Journal of Alzheimer's Disease 12 (2007) 195–206
IOS Press

Lipoic Acid and N-acetyl Cysteine Decrease Mitochondrial-Related Oxidative Stress in Alzheimer Disease Patient Fibroblasts

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Handling editor: Dengshun Wang

Abstract. In this study, we evaluated the effect of lipoic acid (LA) and N-acetyl cysteine (NAC) on oxidative [4-hydroxy-2-nonenal, N^{ε}-(carboxymethyl)lysine and heme oxygenase-1] and apoptotic (caspase 9 and Bax) markers in fibroblasts from patients with Alzheimer disease (AD) and age-matched and young controls. AD fibroblasts showed the highest levels of oxidative stress, and the antioxidants, lipoic acid (1 mM) and/or N-acetyl cysteine (100 μM) exerted a protective effect as evidenced by decreases in oxidative stress and apoptotic markers. Furthermore, we observed that the protective effect of LA and NAC was more pronounced when both agents were present simultaneously. AD-type changes could be generated in control fibroblasts using N-methylprotoporphyrin to inhibit cytochrome oxidase assembly indicating that the the oxidative damage observed was associated with mitochondrial dysfunction. The effects of N-methylprotoporphyrine were reversed or attenuated by both lipoic acid and N-acetyl cysteine. These data suggest mitochondria are important in oxidative damage that occurs in AD. As such, antioxidant therapies based on lipoic acid and N-acetyl cysteine supplementation may be promising.

Keywords: Aging, Alzheimer disease, antioxidants, lipoic acid, mitochondria, N-acetyl cysteine, oxidative stress

INTRODUCTION

Alzheimer disease (AD) is a multifactorial disorder that has many physiological, biochemical, and neurochemical facets. Aging is the major risk factor for AD that coexists with other causes of cognitive decline, particularly vascular dementia [72]. The processes underlying the pathology of AD involve several factors, including mitochondrial dysfunction, abnormal protein aggregation, metal accumulation, inflammation and excitotoxicity. Although the relationship between these factors and the development of AD is multidirectional, oxidative damage is considered a common thread linking some of these factors [40,55].

Increased oxidative damage is a prominent and early feature of vulnerable neurons in AD [71]. Nucleic acid oxidation is marked by increased levels of 8-hydroxy-2-deoxyguanosine (8-OHdG) and 8-hydroxyguanosine (8-OHG) [49–51]. Protein oxidation is marked by elevated levels of protein carbonyl and widespread nitration of tyrosine residues in the susceptible neurons [70,71]. Lipid peroxidation is marked by higher levels of thiobarbituric acid reactive substances (TBARS), malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), isoprostanes and altered phospholipid composition [63]. Modifications to sug-

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ars are also observed via increased glycoxidation and glycation [11,66,69] that are responsible for the formation of advanced glycation endproducts (AGEs) such as N^{ε}-(carboxymethyl)lysine (CML), pentosidine and pyralline.

Mitochondria are essential organelles for neuronal cell function because their limited glycolytic capacity makes them highly dependent on aerobic oxidative phosphorylation for their high energetic demands. However, oxidative phosphorylation is a major source of endogenous toxic free radicals or precursors, including hydrogen peroxide (H_2O_2), hydroxyl ($\bullet OH$) and superoxide ($O_2^{-\bullet}$) radicals that are products of normal cellular respiration [81]. Reactive oxygen species generated by mitochondria have several cellular targets including mitochondrial components themselves (lipids, proteins and DNA). The lack of histones in mitochondrial DNA (mtDNA) and diminished capacity for DNA repair render mitochondria an easy target of oxidative stress events suggesting that these organelles are key elements involved in aging [43] and age-related disorders [2,42,59,75].

The cytopathological significance of oxidative damage is seen by the up-regulation of antioxidant enzymes. Heme oxygenase-1 (HO-1) is an antioxidant enzyme that degrades heme into biliverdin, iron and carbon monoxide and is one of the most sensitive and selective indicators of the cellular oxidative stress response. It has been shown that brains of AD patients present an increase of both HO-1 mRNA and protein [58,65] which co-localizes and parallels the expression of the altered form of tau characteristic of AD [76,77].

Recently, we demonstrated that olfactory epithelium of AD patients [56] and olfactory neuroblasts in culture [23] from cases of AD present high levels of HNE, CML and HO-1 when compared with age-matched controls. Given the proximal role and devastating effect that oxidative stress plays in AD pathogenesis, a therapeutic strategy based on reducing oxidative stress appears reasonable. Both *in vitro* and animal studies suggest that treatment with antioxidant agents may be useful in neurological disorders, including AD [17]. Two agents that have received attention because of their antioxidant capacity are lipoic acid (LA) [38] and N-acetyl cysteine (NAC) [6]. Several studies provide evidence that LA decreases oxidative stress and restores reduced levels of other antioxidants *in vivo* (for review, see [44]). Similarly, it has been reported that NAC acts as a precursor of glutathione synthesis as well as a stimulator of the cytosolic enzymes involved in glutathione regeneration; induces protection by direct reaction between its reducing thiol groups and reactive oxygen species (ROS) and stimulates mitochondrial complexes I and IV (for review, see [6]).

