histograms are mean ± SEM. There was a significant increase in the extent of neuronal 4-HNE immunoreactivity in the global ischemia group compared to controls, $p < 0.0002$, using Student's unpaired *t*-test. Illustrative examples of neuronal 4-HNE immunostaining in a B) control and C) global ischemia case with scores of O and 3 respectively. Scale bar = 50μm.

Apolipoprotein E (apoE, protein; *APOE*, gene) is a polymorphic protein of three isoforms; E2, E3 and E4, encoded by the corresponding alleles ϵ2, ϵ3 and ϵ4. The *APOE* ϵ4 allele is a known risk factor for AD (33), and a determinant of poor outcome after acute brain injury (28, 40). The mechanisms by which the *APOE* ϵ4 allele confers relative vulnerability are unclear. In relation to AD, it has been shown that apoE interacts with amyloid and tau in an apoE-isoform dependent manner with the apoE E4 isoform more likely to promote the formation of amyloid plaques and neurofibrillary tangles (7, 37, 38). Pertinent to acute brain injury, apoE has been proposed to act as an anti-oxidant (9, 15) with the E4 isoform being the least effective (22). We have previously demonstrated an increase in cellular 4-HNE immunoreactivity after global ischemia in apoE deficient mice. Intraventricular infusion of lipid-conjugated apoE ameliorated the extent of 4-HNE immunoreactivity after ischemia providing further evidence that apoE could act as an antioxidant after brain injury (10).

The aims of this study were twofold. First, to determine whether 4-HNE modified proteins were increased after global ischemia in patients who had a cardiorespiratory arrest and subsequently died. This type of brain injury results in a relatively stereotyped pathology in which there is selective neuronal damage, particularly in the hippocampus. Secondly, to determine whether the extent of lipid peroxidation, as defined by 4-HNE immunoreactivity, is *APOE* genotype dependent.

Materials and Methods

Post-mortem human brain tissue. Archival paraffin embedded blocks of medial temporal lobe that included the hippocampus were selected from 56 patients who died after having experienced an episode of global ischemia due to cardiorespiratory arrest (36 males; 20 females; age range 17 to 85 years; mean age 52 ± 2 years). The survival period after the initial episode of global ischemia ranged from 1 hour to 42 days (mean survival, 5.8 ± 1 days). Forty-four control patients (29 males, 15 females; age range from 18 to 82 years, mean age 50 ± 3 years), without clinical or pathological evidence of neurological or psychiatric impairment or cardiovascular disease, were used in this study. As previously described, the *APOE* genotype was determined from the paraffin embedded tissue using the polymerase chain reaction/restriction enzyme analysis technique (29).

4-Hydroxynonenal immunoreactivity. Paraffin embedded sections were de-waxed in an oven for 30 minutes (mins), then in xylene for 20 mins, and then in decreasing concentrations of alcohol, 100%, 90% and 70% for 5 mins each. Sections were put in citric acid buffer (pH6) and microwaved for 10 mins and left in buffer for 40 mins. Endogenous peroxidase was eliminated by incubating with 3% H_2O_2 in methanol for 30 mins, followed by washes in running H_2O (20 mins), and phosphate buffer saline (PBS) (2 × 5 mins). Non-specific sites were blocked with 10% normal goat serum and 0.5% bovine serum albumin in PBS for 1h; then incubated overnight at 4°C in anti-4HNE (1:2000; rabbit polyclonal, Calbiochem) in PBS containing 15% normal goat serum and 1% BSA. After, PBS washing sections were incubated with a biotinylated anti-rabbit IgG and processed with a Vectastain ABC Elite kit.

