UPLC-MS/MS-Based Profiling of Eicosanoids in RAW264.7 Cells Treated with Lipopolysaccharide

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Abstract: While both the pro- and anti-inflammatory effects of several eicosanoids have been widely studied, the degree of inflammation in cells that results from various eicosanoids has yet to be comprehensively studied. The objective of this study was to assess the effect of lipopolysaccharide (LPS) treatment on eicosanoid content in RAW264.7 cells. An Ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS)-based profiling method was used to analyze the eicosanoid contents of RAW264.7 cells treated with different LPS concentrations. The profiling data were subjected to statistical analyses, such as principal component analysis (PCA) and hierarchical clustering analysis. LPS treatment increased nitric oxide production and secretion of pro-inflammatory cytokines, such as tumor necrosis factor-α and interleukin-6, in a concentration-dependent manner. In total, 79 eicosanoids were identified in the cells. RAW264.7 cells treated with different LPS concentrations were well differentiated in the PCA score plot. A heatmap was used to identify the eicosanoids that were up- or down-regulated according to the degree of inflammation and LPS concentration. Thirty-nine eicosanoids were upregulated and seven were down-regulated by LPS treatment in a concentration-dependent manner. Our novel UPLC-MS/MS technique can profile eicosanoids, and can evaluate the correlations between inflammation and eicosanoid metabolism.

Keywords: eicosanoids; inflammation; macrophage; lipopolysaccharide; UPLC-MS/MS

1. Introduction

Eicosanoids, such as prostaglandins (PGs), leukotrienes (LTs), and a number of hydroxyl and epoxy compounds (Figure 1), are bioactive lipid mediators that play vital roles in physiological and pathophysiological conditions [1,2]. They are key mediators and regulators of inflammation and exert both pro- and anti-inflammatory effects [3–5]. Inflammation, a mechanism to protect the host from harmful stimuli, is implicated in the pathogenesis of a number of diseases, including cardiovascular disease, diabetes, allergic diseases, obesity, and cancer [6–10]. Thus, many studies have attempted to identify biomarkers of inflammation, for which the eicosanoids have been targeted as critical metabolites [11,12]. The levels of eicosanoids in biological systems have been assessed to investigate their roles in cell function and pathophysiological events [13,14].
2. Results

2.1. Effect of Lipopolysaccharide (LPS) on RAW264.7 Cells

LPS-stimulated RAW264.7 cells were used to elucidate the correlation between inflammation and eicosanoid metabolism. The cells were treated with 0, 1, 10, 100, 1000 ng/mL LPS for 18 h, and monitored under optical microscopy. LPS is known to induce the differentiation of RAW264.7 cells into dendritic-like cells [30,31]. We observed that LPS-activated RAW264.7 cells had a differentiated form with accelerated spreading and forming pseudopodia (Figure 2). Next, the supernatants were subjected to quantification of nitric oxide (NO) and pro-inflammatory cytokine levels. LPS-induced NO production in RAW264.7 cells changed in a concentration-dependent manner (Figure 3A). These results showed that LPS activated inflammatory signaling pathways.

Figure 1. Chemical structures of typical eicosanoids: prostaglandin E₂ (PGE₂), leukotriene B₄ (LTB₄), 5-hydroxy-eicosatetraenoic acid (5(S)-HETE), and 5,6-epoxy-eicosatrienoic acid (5,6 EET).
were used to optimize the analytical conditions. The addition of each eicosanoid was confirmed by MS scans and were detected primarily as [M – H]⁻ ions by electropray ionization (ESI) in the negative-ion mode. Previously reported MRM transitions for eicosanoids were employed in the current study [32]. Furthermore, source conditions (source temperature (Temp), nebulizer gas (GS1), and heater gas (GS2)) and the compound parameters (collision energy (CE) and declustering potential (DP)) of the mass spectrometer were optimized using eicosanoid standards. Table 1 shows the MRM transitions (precursor m/z (Q1) > product m/z (Q3)), DP, and CE of the eicosanoid standards. Use of a UPLC system with a small-particle-size column enabled effective separation of these standards within 25 min at a flow rate of 0.5 mL/min (Figure 4).