It is well known that fibroblasts from AD patients also show elevations in oxidative markers [12,48]. The goal of this study was to evaluate the effect of LA and NAC in oxidative and apoptotic markers observed in skin fibroblasts obtained from AD, age-matched and young control subjects. Furthermore, to elucidate if the oxidative levels observed were related with mitochondria, we induced mitochondrial dysfunction with N-methylprotoporphyrine IX (NMP) which inhibits cytochrome oxidase assembly. For this purpose, we evaluated oxidative (HNE, CML, HO-1) and apoptotic (Bax and caspase 9) markers. Our data indicate that LA and NAC are highly effective in reducing oxidative and apoptotic changes observed in AD and aged-matched control fibroblasts. These findings provide further support for the use of antioxidants in the treatment of AD and show that mitochondria may play an important role in oxidative damage in disease pathogenesis.

MATERIALS AND METHODS

Skin Fibroblast Cultures

Fibroblasts cultures were obtained from the NIA Aging Cell Culture Repository (Camden, NJ, USA). The clinical diagnosis, age and sex of the donors are listed in Table 1. Cells were cultured at 37°C with 5% CO_2 in 1x DMEM supplemented with 1% (v/v) antibiotic-antimycotic, 1% (v/v) glutamine, and 10% (v/v) heat-inactivated fetal calf serum (Invitrogen, Carlsbad, CA, USA). Stock cultures were split once a week when near confluence. Cells were harvested by trypsinization (0.25% Trypsin, Invitrogen, Carlsbad, CA, USA) for 2 minutes at 37°C.

Table 1

Fibroblast Case	Age	Gender
AD – 1	60	Female
AD – 2	61	Male
AD – 3	67	Male
AD – 4	70	Male
AD – 5	79	Female
Control – 1	35	Male
Control – 2	35	Female
Control – 3	60	Female
Control – 4	68	Male
Control – 5	79	Female

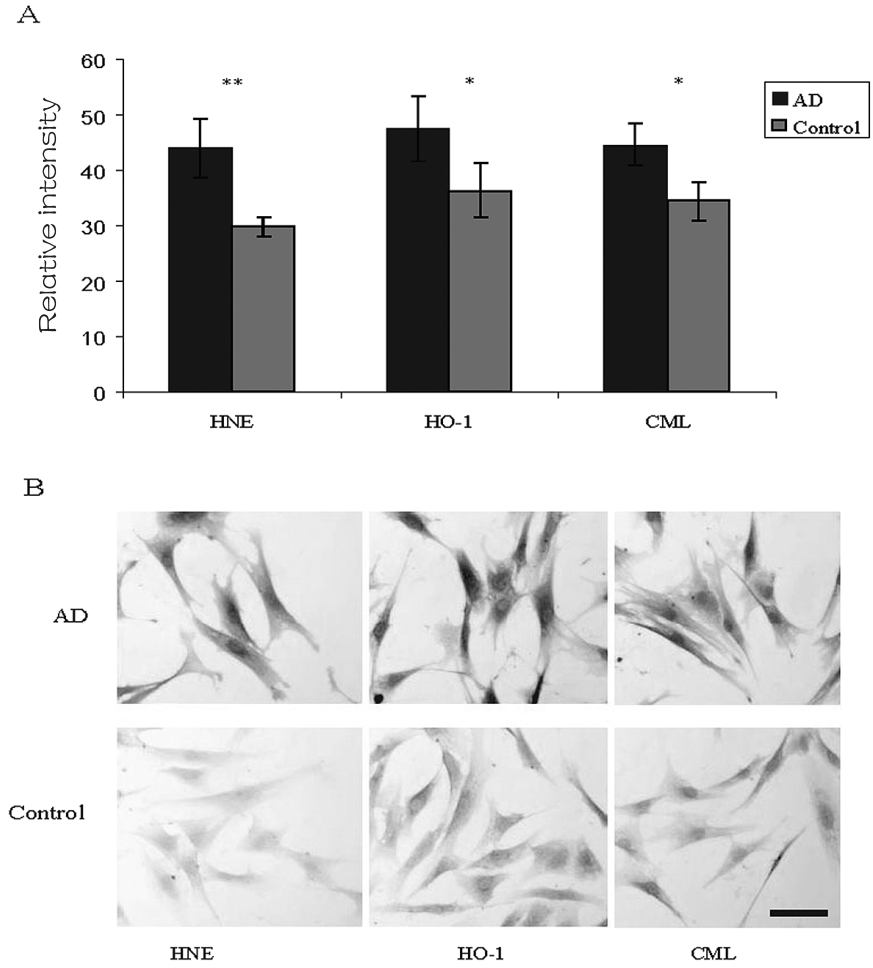

Fig. 1. A) Immunocytochemical quantification of CML, HO-1 and HNE levels. $^{**}p < 0.01$; $^{*}p < 0.05$ when compared with age-matched control fibroblasts. B) Immunostain demonstrating the increased immunoreactivity in AD fibroblasts compared to age-matched controls for HNE, HO-1, and CML. Scale bar = 50 μM.