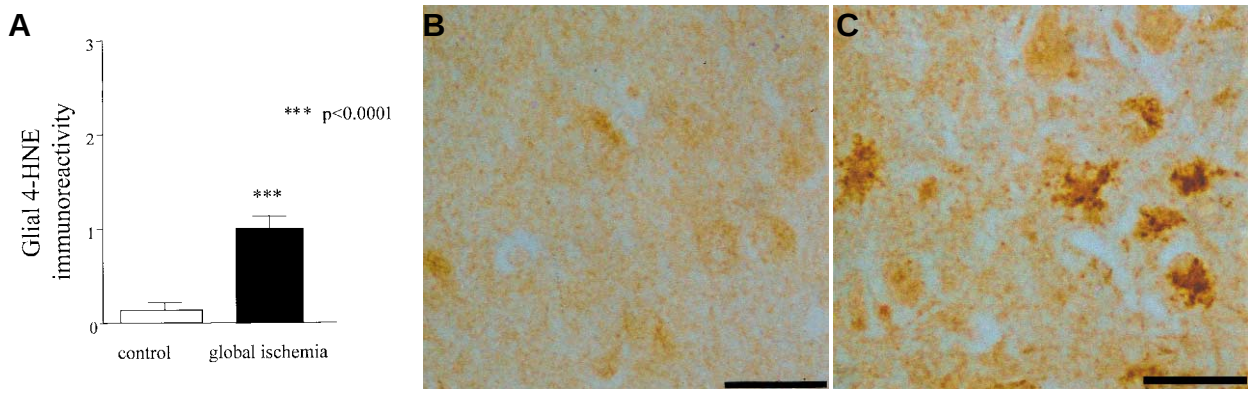

Figure 2. *Glial 4-HNE immunoreactivity in the control and global ischemia group.* **A**) Semi-quantitative assessment of the extent of glial 4-HNE immunoreactivity in the hippocampal formation in the control and global ischemia group. In the control group glial staining was only present in five out of the 44 cases chosen and only one case had intense glial staining the other four had minimal immunoreactivity. Histograms are mean ± SEM There was a significant increase in the extent of glial 4-HNE immunoreactivity in the global ischemia group compared to controls, $p < 0.0001$, using Student's unpaired *t*-test. Illustrative examples of glial 4-HNE immunoreactivity in a **B**) control and **C**) global ischemia case with scores of 0 and 3 respectively. Scale bar = 50μm.

Colour was developed using a 3′3′diaminobenzidine tetrahydrochloride (DAB) kit (Vector Labs). Sections were dehydrated and mounted. For double labelling experiments after the chromagen detection stage, sections were washed in 2 × 10 mins PBS; followed by another blocking period in 10% normal horse serum, 0.5% BSA in PBS (1hr); and then incubated overnight in primary antibody (glial fribrillary acidic protein (GFAP) (1: 1000; mouse monoclonal, Sigma). The following day, tissue sections were rinsed in PBS; incubated with biotinylated anti-mouse IgG; and processed with ABC Vectastain kit; and developed using the chromagen SG (Vector Labs). In the present study, controls for the specificity of the immunostaining included omission of the primary antibody. In addition, a similar cellular pattern of 4-HNE immunoreactivity was detected using a monoclonal 4-HNE antibody to that used in the present study (Japan Institute for the Control of Aging).

Semi-quantification of 4-hydroxynonenal immunoreactivity. Semi-quantitative assessment of 4-HNE immunoreactivity within the hippocampal formation was performed at ×200 magnification. Neuronal immunoreactivity was classified as follows: 0 = no neurons stained, 1 ⩽ 35% neurons stained, 2 = 35-70% neurons stained, 3 ⩾ 70% neurons stained. 4-HNE immunoreactivity was assessed in the dentate gyrus, CA1, CA2, CA3 and CA4 regions. The mean 4-HNE immunoreactivity of these regions in each case was used to calculate the control and global ischemia values. Glial 4-HNE immunoreactivity was similarly classified. Ischemic neuronal damage in haematoxylin and eosin stained sections had previously been semi-quantified in these cases (11). The assessment was similar to that used to define the extent of 4-HNE immunoreactivity except neurons exhibiting the features of ischemic cell damage were defined.

Statistics. Student's unpaired *t*-test was used to assess statistical significance of differences in the degree of neuronal or glial 4-HNE immunoreactivity between control and global ischemia groups. Differences in the degree of 4-HNE neuronal or glial immunoreactivity were compared in cases with and without an *APOE* ϵ4 allele using Student's unpaired *t*-test. Spearman correlation analysis was used to determine whether there was a significant association between the degree of 4-HNE immunoreactivity with survival, or age following global ischemia, or with ischemic neuronal damage.

Results

The control and global ischemia groups were matched for age and male to female ratio The frequency of the *APOE* ϵ4 allele was similar in the control and global ischemia groups. Two controls and three global ischemia cases were *APOE* ϵ4 homozygoytes. Ten controls and thirteen global ischemia cases were heterozygoytes for the *APOE* ϵ4 allele.

