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# Covalent adduct formation between the plasmalogen-derived modification product 2-chlorohexadecanal and phloretin

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ABSTRACT

Hypochlorous acid added as reagent or generated by the myeloperoxidase (MPO)-$H_2O_2$-$Cl^-$ system oxidatively modifies brain ether-phospholipids (plasmalogens). This reaction generates a sn2-acyl-lysophospholipid and chlorinated fatty aldehydes. 2-Chlorohexadecanal (2-ClHDA), a prototypic member of chlorinated long-chain fatty aldehydes, has potent neurotoxic potential by inflicting blood–brain barrier (BBB) damage. During earlier studies we could show that the dihydrochalcone-type polyphenol phloretin attenuated 2-ClHDA-induced BBB dysfunction. To clarify the underlying mechanism(s) we now investigated the possibility of covalent adduct formation between 2-ClHDA and phloretin. Coincubation of 2-ClHDA and phloretin in phosphatidylcholine liposomes revealed a half-life of 2-ClHDA of approx. 120 min, decaying at a rate of $5.9 \times 10^{-3}$ $min^{-1}$. NMR studies and enthalpy calculations suggested that 2-ClHDA-phloretin adduct formation occurs via electrophilic aromatic substitution followed by hemiacetal formation on the A-ring of phloretin. Adduct characterization by high-resolution mass spectroscopy confirmed these results. In contrast to 2-ClHDA, the covalent 2-ClHDA-phloretin adduct was without adverse effects on MTT reduction (an indicator for metabolic activity), cellular adenine nucleotide content, and barrier function of brain microvascular endothelial cells (BMVEC). Of note, 2-ClHDA-phloretin adduct formation was also observed in BMVEC cultures. Intraperitoneal application and subsequent GC–MS analysis of brain lipid extracts revealed that phloretin is able to penetrate the BBB of C57BL/6J mice. Data of the present study indicate that phloretin scavenges 2-ClHDA, thereby attenuating 2-ClHDA-mediated brain endothelial cell dysfunction. We here identify a detoxification pathway for a prototypic chlorinated fatty aldehyde (generated via the MPO axis) that compromises BBB function in vitro and in vivo.


## 1. Introduction

The neurovascular unit physically separates most regions of the brain from the periphery to maintain central nervous system homeostasis [1]. Within this specialized vessel system, brain microvascular endothelial cells (BMVEC) constitute the morphological basis of the blood–brain barrier (BBB) [2]. The formation of tight junctions prevents paracellular transport of molecules and cells and maintains homeostasis of the brain micromilieu via elaborately regulated transport mechanisms.

Under inflammatory conditions BBB function is compromised and can aggravate neuronal dysfunction [3]. Many pathways that compromise BBB and neuronal function in further consequence have been shown to converge on the formation of reactive species [4]. This is of particular importance since tight junction proteins are sensitive to alterations of the intracellular redox status, ultimately resulting in barrier dysfunction [5]. Various effects of reactive oxygen species e.g. inhibition of cerebral blood flow and alterations in barrier integrity have been demonstrated in

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cerebrovascular diseases and stroke [6–8]. Edema formation in stroke causes an increase in cell volume, depolarization, breakdown of ionic gradients, cellular ATP depletion, and finally vasogenic edema in response to BBB breakdown [8].

During our earlier studies we could show pronounced BMVEC barrier dysfunction in response to the fatty aldehyde 2-chlorohexadecanal (2-ClHDA) that is generated during endotoxemia [9,10]. 2-ClHDA is formed by attack of plasmalogens (ether phospholipids) by hypochlorous acid (HOCl) [11,12]. HOCl in turn is generated by the myeloperoxidase (MPO)–$H_2O_2$–$Cl^-$ system of activated phagocytes [13], cells that release MPO at the cerebrovasculature [10]. Under physiological conditions MPO is part of the innate immune system, however, under chronic inflammatory conditions the MPO–$H_2O_2$–$Cl^-$ system is implicated in the development of (neurological) diseases. MPO is abundantly expressed in microglia in and around demyelinated lesions in Multiple Sclerosis in humans and rodents [14]. In line, pharmacological inhibition of MPO reduced the severity of clinical symptoms in a murine model of Multiple Sclerosis [15]. The involvement of MPO in barrier dysfunction was also demonstrated during bacterial meningitis [16,17]. We recently reported significantly elevated MPO levels in mouse brain in response to systemic lipopolysaccharide (LPS) administration [9]. MPO expression was accompanied by a significant decrease of brain plasmalogen content and concomitant formation of 2-ClHDA [9]. In line with deleterious effects of MPO-generated 2-ClHDA we could show that LPS-induced BBB dysfunction was significantly less pronounced in $MPO^{-/-}$ mice as compared to the corresponding littermates [10].

Zhu and colleagues [18] suggested that chemical trapping of reactive aldehydes by polyphenols could provide an attractive approach to prevent cellular dysfunction in response to Schiff's base formation between vital cellular proteins and reactive aldehydes. We recently reported that phloretin, a dihydrochalcone-type polyphenol, prevented 2-ClHDA-induced barrier dysfunction, apoptosis, and ATP depletion in BMVEC in vitro by yet unidentified mechanisms [19]. To reveal the underlying mechanisms of adduct formation between 2-ClHDA and phloretin we applied NMR analysis, theoretical enthalpy calculations, and high-resolution mass spectroscopy (HRMS). To get an indication about altered toxicity profiles after adduct formation, metabolic activity, the cellular adenine nucleotide status, and barrier function was studied in BMVEC. To obtain first evidence about BBB permeability of phloretin in vivo, uptake of i.p. injected phloretin was quantitated in murine brain.