Incubation with Lipoic Acid and N-Acetyl Cysteine

Fibroblasts were plated on LAB TEK II chamber slides (Nalge Nunc International, Rochester, NY, USA) and incubated overnight to allow cells to adhere. After this period, cells were either treated with a final concentration of 0.14% ethanol (control) or with LA (1 mM) (10 mM Stock diluted in 70% ethanol) and/or NAC (100 μM) in phosphate buffered saline (PBS, pH 7.2) for 24 to 48 hours.

Induction of Heme Deficiency

Fibroblasts were plated on LAB TEK II chamber slides and incubated overnight to adhere. After this period cells were either left untreated (control) or treated with 10, 20 or 40 μM NMP (Frontier Scientific, Logan UT, USA) for 5 days. Some NMP treated cells were subsequently treated with LA and/or NAC. The stock solution of NMP was made in 0.1 N NaOH.

Immunocytochemistry

Cells were plated on LAB TEK II chamber slides. After the desired incubation with the experimental conditions previously outlined, cells were rinsed in PBS at 37°C then fixed with methacarn (methanol-chloroform-acetic acid, 6:3:1) for 15 minutes at room temperature. Endogenous peroxidase activity was eliminated by incubation in 3% H_2O_2 in Tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 7.6) for 30 minutes. To reduce non-specific binding, cells were incu-

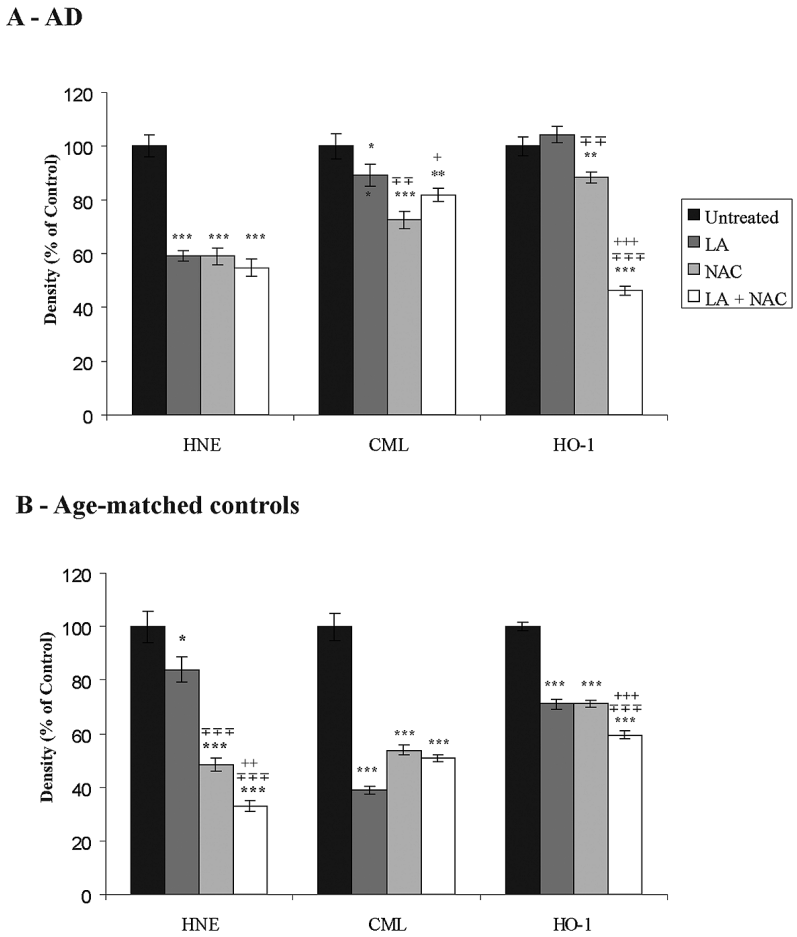

Fig. 2. Effect of LA and/or NAC on HNE, CML and HO-1 immunoreactivity in fibroblasts obtained from AD and age-matched controls. Data are the mean (% of untreated fibroblasts) ± S.E.M. of four independent experiments with each fibroblast line. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ when compared with untreated fibroblasts. $^{\mp\mp\mp}p < 0.001$, $^{\mp\mp}p < 0.01$ when compared with LA condition. $^{+++}p < 0.001$; $^{++}p < 0.01$; $^{+}p < 0.05$ when compared with NAC condition.

bated for 30 minutes with 1% normal goat serum (NGS) in TBS. After rinsing briefly with 1% NGS, cells were incubated overnight with primary antibody. Cells were stained with the peroxidase antiperoxidase method [73] using 3,3'-diaminobenzidine (DAB) as a chromogen (Dako Corporation, Carpinteria, CA, USA).

