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Nanomolar aluminum induces pro-inflammatory and pro-apoptotic gene expression in human brain cells in primary culture

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Abstract

Aluminum, the most abundant neurotoxic metal in our biosphere, has been implicated in the etiology of several neurodegenerative disorders including Alzheimer's disease (AD). To further understand aluminum's influence on gene expression, we examined total messenger RNA levels in untransformed human neural cells exposed to 100 nanomolar aluminum sulfate using high density DNA microarrays that interrogate the expression of every human gene. Preliminary data indicate that of the most altered gene expression levels, 17/24 (70.8%) of aluminum-affected genes, and 7/8 (87.5%) of aluminum-induced genes exhibit expression patterns similar to those observed in AD. The seven genes found to be significantly up-regulated by aluminum encode pro-inflammatory or pro-apoptotic signaling elements, including NF-κB subunits, interleukin-1β precursor, cytosolic phospholipase A_2, cyclooxygenase-2, beta-amyloid precursor protein and DAXX, a regulatory protein known to induce apoptosis and repress transcription. The promoters of genes up-regulated by aluminum are enriched in binding sites for the stress-inducible transcription factors HIF-1 and NF-κB, suggesting a role for aluminum, HIF-1 and NF-κB in driving atypical, pro-inflammatory and pro-apoptotic gene expression. The effect of aluminum on specific stress-related gene expression patterns in human brain cells clearly warrant further investigation.

Keywords: Aluminum; βAPP; COX-2; $cPLA_2$; DAXX; DNA array; HIF-1; Human neural cells; Inflammation; IL-1β, NFκB; Nuclear pore complex; Promoter

The harmful effects of aluminum salts on normal physiological and biochemical systems are many, and over the last 30 years, a considerable body of evidence has emerged concerning the specific actions of aluminum on the structural and functional integrity of the genetic apparatus [1–3]. Multiple adverse biological effects of aluminum on nucleic acids have been repeatedly demonstrated, and instances of highly specialized interactions with nuclear genomic structures illustrate specific aluminum targeting within the neural (neuronal and glial) cells of the central nervous system [1,2]. To further investigate the nature of these interactions, in this study we examined the effects of aluminum sulfate on full-spectrum gene expression in human neural (HN) cells in primary culture using DNA arrays that profile the expression of every human gene. Treatment of 2.5 week old HN cells with 100 nM aluminum sulfate for 3.5 days was found to emulate many of the stress-related gene expression changes as previously reported in human brain tissues affected by late stage Alzheimer's disease (AD) [3–7]. These studies suggest the involvement of soluble aluminum compounds with human brain gene

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expression, and in particular, the molecular processes involved in brain-specific gene transcription.