2.2. Profiling of Eicosanoids by Ultra Performance Liquid Chromatography Coupled to Tandem Mass Spectrometry (UPLC-MS/MS)

To profile the eicosanoid levels in RAW264.7 cells, we established a multiple-reaction monitoring (MRM) method based on UPLC-MS/MS. First, several standards, 5-hydroxy-eicosatetraenoic acid-d8 (5(S)HETE-d8), 14,15-epoxy-eicosatrienoic acid-d11 (14,15EET-d11), leukotriene B₄-d₄ (LTB₄-d₄), prostaglandin E₂-d₄ (PGE₂-d₄), prostaglandin D₂-d₄ (PGD₂-d₄), and arachidonic acid-d₈ (AA-d₈), were used to optimize the analytical conditions. The adduct ion of each eicosanoid was confirmed by MS scans and were detected primarily as [M – H]⁻ ions by electropray ionization (ESI) in the negative-ion mode. Previously reported MRM transitions for eicosanoids were employed in the current study [32]. Furthermore, source conditions (source temperature (Temp), nebulizer gas (GS1), and heater gas (GS2)) and the compound parameters (collision energy (CE) and declustering potential (DP)) of the mass spectrometer were optimized using eicosanoid standards. Table 1 shows the MRM transitions (precursor m/z (Q1) > product m/z (Q3)), DP, and CE of the eicosanoid standards. Use of a UPLC system with a small-particle-size column enabled effective separation of these standards within 25 min at a flow rate of 0.5 mL/min (Figure 4).

Table 1. Optimized multiple-reaction monitoring (MRM) conditions for eicosanoid standards.

| Compounds | Abbreviation  | Ion Mode | MRM Transitions | DP  | CE  |
|-----------|---------------|----------|-----------------|-----|-----|
|           | Q1            | Q3       |                 |     |     |
| Prostaglandin E₂-d₄ | PGE₂-d₄ | Negative | 355 275 | –50 | –25 |
| Prostaglandin D₂-d₄ | PGD₂-d₄ | Negative | 355 275 | –50 | –25 |
| Leukotriene B₄-d₄ | LTB₄-d₄ | Negative | 339 197 | –70 | –22 |
| 14,15 Epoxy-eicosatrienoic acid-d₁₁ | 14,15 EET-d₁₁ | Negative | 330 202 | –50 | –15 |
| 5-Hydroxy-eicosatetraenoic acid-d₈ | 5(S)-HETE-d₈ | Negative | 327 116 | –50 | –20 |
| Arachidonic acid-d₈ | AA-d₈ | Negative | 311 267 | –80 | –20 |

Q1, precursor m/z; Q3, product m/z; DP, Declustering potential; CE, Collision energy.
2.3. Quantification of Eicosanoids in LPS-Treated RAW264.7 Cells

The above UPLC/MS/MS-based method was used to quantify eicosanoids in RAW264.7 cells treated with 0, 1, 10, 100, and 1000 ng/mL LPS. Assays were performed in triplicate for each LPS concentration. Eicosanoids were extracted using 10% methanol. In this study, a total of 150 MRM transitions were used to profile eicosanoids. A total of 79 eicosanoids were identified in RAW264.7 cells (Table 3). To ensure reliable quantification, we used deuterated compounds, including 5(S)-HETE-d8, 14,15-EET-d11, AA-d8, LTB4-d4, and PGE2-d4, as ISs to normalize the data. The normalized data (peak areas of compounds/peak area of IS) were subjected to statistical analyses.

Table 2. Validation of the eicosanoid profiling method based on UPLC-MS/MS and the LOD and LOQ of each compound.

| Eicosanoids   | RT (min) | RSD (n = 6) (%) | Correlation (R²) | Linear Range (pg) | LOD (pg) | LOQ (pg) |
|---------------|----------|-----------------|------------------|-------------------|----------|----------|
|               |          |                  |                  |                   |          |          |
| PGE2-d4       | 8.06     | 0.25             | 2.85             | 0.9978            | 30–10,000| 3        |
| PGD2-d4       | 8.43     | 0                | 4.44             | 0.9982            | 30–10,000| 3        |
| LTB4-d4       | 12.77    | 0.20             | 5.39             | 0.9969            | 100–10,000| 60       | 100     |
| 14,15 EET-d11 | 17.70    | 0                | 6.08             | 0.9964            | 10,000–100,000| 3000 | 10,000 |
| 5(S)-HETE-d8  | 17.85    | 0                | 5.01             | 0.9972            | 30–10,000| 6        |
| AA-d8         | 21.53    | 0                | 7.84             | 0.9959            | 3–6000   | 0.1      | 3       |

UPLC-MS/MS, Ultra performance liquid chromatography coupled to tandem mass spectrometry; LOD, The limit of detection; LOQ, The limit of quantification; RT, Retention time; RSD, Relative standard deviation.
Table 3. Eicosanoids identified in RAW264.7 cells.