## 2. Materials and methods

Cell culture supplies were from Gibco (Vienna, Austria), PAA Laboratories (Linz, Austria), Costar (Vienna), or VWR (Vienna). Dulbecco's modified Eagle's medium (DMEM) Ham's F12, hydrocortisone, *N*-chlorosuccinimide, DL-proline, dipalmitoylphosphatidylcholine (DPPC), phloretin (3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl) propan-1-on), resveratrol, pentafluorobenzyl (PFB) hydroxylamine, dimethyl sulfoxide (DMSO), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma-Aldrich (Vienna). Electrical cell-substrate impedance sensing (ECIS) electrode arrays (8W10E+) were from Ibidi (Martinsried, Germany). Hexadecanal was from Toronto Research Chemicals (Toronto, Canada). *N*-Methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) was from ABCR (Karlsruhe, Germany), trimethylchlorosilane (TMCS) and pyridine were from Pierce (Rockford, IL, USA). Silica 60 gel and silica 60 TLC plates were from Merck (Darmstadt, Germany). Poly-Prep® chromatography columns were from Bio-Rad (Vienna). Phosphospecific rabbit polyclonal anti-pp44/42 MAPK (Thr202/Tyr204), anti-pSAPK/JNK1/2 (Thr183/Tyr185), pan-specific rabbit monoclonal anti-p44/42, and rabbit polyclonal anti-SAPK/JNK1/2 antibodies were from Cell Signaling Technology (Beverly, MA). HRP-labeled secondary goat anti-rabbit IgG was from Pierce (Rockford, IL). All solvents and other reagents of analytical grade were from Merck, Sigma-Aldrich, or Roth.

### 2.1. Synthesis of 2-chlorohexadecanal (2-ClHDA)

2-ClHDA was synthesized by organocatalytic α-chlorination of hexadecanal [20]. DL-Proline (0.2 equ.) followed by *N*-chlorosuccinimide (1.5 equ.) were added to a solution of hexadecanal (1 equ.) in 4 ml $CH_2Cl_2$ at 0 °C. The suspension was allowed to warm to ambient temperature and was stirred over night. Hexane (15 ml) was added to the reaction mixture and the precipitate was filtered. 2-ClHDA was purified using a Silica 60 column and hexane/diethyl ether (90:10, v/v) as eluent (yield = 51%). 2-Cl[$^{13}C_8$]HDA used as internal standard for gas chromatography–mass spectroscopy (GC–MS) analysis was synthesized and purified as described [9].

### 2.2. Analytical procedures

DPPC liposomes containing equimolar concentrations of phloretin and 2-ClHDA were prepared by dispersing a mixture of 2.5 mg DPPC (0.7 mM), 350 μg phloretin (0.3 mM), and 350 μg 2-ClHDA (0.3 mM) in 2 ml phosphate-buffered saline (PBS, pH 7.4). The mixture was sonicated on ice for 3 × 10 s. The reaction was started by gently stirring the mixture at 37 °C in the dark under an argon atmosphere. At the indicated time points 100 μl aliquots (approx. 18 nmol of reactants) were removed and 250 ng 2-Cl[$^{13}C_8$]HDA [9] was added. Samples were immediately extracted twice with ethyl acetate (2 ml) followed by a Folch extraction. Extracts were stored at −70 °C until analysis. After conversion to the corresponding PFB-oxime derivatives 2-ClHDA was quantitated by negative ion chemical ionization (NICI)–GC–MS. Alternatively adduct formation was studied in acetonitrile, tetrahydrofurane (THF; both solvents containing 1% triethylamine; 240 μM 2-ClHDA and phloretin), or high-density lipoproteins (HDL; 80 μM 2-ClHDA and phloretin in PBS containing 250 μg HDL protein/ml). HDL was isolated from plasma as described [21]. Following extraction, phloretin and adduct concentrations were quantitated by HPLC (see below). A one-phase exponential decay model ($C_t = C_0 \times e^{-kt}$) was used to fit experimental data using the Prism 5.0 software (GraphPad, San Diego, CA, USA).

### 2.3. Derivatization for GC–MS

Preparation of PFB-oxime derivatives of 2-ClHDA and 2-Cl[$^{13}C_8$]HDA for NICI–GC–MS was performed as described above. Phloretin and resveratrol were converted to the corresponding trimethylsilyl (TMS)-ether derivatives in 100 μl MSTFA/pyridine (2:1; v/v) containing 1% (v/v) TMCS at room temperature (RT) for 30 min immediately before electron impact (EI)–GC–MS analysis due to putative phloretin keto-enol tautomerism.

### 2.4. NICI–GC–MS and EI–GC–MS

Samples were separated on a Thermo Scientific Trace GC Ultra (helium was used as carrier gas, 2 ml/min) with a Zebron ZB-AAA capillary column (15 m, 0.25 mm inner diameter, Phenomenex®) and analyzed using a DSQII mass spectrometer (Thermo Scientific).

For NICI–GC–MS the injector temperature was set to 280 °C. The oven temperature was maintained at 100 °C for 5 min, increased during the first ramping step at a rate of 20 °C/min to 175 °C, and held at 175 °C for 5 min. In the second ramping step the temperature was raised at a rate of 15 °C/min to 280 °C and held