The antisera to the following markers were used HO-1 (1:00; [65]); HNE (1:50; [63]); CML (1:00; [11]); Bax (1:100; StressGen, San Diego, CA, USA) and Caspase 9 (1:100, StressGen, San Diego, CA, USA).

Quantification

The intensity of the immunoreaction for each antiserum used was measured using an Axiocam digital camera and KS300 image analysis software (Carl Zeiss, Inc., Thornwood, NY, USA). The cells were manually outlined and the computer-generated optical density values determined. Background values, samples similarly processed but lacking the primary antibodies, were subtracted and the mean densities determined for each case.

Statistical Analysis

Results are presented as raw data or as relative intensity (% of control or untreated condition) ± SEM of the indicated number of experiments. Relative intensity is utilized for those experiments where the control fibroblasts were stained for an increased incubation time with DAB in order to visualize a difference between treatments. Statistical significance was determined using the one-way ANOVA test for multiple comparisons, followed by the post-hoc Tukey-Kramer test.

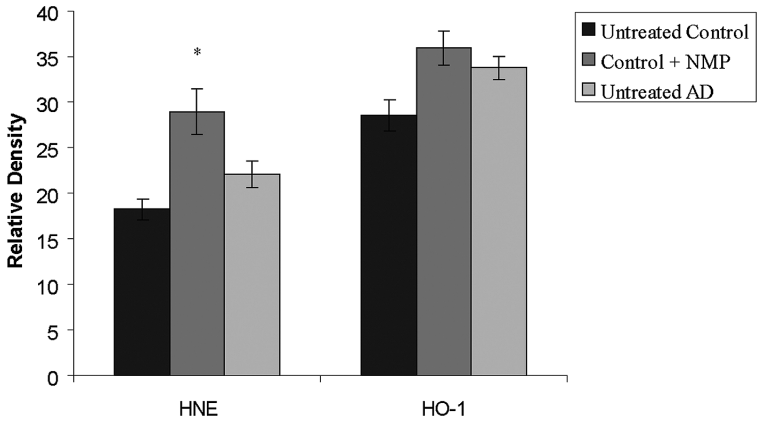

Fig. 3. Effect of 20 μM NMP on age-matched control fibroblasts. $^{*}p < 0.05$ when compared with untreated control fibroblasts.

RESULTS

LA and/or NAC Prevent the Increase in Oxidative Stress Marker Levels

AD fibroblasts showed higher levels of oxidative markers (HNE, CML and HO-1) when compared to age-matched controls (Fig. 1). Importantly, co-incubation of AD fibroblasts with LA or NAC reduced the levels of all oxidative markers (Fig. 2), consistent with the notion that both compounds have antioxidant properties. Interestingly, co-incubation of LA and NAC afforded a higher protection than that promoted by each agent alone (Fig. 2) supporting the notion that a combination of antioxidants is more effective than a single agent.

Mitochondrial-Associated Oxidative Stress is Reversed/Attenuated by LA and/or NAC

To determine if oxidative stress was related to mitochondrial dysfunction and whether the protective effect of LA and NAC occur at the mitochondrial level, we induced heme deficiency by inhibition of ferrochelatase with NMP. NMP mimics protoporphyrin IX, the substrate for ferrochelatase, except that a methyl group is added to a nitrogen group. NMP binds ferrochelatase with affinity similar to protoporphyrin IX, but the methyl group prevents iron from being inserted into NMP [14]; thus, it is a selective and specific inhibitor for ferrochelatase [22] and has been used previously in several studies to inhibit heme synthesis [4,5,78]. We observed that NMP induced a concentration-dependent decrease in cytochrome oxidase content when compared with control conditions (data not shown) indicating that the compound is effective in inducing heme deficiency. Interestingly, we observed that age-matched control fibroblasts in the presence of NMP increased oxidative stress to levels similar or above those of AD fibroblasts (Fig. 3). These findings suggest that mitochondrial dysfunction is a key source of oxidative stress. The increase in oxidative levels due to NMP was reversed/attenuated with the presence of LA and/or NAC (Figs 4 and 5) suggesting that both compounds act preferentially on mitochondria to attenuate oxidative stress.

LA and/or NAC Protect Against the Increase in Mitochondrial-Associated Apoptotic Markers

Caspases are a family of proteins that are one of the main effectors of apoptosis. They are a group of cysteine proteases that exist within the cell as inactive proforms or zymogens. These zymogens can be cleaved to form active enzymes following the induction of apoptosis. Bax is a member of the Bcl2 family of proteins and is a critical regulator of apoptotic cell death (pro-apoptotic protein). NMP induced an increase in the levels of Bax and Caspase-9 in fibroblasts of AD, young and aged-matched controls (Fig. 6) which is in accordance with the increase in oxidative stress levels. Levels of apoptotic markers were reversed /attenuated by LA and/or NAC (Fig. 6), again supporting the idea that metabolic antioxidants are capable of preventing, or at least attenuating mitochondrial-associated oxidative stress and, consequently, apoptotic cell death.