Previous reports examining the effects of aluminum on run-on gene transcription, based on the incorporation of [α-^{32}P]-uridine triphosphate into newly synthesized messenger RNA, showed that in isolated human brain cell nuclei, 100 nM ambient aluminum had potent modulatory effects on RNA polymerase II-directed processes [1,3]. Therefore, in the experiments described here, the effects of 100 nM aluminum on total gene expression patterns were examined in greater detail. Human neural (HN) cells, a primary, untransformed co-culture of neurons and glia (Clonetics CC-2599; Bio-Whittaker/Cambrex Cell Biology, Walkersville MD) were grown for 3 weeks as previously described [4,5]. At 3 weeks of development this cell culture contains approximately equal populations of differentiated human neurons and glia [4]. During HN cell growth, cell culture maintenance medium was changed every 3.5 days; during the last 3.5 days of culture, cells were incubated in serum-free medium that contained either 100 nM magnesium sulfate (control; $MgSO_4$) or 100 nM aluminum sulfate (treated; $Al_2(SO_4)_3$) from freshly prepared stock solutions [3]. After three full weeks of culture, four control plates and four treated plates of HN cells were subjected to total RNA and protein isolation using Trizol reagent (Invitrogen, Carlsbad CA), as previously described [4,6]. Total RNA concentration and spectral purity were determined using RNA Nano Labchip analysis and a 2100 Spectral Bioanalyzer (Caliper Technologies, Mountainview, CA; Agilent Technologies, Palo Alto, CA) [5,6]. 28S/18S ratios consistently exceeded 1.4 and the A_{260}/A_{280} of total RNA (based on peak area) was typically $\geqslant 1.8$. Poly A+ messenger RNA was found to range in size from 0.2 to 8 kb [7]. No significant differences in the spectral purity, molecular size or yield of total RNA between control or treated HN cells were noted. The SuperScript Choice System (Invitrogen) was used for RNA probe synthesis [4,6,7]. Briefly, eight Test3 DNA arrays (900341; Affymetrix) and eight human genome U133 plus 2.0 analytical Genechips (900466; Affymetrix; containing 33,000 gene targets) were used in total genome expression analysis. DNA arrays were scanned, pixel intensities and gene expression signals were quantified, and features were extracted using Microarray Suite ver 5.0, (Affymetrix) and Genespring ver 7.2 algorithms (Silicon Genetics, Redwood City, CA) [5–7]. Statistical significance of treated gene expression levels over controls was analyzed using a two-way factorial analysis of variance (p, ANOVA; Statistical Analysis System; SAS Institute, Cary, NC). Gene promoter maps identifying potential DNA binding sites in specific pro-inflammatory and pro-apoptotic gene promoters were created and displayed using DNASIS MAX algorithms (Mirai-Bio Inc, Hitachi Genetic Systems, Alameda, CA).

Data analysis for gene expression in HN cells in the presence of aluminum is compared in Table 1 against 24 of the most significant gene expression levels found to be altered by a factor of $\geqslant$3-fold ($p < 0.05$) in AD brain [5–7]. The results indicate that in AD, of the 24 gene expression levels found to be altered by a factor of $\geqslant$3-fold ($p < 0.05$), 16 of 24 (66.7%) were found to be down-regulated. Of this group, 11/16 (68.8%) were also found to be down-regulated, at the level of gene expression, in aluminum sulfate-treated HN cells when compared to controls. Interestingly, of the up-regulated genes in AD, 7/8 (87.5%) exhibit similar expression patterns to those observed in aluminum-treated HN cells. Because gene expression patterns are a reflection of the physiological status of the cell, these data suggest that pathological processes directed by these atypical gene expression profiles, such as inflammation and apoptosis, are common to both AD and aluminum-treated HN cells. To further investigate the possibility of coordinate up-regulation of these pro-inflammatory and pro-apoptotic genes, the immediate promoters of 5/7 up-regulated genes were screened, analyzed and mapped for TF-DNA binding sites and the analysis is shown in Fig. 1. Gene promoters were analyzed using DNASIS DNA Sequence analysis software capable of recognizing ~360 consensus and relaxed TF-DNA recognition motifs. Preliminary analysis shows that these up-regulated genes contain multiple binding sites for the relatively rare, oxidative-stress sensitive TFs HIF-1 (5′-R*CGTG*-3′) [8,9] and NF-κB (5′-GG*GACT*TT-3′) [8,10] in their immediate promoters (core recognition sequences italicized). The correlation of an occurrence of an HIF-1- or NF-κB-DNA binding site in each pro-inflammatory or pro-apoptotic gene promoter with the abundance of that specific RNA signal intensity was found to be highly significant ($r^2 = 0.88$; $p < 0.05$).