4-HNE immunoreactivity. There was minimal neuronal 4-HNE immunostaining present in all of the cases in the control group; only 5 out of 44 control cases had evidence of glial 4-HNE immunoreactivity. However,

there was evidence of increased 4-HNE immunoreactivity within neuronal perikarya in the hippocampus of all the global ischemia group compared to the controls. Glial 4-HNE immunostaining was found in 35 out of 56 global ischemia cases. The pattern of 4-HNE immunoreactivity varied between each global ischemia patient. Immunostaining within neuronal perikarya was restricted to the pyramidal layer of CA4, CA3, CA2 and CA1 regions, and granule cells in the dentate fascia. 4-HNE immunostaining of glial cells was also primarily detected within the hippocampal formation, but was also prominent within the neocortex in the global ischemia compared to control cases.

Neuronal 4-HNE immunoreactivity. There was a significant increase in the extent of 4-HNE immunopositive neurons within the global ischemia group, 1.63 ± 0.09 compared to controls 1.18 ± 0.09 $p < 0.0002$) (Figure 2). Neuronal 4-HNE immunostaining was present in both ischemic and non-ischemic neurons and was not correlated to the extent of ischemic cell damage in these patients ($r^2 = 0.0388$, $p > 0.05$) (11). Possession of an *APOE* ϵ4 allele did not influence the extent of 4-HNE immunoreactivity in either the control or global ischemia group (Figure 4A). Survival of the patient after global ischemia was significantly and negatively correlated with the amount of neuronal 4-HNE immunostaining ($r^2 = 0.0801$, $p < 0.05$) (Figure 5A) such that increasing survival was associated with a reduction in the extent of neuronal 4-HNE immunostaining. There was no correlation between the amount 4-HNE immunoreactivity with age in the control ($r^2 = 0.05778$; $p > 0.05$) or global ischemia group ($r^2 = 0.0615$, $p > 0.05$).

Glial 4-HNE immunoreactivity. Glial cell bodies and processes were intensely immunostained with 4-HNE in the hippocampal formation and neocortex. Semi-quantitative assessment of the extent of 4-HNE immunoreactivity demonstrated that there was a significant increase in the amount of 4-HNE immunopositive glia in the global ischemia group compared to the control group (1 ± 0.086 versus 0.175 ± 0.086, $p < 0.0001$) (Figure 2A). There was minimal glial immunostaining in the control group (Figure 2B) with the exception of one case in which glial immunostaining was prominent (semi-quantitative score of 3). Within the global ischemia group, glial immunoreactivity ranged from minimal (a score of 1) to extensive (a score of 3) (Figure 2C). Double labelling with antibodies to 4-HNE and GFAP illustrated that the majority of 4-HNE positive glial cells were astrocytes (Figure 3A). GFAP positive glial cells that were 4-HNE negative were also present within the hippocampal formation (Figure 3B). The extent of glial 4-HNE immunostaining did not correlate with neuronal 4-HNE immunoreactivity in either the control or ischemic group. Glial immunostaining was