DISCUSSION

In this study we show that AD fibroblasts possess high levels of oxidative markers (HNE, CML and HO-

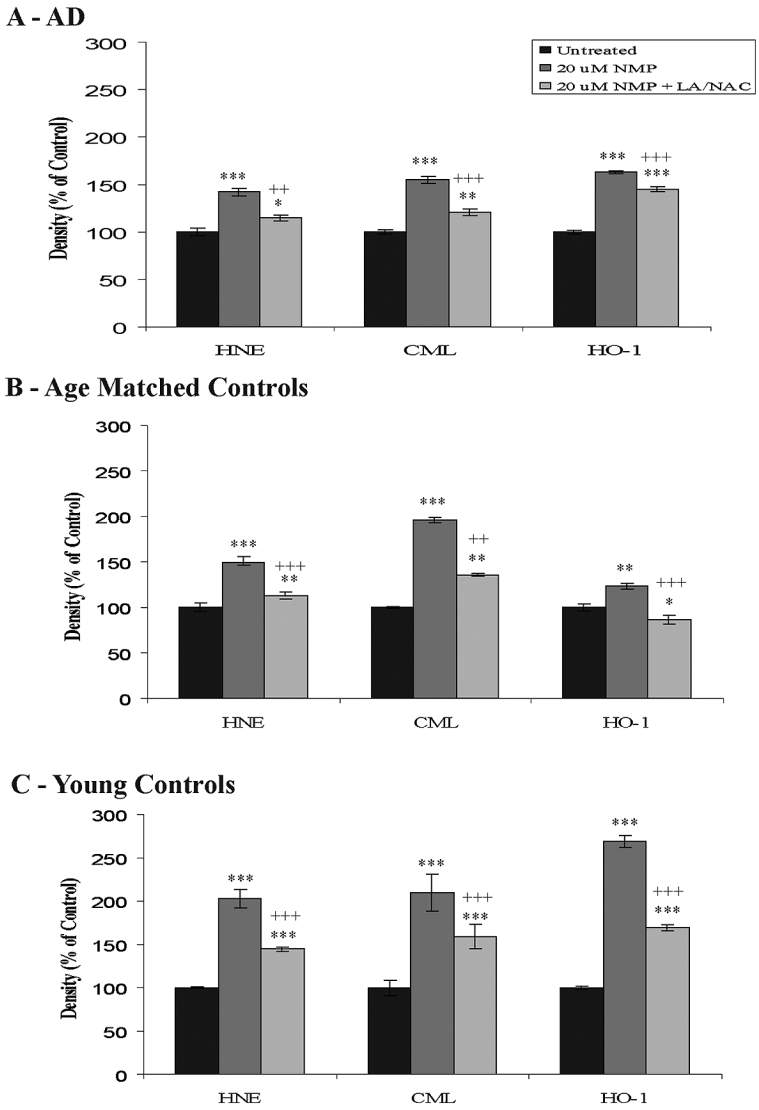

Fig. 4. Effect of LA and/or NAC in the increase of oxidative stress markers immunoreactivity induced by NMP. The data are means (% of control) ± S.E.M. of four independent experiments with each fibroblast line. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ when compared with untreated fibroblasts. $^{+++}p < 0.001$; $^{++}p < 0.01$ when compared with NMP-treated fibroblasts.

1) when compared with young and age-matched controls. These results are in accordance with previous studies from others as well as from our laboratory showing that fibroblasts [12,48], olfactory epithelium [56] and olfactory neuroblasts [23] from cases of AD have higher levels of oxidative markers when compared with age-matched controls. We also demonstrate that the inhibition of cytochrome oxidase assembly potentiates the increase in oxidative and apoptotic (Bax and caspase 9) markers indicating that mitochondria are key elements involved in oxidative stress occurring in aged and AD fibroblasts. However, the key finding of this study is that LA and NAC exert substantial protection against age- and AD-associated oxidative stress and that this protection was more pronounced when both agents are present simultaneously.