The vast majority of all cases of AD are sporadic and of unknown origin, and environmental toxins injurious to normal brain function have been strongly implicated [1–3,12–14]. Notably, expression of the familial Alzheimer genes encoding presenilin-1, presenilin-2 and the apolipoprotein E4 isoform were found not to be overtly affected by aluminum in these experiments. The major risk factor for the development of AD is aging, suggesting an accumulation of biological insults over decades of life. Specific compartments of the brain appear to accumulate aluminum from the environment as a function of aging [1–3]. This is the first study describing the effects of aluminum sulfate, at 100 nM concentrations, on total gene expression patterns in untransformed human brain cells in primary culture using DNA arrays. The molecular biology, transport and in vivo speciation of aluminum in genetic systems is not well understood, however, as little as 50 nM ambient aluminum has been shown to dramatically perturb RNA polymerase II-direct gene transcription in isolated mammalian brain cell

Table 1
Trends in gene expression in AD compared to aluminum-treated HN cells

Signal Intensity	Genbank Accession	Protein/Gene Description	Alzheimer			Al-HN
			TF	SN	PI	
-65391	X06389	synaptophysin (SYNp)		■		■
-53732	NM006086	class III β-tubulin (CIIIBT)		■		
-48172	AF040991	ROBO2; axon guidance receptor		■		■
-42348	NM006473	PCAF-associated factor 65β	■		■	
-38115	U45448	P2X purinoceptor 3; ATP receptor P2X3		■		■
-34550	X78710	metal-regulatory TF (MRTF-1)		■		■
-32732	U41766	metalloprotease / disintegrin (MDC9)		■		■
-32268	XM046146	choline O-acetyltransferase (CHAT)		■		■
-28440	M58378	synapsin; synaptic vesicle phosphoprotein		■		■
-25508	NM024342	glucocorticoid receptor represor factor-1			■	■
-24320	M22637	LYL-1 (lineage/differentiation protein)	■			
-22992	X05608	neurofilament light chain (NFL) protein		■		■
-21750	M77810	endotelial transcription factor; GATA2		■	■	■
-21424	X60201	brain- derived neurotrophic factor (BDNF)		■	■	
-20670	M95809	basic transcription factor 62 (BTF2)		■	■	■
-20492	AF059274	neuroglycan C precursor protein	■		■	
+15455	M58603	NF-kB p100 subunit, pro-inflammatory	■		■	■
+22008	NM003998	NF-kB p52 subunit, pro-inflammatory	■		■	■
+28851	NM000576	interleukin-1beta (IL-1β) precursor; catabolin			■	■
+36745	NM001814	dipeptidyl-peptidase I precursor; cathepsin C			■	
+40336	U04636	cyclooxygenase-2 (COX-2)			■	■
+41256	X12751	beta amyloid A4 protein precursor (βAPP)			■	■
+48296	D38178	cytosolic phospholipase A_2 ($cPLA_2$)		■	■	■
+61668	AF015956	DAXX (pro-apoptotic, death-associated protein)	■		■	■

Values under 'signal intensity' indicate magnitude of change; "−" values indicate gene down-regulation and "+" values indicate gene up-regulation in Alzheimer's disease (AD) brain with a $p < 0.05$ [3–7]. Signal intensities are arranged from the most down-regulated to the most up-regulated genes found in AD brain [4–7]. 'Genbank accession' numbers are for human-specific sequences, and complete descriptive data for these proteins/genes can be accessed at the www.ncbi.nlm.nih.gov website. 'Protein/gene description' column indicates specific transcripts affected. These genes generate RNA messages encoding transcription factors (TF), synaptic signaling/cytoskeletal/neurotrophic (SN) elements and proteins involved in pro-inflammatory/pro-apoptotic (PI) signaling. Shaded boxes beneath column marked 'Alzheimer' indicate category of each gene transcript in AD (some have multiple functions), and beneath 'Al-HN' indicate preliminary data on similar gene expression patterns observed in aluminum-treated HN cells.

