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Docosahexaenoic acid attenuates in endocannabinoid synthesis in RAW 264.7 macrophages activated with benzo(a)pyrene and lipopolysaccharide

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HIGHLIGHTS

- Differences in FA in RAW 264.7 cells upon BaP and LPS activation were observed.
- Co-treatment with LPS and BaP resulted in overexpression of COX-2 and CB2 in macrophages.
- Significant interactions were noted after DHA and LPS + BaP treatment in ECBs synthesis.
- Synthesis of ECBs suggested anti-inflammatory properties of DHA under BaP exposure.
- A novel environmental stress reaction in LPS activated macrophages is suggested.

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ABSTRACT

Endocannabinoids are synthetized as a results of demand from membrane phospholipids. The formation and actions of these lipid mediators depend to a great extent on the prevalence of precursor fatty acid (FA), and can be influenced by diet or supplementation. The purpose of this study was to evaluate the interactive effects of lipopolysaccharide (LPS) and benzo(a)pyrene (BaP) in RAW 264.7 cells supplemented with docosahexaenoic acid (DHA).

After LPS and/or BaP treatment in macrophages pre-incubated with DHA, a significant decrease in the amount of fatty acid was observed. The highest content of monounsaturated fatty acids was detected in RAW 264.7 cells co-treated with LPS and BaP.

Significant interactions between LPS and BaP co-treatment in terms of endocannabinoid levels were observed in RAW 264.7 cells after DHA supplementation. The highest amount of endocannabinoids was detected in macrophages supplemented with DHA and co-treated with BaP and LPS: arachidonoyl ethanolamine AEA (5.9 μg/mL), docosahexaenoyl ethanolamide DHEA (10.6 μg/mL) and nervonoyl ethanolamide NEA (16.5 μg/mL).

The highest expression of cyclooxygenase (COX-2) and cannabinoid receptor 2 (CB2) was noted in macrophages supplemented with DHA and activated with LPS and BaP.

Our data suggested a novel, CB2 receptor-dependent, environmental stress reaction in macrophages co-treated with LPS and BaP after supplementation with DHA. Despite the synergistic LPS and BaP action DHA potentiates the anti-inflammatory response in RAW 264.7 cells.

1. Introduction

Adverse health effects such as inflammation, metabolic disorders, and lung and cardiovascular diseases as well as cancer may be attributed to exposure to air pollution and consumption of chemically contaminated food. This leads to a larger intake and retention of lipophilic toxic chemicals in the body with an increase

Abbreviations: AA, arachidonic acid; AEA, N-arachidonoyl ethanolamine anandamide; BaP, benzo(a)pyrene; CB, cannabinoid receptor; DHEA, N-docosahexaneoyl ethanolamide DHA docosahexaenoic acid; ECBs, endocannabinoids; EAE, eicosapentaenoyl ethanolamide; EPA, eicosapentaenoic acid; FAs, fatty acids; LPS, lipopolysaccharide; MUFAs, monounsaturated fatty acids; NEA, nervonoyl ethanolamide; PUFAs, polyunsaturated fatty acids UNSFAs unsaturated fatty acids.

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in risks to human health (Walker et al., 2007; Diggs et al., 2013; Banks et al., 2016).

Polycyclic aromatic hydrocarbons (PAHs) are a large family of toxic compounds generated from the combustion of organic materials, diesel exhaust, and industrial waste. Some PAHs are classified as human carcinogens, and also elicit a broad spectrum of toxic responses in human and animals (Kelley et al., 1997; Hanzalova et al., 2010; Kim et al., 2015; Gdula-Argasińska et al., 2015a, 2016). Benzo(a)pyrene (BaP) is a principle indicator of carcinogenic PAHs.

Rapid metabolism of polycyclic aromatic hydrocarbons and generation of epoxide may propagate oxidative stress. Oxidative stress caused by lipid peroxidation through free radicals is believed to be one of the key factors underlying chronic diseases (Kim et al., 2015; Gdula-Argasińska et al., 2013; Gdula-Argasińska et al., 2015a). Further genetic and biochemical studies are necessary to characterize the molecular mechanisms involved in PAH-induced modulation of gene expression and to identify their effects on human health (Kim et al., 2015; Banks et al., 2016; Gdula-Argasińska et al., 2016).

Interaction between polycyclic hydrocarbons and lipids has suggested that PAHs-lipid associations may represent critical factors in carcinogen transport and carcinogen modification of biological structures (Diggs et al., 2013; Kim et al., 2015; Gdula-Argasińska et al., 2013, 2015a, 2016).

Bioactive metabolites of unsaturated fatty acids (UNSFAs) are synthetized in response to various stimuli. UNSFAs are released from membranes or storage lipids and transformed enzymatically into lipid mediators including eicosanoids and endocannabinoids (ECBs) (Han et al., 2009; Balvers et al., 2010, Kendall and Nicolaou, 2013; Kim and Watkins, 2014). The formation and actions of these lipid mediators depend to a great extent on the prevalence of precursor fatty acid (FA), and can be influenced by diet or supplementation (Clària et al., 2011; Serhan and Petasis, 2011; Brown et al., 2013; Gdula-Argasińska et al., 2013, 2015a, 2016). Many of the downstream products of UNSFA are bioactive compounds controlling physiological functions, metabolism, homeostasis, and are also involved in the inflammation and immunity that relates to the pathology of various diseases. Lipid mediators also play a fundamental role as signaling molecules of a variety of cellular biochemical cascades including free radicals (Serhan and Petasis, 2011; Davidson et al., 2012; Spite et al., 2014).