An accumulating body of knowledge suggests that oxidative stress, and subsequent oxidative damage [55, 57] occurs early in the progression of AD, significantly before the development of the pathologic hallmarks, neurofibrillary tangles and senile plaques [50–52,71]. In diseased neurons the interaction of abnormal mitochondria, redox transition metals, and oxidative stress response elements contributes to the generation

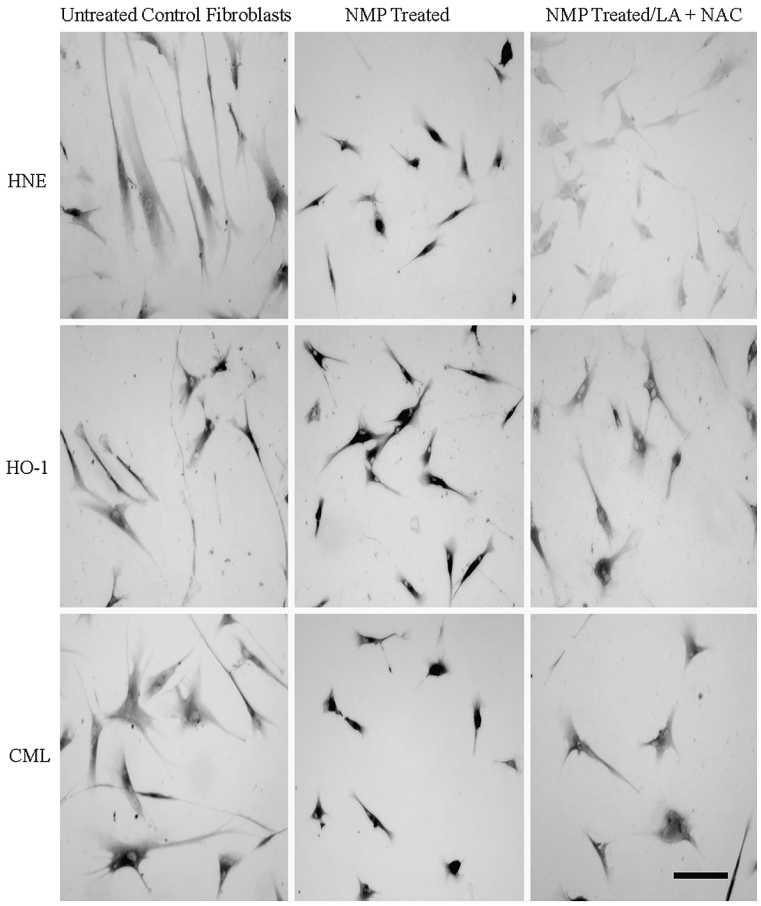

Fig. 5. Immunostain of control fibroblasts demonstrating the increase in oxidative stress markers immunoreactivity after NMP treatment followed by a decrease in immunoreactivity after LA and NAC treatment. Scale bar = 50 μM.

of ROS [55].

Accumulation of AGEs in the brain is a feature of aging [45,67] and the Maillard reaction is implicated in the development of pathophysiology in age-related diseases such as diabetes mellitus, atherosclerosis and AD [46,61,79]. CML, the predominant AGE that accumulates *in vivo* [18,60] along with its glycation-specific precursor hexitol-lysine are increased in neurons, especially those containing intracellular neurofibrillary pathology in cases of AD [11]. Using immunocytochemical methods, Girones et al. [24] examined the distribution of CML in brain tissue from AD and diabetes mellitus subjects and aging controls. They observed that CML reactivity was more evident in brains from patients suffering from both AD and diabetes mellitus, followed by AD, diabetes mellitus, and aging controls. Accordingly, we observed the highest levels of CML in AD fibroblasts (Fig. 1). Co-localization of CML with adducts derived from products of lipid peroxidation, HNE and MDA, supports the concept that lipid peroxidation itself, in addition to and apart from advanced glycation, triggers the formation of CML [21]. Recently, Dei et al. [16] reported that while both MDA and CML accumulate under oxidative stress, CML accumulation is largely limited to neurons, in normal aging, while MDA also accumulates in glia. However, in AD, both MDA and CML are deposited in both astrocytes and neurons. Data from the literature indicates that HNE is increased in brain tissue [39,63,77,82] and cerebrospinal fluid [36] of AD patients. These findings are supported by our results showing that AD fibroblasts present higher levels of oxidative damage stress when compared with control fibroblasts (Fig. 1).

HO-1, the rate-limiting step in heme catabolism, plays an important role in AD [65]. The vasoactive molecule carbon monoxide and the potent antioxidant biliverdin, products of an HO-1-catalyzed reaction, represent a protective system, potentially active against