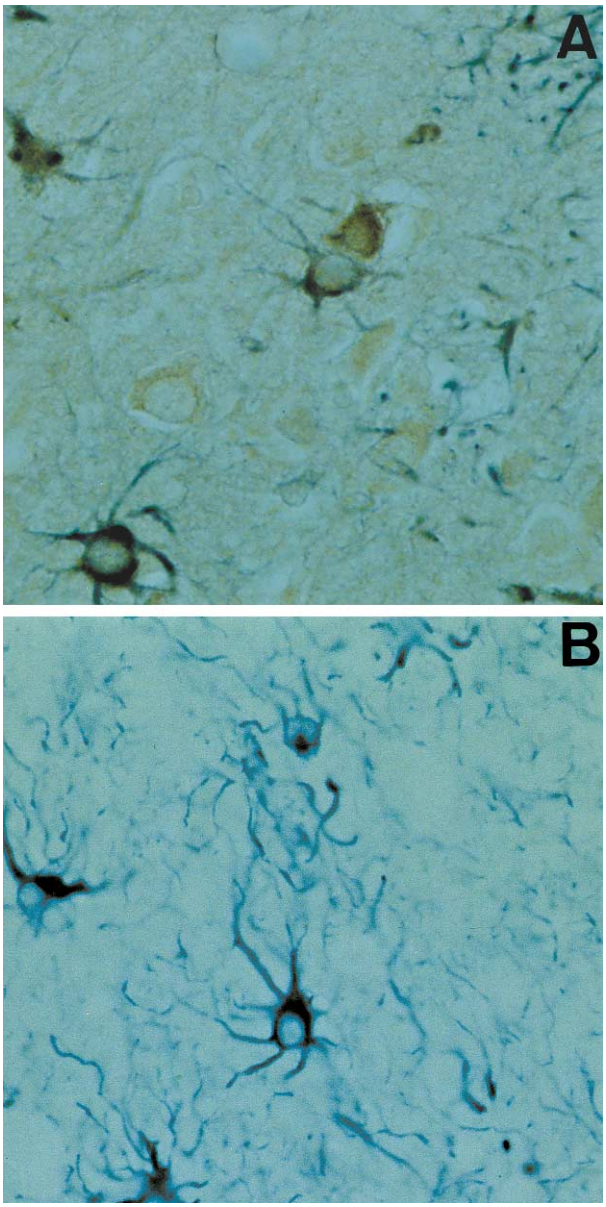

Figure 3. *4-HNE immunoreactivity is present in GFAP positive cells.* Sections from the global ischemia and control group that were positive for 4-HNE immunoreactivity in glial cells were double labelled for 4-HNE and GFAP. GFAP positive cells and 4-HNE positive glial cells are shown in **A**). There were also GFAP positive/4-HNE negative cells present within the hippocampal formation shown in **B**).

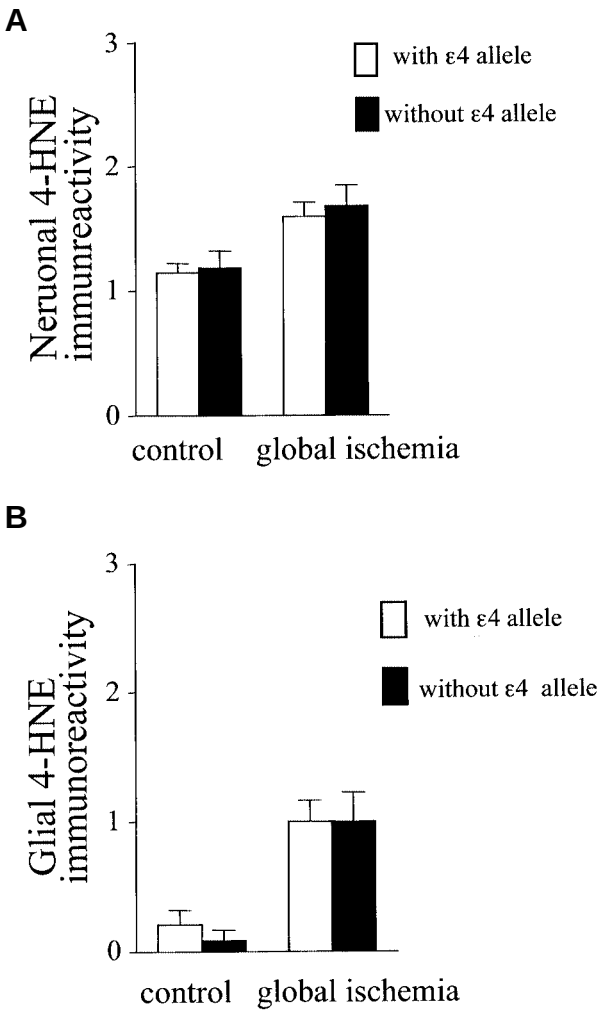

Figure 4. *APOE genotype does not influence neuronal or glial 4-HNE immunoreactivity in either the control or global ischemia group.* The control and global ischemia groups were subdivided into those with and without an *APOE* ϵ4. Histograms are mean ± SEM. The data show that the presence of an apoE ϵ4 allele does not significantly influence the amount of **A**) neuronal or **B**) glial 4-HNE immunoreactivity in either the control or global ischemia group, $p > 0.05$ using Student's unpaired *t*-test.

not related to possession of an *APOE* ϵ4 allele (Figure 4B), or correlated with age ($r^2 = 0.0097$, $p > 0.05$) in the control group or the global ischemia group ($r^2 = 0.0262$, $p > 0.05$). The length of time a patient survived after the global ischemia demonstrated a significant and positive correlation with the amount of glial 4-HNE immunoreactivity ($r^2 = 0.2958$, $p < 0.0001$) (Figure 5B).

