INTRODUCTION

Disulfiram (antabuse), an inhibitor of acetaldehyde dehydrogenase, is a drug used extensively in the treatment of alcohol addiction. This drug is generally safe but does have some rare, serious adverse effects. The most serious of these side-effects are various neurotoxicities including optic neuropathy, peripheral neuropathy and basal ganglia lesions. These toxicities generally occur with long-term dosing [1-3], in over-dose situations [4], and are irreversible in some cases. However, they can also occur with short dosing periods of several weeks in some individuals [5-6].

Efforts to study the mechanisms of these neurotoxicities are complicated by the many metabolites of disulfiram [7]. The predominant hypothesis is that metabolites of disulfiram induce oxidative stress resulting in demyelination; however, animal studies do not support this hypothesis [8]. We therefore decided to examine the effects of disulfiram on human lymphoblasts which are useful for studying human lipid pathways [9] and undertook a shotgun lipidomics analysis [10] utilizing an analytical platform that measures over 900 individual lipids across 27 lipid subfamilies [11-14].

MATERIALS AND METHODS

Tissue Culture

Human lymphoblasts (GM07210, Coriell Cell Repositories) were grown as suspension cultures in 12 well plates with HyClone RPMI-1640 medium containing 10% fetal bovine serum, 25 mM HEPES, 2 mM glutamine, and 100 μg/ml penicillin/ streptomycin in an incubator at 37°C and 5% CO₂. Lymphoblasts were incubated with disulfiram dissolved in dimethyl sulfoxide (DMSO) such that the final DMSO concentration was 0.1%. For lipid replacement experiments, krill oil (Now Foods, Neptune krill oil) was dissolved in ethanol such that the final ethanol concentration was 0.1%. Cells were harvested by centrifugation at 4,500 × g for 10 min with the cell pellet washed once with cold phosphate-buffered saline.
Lipid Extraction and Analysis

Lipids were extracted with methy-tert-butyl ether and methanol containing [1H]arachidonic acid, [1H]hexacosanoic acid, [1C18]stearic acid, [1H32]DC 16:0, [1H32]cholesterol sulfate, [1H18]MAG 18:1, [1C36]DG 36:2, [1H]TAG 48:0, [1H7]PtdE 34:1, [1H7]PtdE 28:0, [1H7]PtdC 34:1, [1H7]PtdC 28:0, [1H5]PtdC 32:0, [1H]PS 36:1, [1H7]PA 34:1, [1H]PG 32:0, [1H]camitine 18:0, [1H]LPC, CL(56:0), glyburide, and bromocriptine as internal standards. Extracts were dried by centrifugal vacuum evaporation and dissolved in isopropanol:methanol:chloroform (4:2:1) containing 15 mM ammonium acetate. Shotgun lipidomics (5 μL per min) were performed utilizing high-resolution (140,000 at 200 amu) data acquisition, with sub-millimass accuracy on an orbitrap mass spectrometer (Thermo Q Exactive) with successive switching between polarity modes [10-12]. Washes (500 μL) with methanol followed by hexane/ethyl acetate (3:2), between samples, were used to minimize ghost effects.

In negative ion ESI, the anions of ethanolamine plasmalogens (PlsE), phosphatidylethanolamines (PtdE), lysophosphatidylethanolamines (LPE), lysoalkenyl-acyl glycerophosphoethanolamines (LPEp), phosphatidylglycerols (PG), lysophosphatidylglyceroles (LP), phosphatidic acids, lysophosphatidylinositolss (PI), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinositolss (PS), lysophosphatidylinosito...
Incubation of disulfiram-treated cells with krill oil resulted in augmentation of ethanolamine glycerophospholipids but not PI, PS, or PG [Figure 6].

**DISCUSSION**

Disulfiram is an important drug in the treatment of alcohol abuse and is under clinical evaluations in a number of other addictions [15]. However, a number of serious neurotoxicities can develop with this drug [1-6]. Our data are the first to demonstrate that these neurotoxicities involve dramatic decrements in critical structural glycerophospholipids and sphingolipids. The large dose- and time-dependent decreases in membrane diacyl-glycerophospholipids are complicated in that PtdE, PS, PI, and PG levels are lowered while PtdC levels are unaffected. Since these lipids have common precursor molecules [Figure 7], it is most likely that the metabolism of the affected lipids is occurring at a more rapid rate than synthesis can keep pace with. The demands on synthesis are further validated by our observations of decreased levels of DG, precursors to glycerophospholipids [Figure 7]. Furthermore, the observed increases in MG suggest that acylation of MG to DG may be
inhibited by disulfiram, in addition to inhibition of degradation of the MG pool by MG lipase [16]. The DG pool, in addition to supplying precursors for structural glycerophospholipids and TG, also function as signal transduction mediators and are essential structural lipids of the nuclear envelope and nucleoplasmic reticulum [17,18]. Therefore, it is prudent to also speculate that DG-dependent alterations in nuclear function may contribute to the toxicity of disulfiram.

The decrements that we noted in lysophosphoethanolamines further support the suggestion of augmented glycerophospholipid degradation. Maintenance of PtdC levels may involve decreased degradation along with decreased base exchange to generate PS [19] and by increased methylation of PtdE to maintain the PtdC pool [20]. Our experiments with krill oil, which contains a large variety of glycerophosphocholines, glycerophosphoethanolamines, and their precursors [21], further demonstrate that synthetic pathways are intact and can be augmented with metabolic precursors.

Plasmalogens, another class of critical membrane lipids [22], also were decreased in the case of PIsE but not PIsC. These data suggest that PIsE may be utilized to support choline plasmalogen levels. The biosynthesis of very-long-chain dicarboxylic acids involves the ω-oxidation of very-long-chain fatty acids. This is a complicated pathway involving fatty aldehyde dehydrogenase, an enzyme inhibited by disulfiram [23-25]. Our data also
demonstrate that disulfiram is a potent inhibitor of fatty aldehyde dehydrogenase in that we monitored dramatic reductions in very-long-chain dicarboxylic acids. In addition, since fatty aldehyde dehydrogenase metabolizes fatty aldehydes generated during plasmalogen metabolism [26], accumulation of these reactive aldehydes could lead to cytotoxicity [27].

In the case of structural sphingolipids, large decreases in the levels of SM were measured while Sulf and Cer were minimally affected by disulfiram. Since the ceramide pool provides precursors for both SM and Sulf, these data also suggest that the enhanced metabolism of SM is responsible for the decreases in these sphingolipids.

CONCLUSION

We observed dramatic decrements in structural lipids after treatment of human lymphoblasts with disulfiram. Our data suggest that catabolic pathways are activated and that this mechanism may be responsible for the clinical neurotoxicities of disulfiram.

ACKNOWLEDGMENTS

This research was funded by Lincoln Memorial University, DeBusk College of Osteopathic medicine (LMU-DCOM). RSA and CCF received summer medical student awards from LMU-DCOM.

REFERENCES

1. Boukriche Y, Weisser I, Aubert P, Masson C. MRI findings in a case of LMU-DCOM.