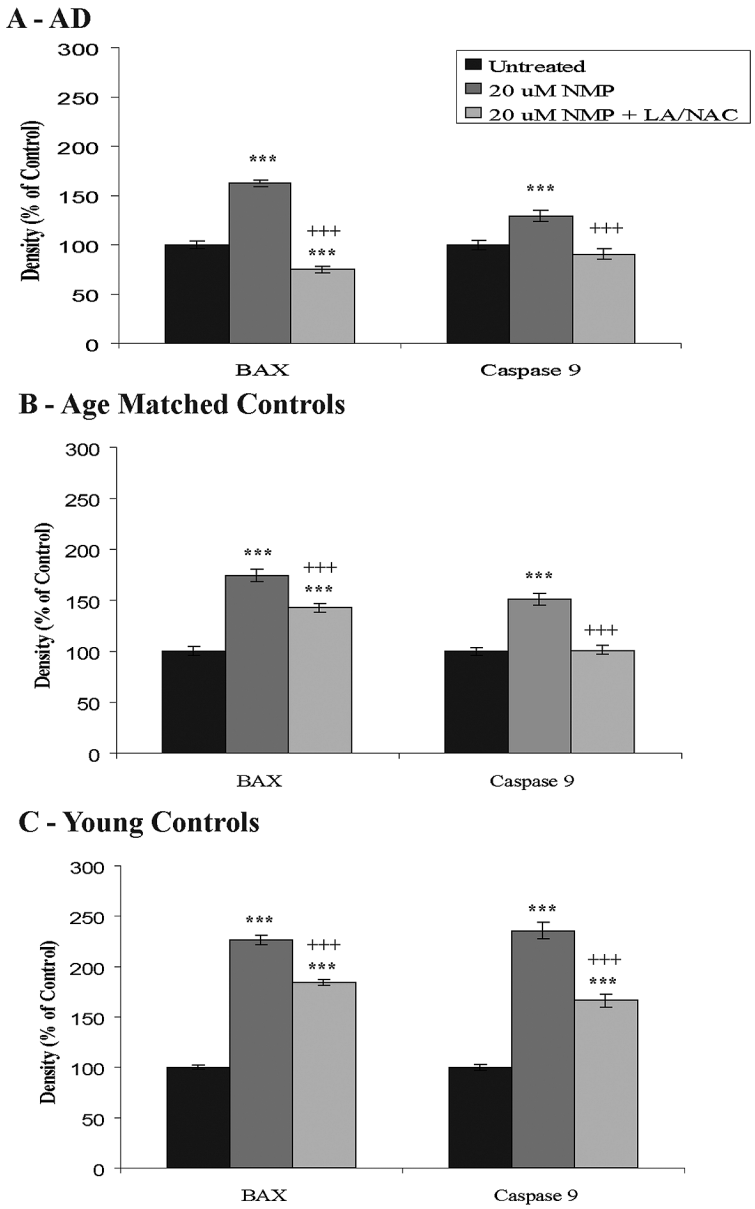

Fig. 6. Effect of LA and/or NAC in the levels of apoptotic markers (caspase 9 and Bax) induced by NMP. The data are means (% of control) ± S.E.M. of four independent experiments with each fibroblast line. $^{***}p < 0.001$ when compared with untreated fibroblasts. $^{+++}p < 0.001$ when compared with NMP-treated fibroblasts.

brain oxidative injury. In accordance with previous results from our laboratory for olfactory neuroblasts [23, 56], we observed that AD fibroblasts present significantly higher levels of HO-1 when compared with age-matched controls (Fig. 1). Studies in a transgenic mouse model of AD showed that the expression of CuZn superoxide dismutase and HO-1 is significantly higher when compared with control mice [54]. Using immunolabeling or PCR techniques, a robust overexpression of HO-1 proteins, or mRNA in brain samples of sporadic AD when compared with age-matched controls was observed [58,64,66,68].

Mitochondrial dysfunction is characteristic of aging and several neurodegenerative conditions including AD [30]. In the present study we induced mitochondrial dysfunction through heme deficiency promoted by NMP. This compound interferes with the assembly of mitochondrial cytochrome oxidase by inhibition of

heme formation by ferrochelatase. A previous study showed that NMP reduces the activity of ferrochelatase by 15% [78]. Thus, inhibition of ferrochelatase leaves the cell with a shortage of protohemes. Cytosolic and mitochondrial enzymes catalyze the maturation of protoheme to heme-a [7,33], suggesting that heme-a shuttles from the cytosol to mitochondria to be incorporated into subunit I of cytochrome oxidase. Atamna et al. [4] studied the effect of heme deficiency in young and old normal human fibroblasts. They observed that regardless of age, heme deficiency increases the steady-state levels of oxidants and lipid peroxidation and sensitizes the cells to fluctuations in intracellular calcium. They reported also a 95% decrease in the activity and protein content of mitochondrial complex IV. The same group reported that heme deficiency in brain cells decreases mitochondrial complex IV activity, activates nitric oxide synthase, alters amyloid-β protein precursor and corrupts iron and zinc homeostasis [5]. In accordance, our results show that NMP induces a concentration-dependent decrease in cytochrome oxidase content (data not shown) and an increase in oxidative and apoptotic markers (Figs 3–6). Interestingly, age-matched control fibroblasts in the presence of NMP present levels of HNE and HO-1 similar to that of AD fibroblasts at basal conditions (Fig. 3), which suggests that the oxidative stress phenomena occurring in AD fibroblasts under basal conditions results from dysfunctional mitochondria.