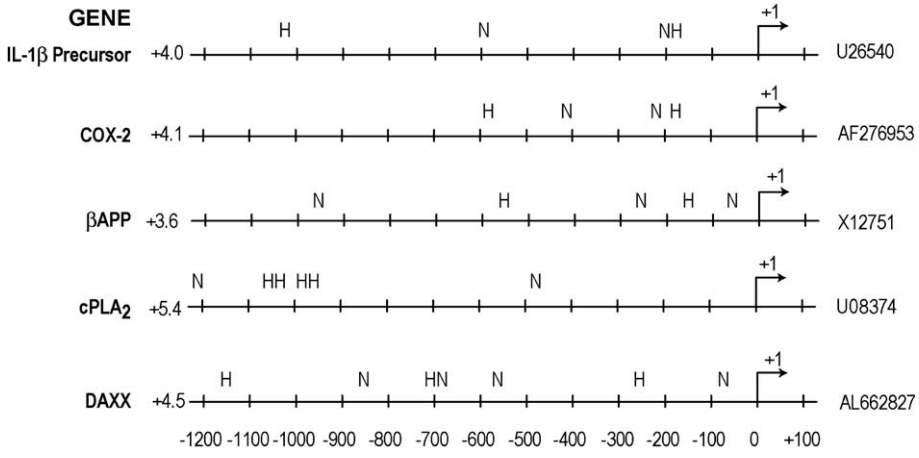

Fig. 1. Human gene promoter map analysis for five of seven genes up-regulated in AD and in aluminum-treated HN cells for which DNA sequence data is available. The immediate promoters of these genes (−1200 to +100 bp region) contain multiple DNA binding sites for the relatively rare stress-responsive transcription factor HIF-1 (H) and NF-κB (N) [7–10]. The correlation of the occurrence of H or N sites in these gene promoters with abundance of their respective RNA signal intensities (Table 1) reaches an $r^2 = 0.88$ ($p < 0.05$). The transcription start site is at +1 (arrow). Each of these genes also contains TATA-type transcription start sequences [4,7]. Genbank Accession numbers shown at right are for human-specific gene promoter sequences.

nuclei [1–3]. Metal-mediated oxidative stress, such as that induced by aluminium, may have additional effects on messenger RNA abundance and speciation including the generation of aberrantly spliced RNA isoforms [14]. Other aluminum salts, such as aluminum chloride, have shown similar effects on gene expression patterns in

cultured HN cells (unpublished observations). A major finding in this study was that aluminum-treated HN cells emulate many of the gene expression patterns observed in AD brain, including a significant up-regulation of a family of genes known to drive pro-inflammatory and pro-angiogenic neuropathology. The promoters of these up-regulated genes are enriched in DNA binding sites for two stress-related transcription factors, HIF-1 and NF-κB, DNA binding proteins known to contribute to pathogenic processes associated with AD-type neural degeneration [10–12]. Confirmation of these stress-related gene expression changes by Northern and Western analyses is essential, and this work is currently in progress.

Finally, as determined by fluorescence microscopy, the nuclear pore complexes (NPCs) of HN cells are somewhat unusual in that HN NPCs are often tightly juxtaposed against HN cell plasma membranes (unpublished observations). Therefore, in these in vitro experiments, the distance for externally applied aluminum to access the core of the HN cell genetic material, and its high phosphate density, may be as little as 15–20 nm [15]. Removal of neuroprotective components of the blood–brain barrier in our assay system may therefore render cultured HN cells especially vulnerable to the genotoxic effects of aluminum. Moreover, while 100 nM of exogenously applied aluminum may seem like a very small amount, it still represents about 6×10^{16} atoms of aluminum being rapidly introduced into the highly dynamic and easily perturbed DNA-directed copying process of gene transcription [1–3,8]. Further research is required to examine the adverse effects of other environmentally abundant aluminum compounds (fluorides, lactates, maltolates, nitrates, etc.), along with other potentially neurotoxic metals (such as iron and zinc), on specific stress-related gene expression patterns in human brain cells.

Abbreviations

AD	Alzheimer's disease
HIF-1	hypoxia inducible factor
HN	human neural
NF-κB	nuclear factor for kappa B

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