Discussion

The present study indicates that the extent of lipid peroxidation, as defined by 4-HNE immunoreactivity, is markedly increased in neurons and glial after global ischemia in human hippocampus. We hypothesised that the extent of 4-HNE immunoreactivity would be *APOE* genotype dependent, but there was no evidence in the present study that *APOE* genotype influences the extent of neuronal or glial 4-HNE immunoreactivity.

Lipid peroxidation is a major contributor to ischemic damage, but the underlying mechanisms are poorly understood. This process results in the generation of aldehyde by-products, including 4-HNE. The presence of 4-HNE is therefore indicative that lipid peroxidation has taken place. Previous studies have indicated an increase in 4-HNE immunoreactivity in animal models of brain injury; a marked increase in cellular 4-HNE immunoreactivity was described after focal cerebral ischemia in rats (21, 42) and global cerebral ischemia in mice (10) and fluid percussion brain injury in rats (45). The present study is the first to present evidence of increased 4-HNE in neurons and glial after cerebral ischemia in human brain.

Global ischemia, as a result of cardiac arrest in humans, results in a relatively stereotyped pathology in which there is selective neuronal damage in the hippocampus and neocortical regions. However, we noted that 4-HNE was present in neurons that were both morphologically normal and neurons that exhibited the characteristics of ischemic cell change. 4-HNE binds to cytoskeletal proteins such as neurofilaments and microtubule associated proteins that are essential for structural integrity (20, 23, 27). Conformational changes in the structure of β–tubulin, a major component of microtubules, were induced by exposure of synaptosomes to 4-HNE (39). Therefore, it is possible that the presence of 4-HNE in morphologically normal neurons could be an indicator of an early response of the neuron to the ischemic insult. We did not find a correlation between the extent of 4-HNE immunoreactivity and ischemic neuronal damage (11). This may reflect the different time courses of the response to injury. The production of free radicals and subsequent lipid peroxidation is an initial event which precedes and eventually culminates in neuronal damage. Thus, it is likely that the 4-HNE is increased in cells which are not yet exhibiting histological evidence of ischemic damage.

The data show that the amount of 4-HNE immunoreactivity in neurons decreased with patient survival time, whereas the extent of 4-HNE within glial cells increased with survival time. There was no association between the extent of neuronal with glial 4-HNE immunostaining. The neuronal response may be attributed to the initial surge in metabolic activity within neurons and ele-

vated levels of free radicals initiating lipid peroxidation and generation of 4-HNE. As indicated above, this is an early response to injury, and one which will decline with time. However, the increasing 4-HNE within glia cells with time may simply reflect the process of gliosis as a consequence of the initial neuronal injury and this is a delayed response to injury.

Oxidative stress and lipid peroxidation has been suggested to contribute to the pathogenesis in Alzheimer's disease (AD) (16). In view of the increasing interest in the relationship between *APOE* genotype and risk factors of neurodegenerative diseases such as AD and head injury, we sought to determine whether *APOE* genotype influenced the extent of 4-HNE immunoreactivity in our population of cardiac arrest patients compared to controls. It has also been demonstrated that cellular 4-HNE immunoreactivity is increased in post-mortem AD tissue compared to controls. ApoE has an important role in AD, the mechanism of which is unclear. ApoE, has been proposed to have an anti-oxidant effect (9, 15). There is now *in vitro* evidence to indicate that lipid peroxidation induced damage may be *APOE* genotype dependent (22). We have previously shown that following global ischemia in apoE deficient mice there was increased neuronal 4-HNE immunoreactivity compared to wild type mice (10). This response was significantly reduced after intraventricular infusion of lipid conjugated human apoE (10). There was no correlation in this study between 4-HNE immunoreactivity and possession of an *APOE* ϵ4 allele in either the control or global ischemia group. This is similar to another study by Sayre and colleagues (34) who stained post-mortem AD tissue of known *APOE* genotype with two polyclonal 4-HNE antibodies. 4-HNE immunoreactive adducts were present in both neurofibrillary tangles and morphologically normal neurons (34). However, in contrast, Montine *et al* have demonstrated an association between 4-HNE pyrolle adducts and *APOE* ϵ4 homozygosity (24, 26). In another study this group also found that glial 4-HNE immunostaining was exclusively restricted to AD patients with an *APOE* ϵ3 allele (26). In our study, the extent of glial 4-HNE immunostaining was not attributed to either the *APOE* ϵ3 or ϵ4 allele. *In vitro* binding studies have demonstrated a preferential association between 4-HNE and *APOE* ϵ2, ϵ3 alleles, but not the ϵ4 allele (30). The authors propose that the presence of cysteine residues on the different isoforms could account for the differential binding properties of the apoE isoforms. The ϵ4 allele does not possess any cysteine residues and, therefore, will not bind to 4-HNE via Michael addition. The antibody used in our study only