The specific molecular mechanism for health benefits from *n*-3 and *n*-6 UNSFA still remain unclear. Lipid mediators from *n*-3 and *n*-6 UNSFA represent an as yet not fully elucidated class of molecules that have been proven to be critical to inflammation or environmental stress (Serhan and Petasis, 2011; Spite et al., 2014; Calder, 2015).

Endocannabinoids are most frequently considered to be as both presynaptic and postsynaptic neuromodulators, but they have an emerging role in the immune system and inflammation. ECBs have anti-inflammatory properties, and mainly modulate inflammation through cannabinoid receptor 2 (CB2) activation (Brown et al., 2013; Crowe et al., 2014; Kim and Watkins, 2014).

Recent studies have positioned cyclooxygenase-2 (COX-2) as a key regulator of the endocannabinoid system (Han et al., 2009; Kendall and Nicolaou, 2013; Kim and Watkins, 2014; Urquhart et al., 2015). It is not clear if the docosahexaenoic (DHA) acid influences ECBs and COX protein levels in cells. The molecular basis for DHA to modulate COX-2 and potentially mitigate inflammation is most likely multifactorial and remains incompletely understood (Nagarkatti et al., 2009; Calder, 2015; Urquhart et al., 2015).

Therefore, the purpose of the present study was to analyze fatty acid and endocannabinoid contents in murine RAW 264.7 macrophages after incubation with DHA and after co-treatment with LPS and BaP.

2. Materials and methods

2.1. Reagents

All used chemicals were of analytical grade. Methanol, chloroform, formic acid, acetonitrile, ethanol and water were from Merck (Darmstadt, Germany). Arachidonoyl ethanolamine (AEA), AEA-d4, eicosapentaenoyl ethanolamide (EPEA), EPEA-d4, docosaheaenoyl ethanolamide (DHEA), DHEA-d4 and nervonoyl ethanolamide (NEA) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Docosahexaenoic acid (DHA), benzo(a)pyrene (BaP), lipopolysaccharide from *E. coli* (LPS), butylated hydroxytoluene (BHT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

2.2. Cultured cells

Mus musculus murine macrophages (American Type Cell Culture, RAW 264.7, TIB-71) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS and 1% antibiotic solution: 100 IU/mL penicillin and 0.1 μg/mL streptomycin (ATTC, Manassas, VA, USA). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO2 in air and were finally seeded into 75 cm^3 flasks (Sarstedt, Nümbrecht, Germany) at a density of 1×10^6 cells in 10 mL of medium. Cell morphology was investigated with an inverted light microscope (Olympus, Tokyo, Japan). Cell viability during culturing was assessed with the Trypan Blue Exclusion Test. 24 h after seeding, macrophages were supplemented with 10 μmol of DHA for 48 h. After this time, cells were activated by LPS (1 μg/mL) for 24 h and/or treated with 100 nmol of BaP for 2 h.

2.3. Lipid extraction

Cells were collected and acidified with 2 μL of 0.01% formic acid. One microliter of 0.001% butylated dihydroxytoluene (BHT) was added to prevent lipid oxidation. Lipids were extracted with chloroform–methanol solution (2:1 v/v). Two microliters of internal standards were used throughout (DHEA-d4 and EPEA-d4 at concentration 10 μg/mL). Samples were vortexed for 30 s and centrifuged for 10 min at 1000×*g*. Organic phases were collected and dried under a stream of nitrogen. The residue was dissolved in 40 μL of acetonitrile.

2.4. Fatty acid analysis

Cell membranes were prepared by hypotonic hemolysis at 4 °C in 10 mmol Tris with pH 7.4 and then isolated by centrifugation (10,000×*g*, 15 min) according to the method proposed by Graham (2006). Lipid extraction from the cell membranes was carried out using a solution of chloroform/methanol (2:1 v/v). The synthesis of fatty acid methyl esters was carried out with 14% BF3 in methanol. FAME were analyzed using gas chromatography (Agilent 6890N) with a DB-23 (60 m, 0.25 mm) column, as described earlier (Gdula-Argasińska et al., 2015b). FAME were identified according to standards (Sigma–Aldrich). The data were analyzed using ChemStation. Results were expressed relative percentages of the sum of saturated (SFA), unsaturated (UNSFA), monounsaturated (MUFA), *n*-3 and *n*-6 FA.

2.5. LC–MS/MS conditions

LC was performed using an Agilent 1100 LC system (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was carried out with a Thermo Scientific BDS HYPERSIL C18 column (100 × 3 mm I.D., 3 μm particle size). The advance column,