It is now well established that mitochondria might also regulate and promote apoptosis by releasing cytochrome c or other protease zymogens from the mitochondrial intermembrane space into the cytosol [25] and, through the activation of caspase-9 and -3, eventually lead to apoptosis [34]. Accordingly, we observed that mitochondrial dysfunction induced by NMP leads to an increase in caspase 9 immunoreactivity (Fig. 6). Furthermore, we also observed that the presence of NMP promotes an increase in Bax levels (Fig. 6). Bax is a member of the Bcl-2 family of proteins, which can promote apoptosis by forming oligomers in the mitochondrial outer membrane and creating a channel for the release of cytochrome c and other apoptotic substances [3,19]. Another study indicated that Bax can bind to the voltage-dependent anion channel (VDAC) and promote the release of cytochrome c through this channel [80]. Bax translocation onto the mitochondrial membrane therefore becomes one of the important indicators for the onset of mitochondria-mediated apoptosis.

By acting as a cysteine donor, NAC maintains intracellular glutathione levels and is neuroprotective for a range of neuronal cell types against a variety of apoptotic stimuli *in vitro* [20,41,47]. NAC may therefore reduce neuronal death by blocking attempted entry into the cell cycle, by improving free radical surveillance, or by preserving mitochondrial function. In turn, LA can also scavenge ROS [8,9,53], regenerate endogenous antioxidants [32], repair oxidative damage [10] and chelate metals [32]. Furthermore, LA is a coenzyme for mitochondrial pyruvate and α-ketoglutarate dehydrogenases. We observed that pre-treatment of fibroblasts with LA and NAC leads to a decrease in age- and AD-associated oxidative levels (Fig. 2). Furthermore, these compounds protect against the increase in oxidative and apoptotic markers induced by NMP (Figs 4–6). *In vitro* studies showed that pretreatment of dissociated primary hippocampal cultures with LA promote a significant protection against amyloid-β and iron/H_2O_2 toxicity [37]. Furthermore, it has been shown that old rats supplemented with (R)-α-lipoic acid showed an improvement of mitochondrial function, decreased oxidative damage, and increased metabolic rate [26]. Accordingly, Suh et al. [74] reported that old rats injected with (R)-α-lipoic acid presented an improvement in GSH redox status of both cerebral and myocardial tissues when compared with control rats. Hager and collaborators [28] reported that the administration of 600 mg α-lipoic acid/day to nine patients with AD for an average of 337 days promoted the stabilization of cognitive measures. Recently, Hart et al. [29] reported that NAC preserves mitochondrial function and protects sensory neurons after nerve injury. Furthermore, administration of NAC protects the brain from free radical injury, apoptosis, and inflammation [15, 31]. Cocco et al. [13] reported that old rats treated with NAC showed a slight brain-specific improvement of mitochondrial energy production efficiency, mostly with NAD-dependent substrates, together with a decrease in carbonyl protein content and an increase in the amount of protein thiols of brain cytosolic fraction when compared with untreated animals. Adair et al. [1] performed a clinical trial where NAC or placebo was administered in a double-blind fashion to patients with probable AD. They observed that NAC has a positive effect on nearly every outcome measure, although significant differences were obtained only for a subset of cognitive tasks.

Interestingly, we observed that co-incubation of fibroblasts with LA and NAC exert a more pronounced protective effect (Fig. 2). Accordingly, a previous study reported that acetyl-L-carnitine (ALCAR) plus LA partially reversed the age-related decline in aver-

age mitochondrial membrane potential and significantly increased hepatocellular O_2 consumption, indicating that mitochondrial-supported cellular metabolism was markedly improved by the presence of both compounds [2]. The same study indicates that ALCAR plus LA also increased ambulatory activity in both young and old rats, with this effect being significantly higher when compared with old rats fed ALCAR or LA alone [27]. Liu et al. [35] reported that dietary administration of ALCAR and/or LA to old rats improve performance on memory tasks by lowering oxidative damage and improving mitochondrial function. Recently, it has been shown that co-supplementation of LA and carnitine has a beneficial effect in reversing the age-related abnormalities seen in aging. This effect was associated with the decrease in free radical production and rise in antioxidant levels by carnitine and lipoic acid, thereby lowering oxidative stress [62].

In conclusion, our results show that LA and NAC decrease the levels of oxidative and apoptotic markers via protection of mitochondrial function. The combination of both LA and NAC maximizes0 the protective effect suggesting that the combination of both agents may prevent mitochondrial decay associated with aging and age-related disorders such as AD. Antioxidant therapies based on LA and NAC seem promising since they can act on mitochondria, one key source of oxidative stress in aging and neurodegeneration.

ACKNOWLEDGMENTS

Work in the authors' laboratories is supported by the National Institutes of Health, the Alzheimer's Association and Philip Morris USA Inc. and Philip Morris International.

Dr. Smith is, or was, a consultant to, owns equity in, and has received research support from Voyager Pharmaceutical Corporation, Neurpharm, Neurotez, and Panacea Pharmaceutical Corporation. Dr. Perry owns equity in Voyager Pharmaceutical Corporation and Panacea Pharmaceutical Corporation.

This manuscript was managed by Dr. Dengshun Wang, Associate Editor of the Journal of Alzheimer's Disease, who independently solicited peer review and handled the decision process.

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