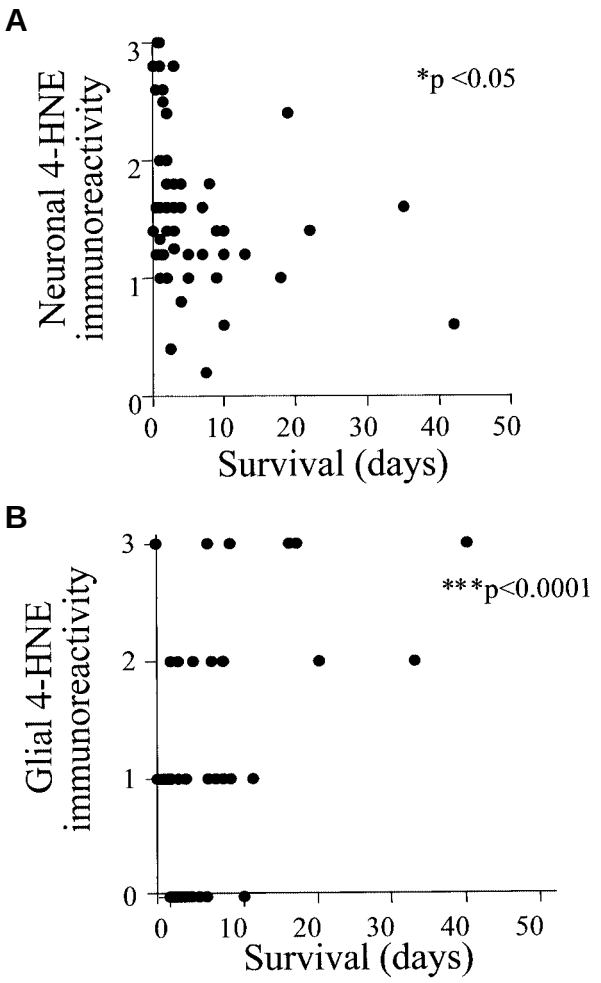

Figure 5. *Correlation between 4-HNE immunoreactivity and survival time in the global ischemia group.* **A**) The amount of neuronal 4-HNE immunoreactivity in the global ischemia group was significantly correlated to the length of time the patient survived after cardiorespiratory arrest $r^2 = 0.2832$; $p < 0.05$. **B**) Glial 4-HNE immunoreactivity was significantly related to survival time, $r^2 = 0.5438$; $p < 0.0001$.

detects 4-HNE that that forms covalent bonds with lysine, histidine and cysteine residues via Michael nucleophillic addition (41). Therefore, further studies with antibodies raised against the pyrolle adducts should be carried out to determine if there is an association between *APOE* genotype and oxidative stress following cardiorespiratory arrest.

The data does demonstrate that following global ischemia lipid peroxidation does occur, and it may be involved early in the ischemic cascade due its presence in morphologically normal neurons and the intensity of

4-HNE immunoreactivity in neurons declining with survival time. The limitations of the study, in terms of variation in severity of insult and survival period after global ischemia, may have precluded detectable *APOE* genotype differences. The recent development of transgenic mice which express human *APOE* alleles $\epsilon 2$, $\epsilon 3$, $\epsilon 4$ will allow isoform-specific differences to be determined (43, 44). Thus, further analysis has to be carried out in reproducible models of injury to enable us to establish whether *APOE* genotype influences the extent of oxidative damage following brain injury. The possibility still remains that oxidative injury, after global ischemia, is not under the influence of *APOE* genotype.

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