| No. | Compound Name                          | Abbreviation | MRM Transitions | DP  | CE  | RT (min) | IS          | Alteration by LPS Treatment |
|-----|----------------------------------------|--------------|-----------------|-----|-----|---------|-------------|-----------------------------|
| 1   | 12S-hydroxy-heptadecatrienoic acid     | 12-HHT       | 279 163         | −30 | −30 | 14.3    | 5(S)HETE-d8 | Up                          |
| 2   | 13-hydroxy-g-octadecatrienoic acid     | 13-HOTre-g   | 293 193         | −70 | −20 | 15.5    | 5(S)HETE-d8 | Up                          |
| 3   | 9-hydroxy-octadecadienoic acid         | 9-HODE       | 295 171         | −60 | −25 | 16.6    | 5(S)HETE-d8 | Up                          |
| 4   | 13-hydroxy-octadecadienoic acid        | 13-HODE      | 295 195         | −60 | −20 | 16.3    | 5(S)HETE-d8 | –                           |
| 5   | 9,10-hydroxy-octadecadienoic acid      | 9,10-diHOME  | 313 201         | −60 | −30 | 14.2    | 5(S)HETE-d8 | –                           |
| 6   | 12,13-hydroxy-octadecadienoic acid     | 12,13-diHOME | 313 183         | −60 | −30 | 13.5    | 5(S)HETE-d8 | –                           |
| 7   | 9-hydroxy-eicosapentaenoic acid        | 9-HEPE       | 317 149         | −75 | −20 | 15.7    | 5(S)HETE-d8 | Up                          |
| 8   | 13-hydroxy-eicosapentaenoic acid       | 13-HEPE      | 317 115         | −40 | −17 | 16.6    | 5(S)HETE-d8 | Down                       |
| 9   | 5-hydroxy-eicosapentaenoic acid        | 5-HEPE       | 317 219         | −60 | −20 | 15.5    | 5(S)HETE-d8 | Up                          |
| 10  | 15-hydroxy-eicosapentaenoic acid       | 15-HEPE      | 317 127         | −70 | −25 | 15.5    | 5(S)HETE-d8 | –                           |
| 11  | 11-hydroxy-eicosapentaenoic acid       | 11-HEPE      | 317 121         | −70 | −24 | 15.7    | 5(S)HETE-d8 | Up                          |
| 12  | 12-hydroxy-eicosapentaenoic acid       | 12-HEPE      | 317 179         | −70 | −20 | 15.9    | 5(S)HETE-d8 | –                           |
| 13  | 18-hydroxy-eicosapentaenoic acid       | 18-HEPE      | 317 215         | −50 | −15 | 16.7    | 5(S)HETE-d8 | Up                          |
| 14  | 11-hydroxy-eicosatetraenoic acid       | 11-HETE      | 319 167         | −60 | −20 | 17.1    | 5(S)HETE-d8 | Up                          |
| 15  | 9-hydroxy-eicosatetraenoic acid        | 9-HETE       | 319 151         | −60 | −20 | 17.0    | 5(S)HETE-d8 | Up                          |
| 16  | 5-hydroxy-eicosatetraenoic acid        | 5-HETE       | 319 115         | −60 | −20 | 17.9    | 5(S)HETE-d8 | Down                       |
| 17  | 8-hydroxy-eicosatetraenoic acid        | 8-HETE       | 319 155         | −60 | −20 | 17.3    | 5(S)HETE-d8 | –                           |
| 18  | 15-hydroxy-eicosatetraenoic acid       | 15-HETE      | 319 219         | −50 | −15 | 16.7    | 5(S)HETE-d8 | Up                          |
| 19  | 12-hydroxy-eicosatetraenoic acid       | 12-HETE      | 319 179         | −60 | −20 | 17.3    | 5(S)HETE-d8 | Up                          |
| 20  | 18-hydroxy-eicosatetraenoic acid       | 18-HETE      | 319 261         | −80 | −25 | 15.9    | 5(S)HETE-d8 | –                           |
| 21  | 17-hydroxy-eicosatetraenoic acid       | 17-HETE      | 319 247         | −80 | −25 | 15.9    | 5(S)HETE-d8 | –                           |
| 22  | 16-hydroxy-eicosatetraenoic acid       | 16-HETE      | 319 189         | −80 | −25 | 16.0    | 5(S)HETE-d8 | –                           |
| 23  | 5-hydroxy-eicosatetraenoic acid        | 5-HETE       | 321 115         | −70 | −19 | 19.1    | 5(S)HETE-d8 | Down                       |
| 24  | 15-hydroxy-eicosatetraenoic acid       | 15-HETE      | 321 221         | −70 | −21 | 17.4    | 5(S)HETE-d8 | Up                          |
| 25  | 5,6-dihydroxy-eicosatetraenoic acid    | 5,6-DHET     | 337 145         | −75 | −25 | 16.5    | 5(S)HETE-d8 | Down                       |
| 26  | 8,9-dihydroxy-eicosatetraenoic acid    | 8,9-DHET     | 337 127         | −60 | −30 | 15.5    | 5(S)HETE-d8 | –                           |
| 27  | 11,12-dihydroxy-eicosatetraenoic acid  | 11,12-DHET   | 337 167         | −60 | −25 | 15.8    | 5(S)HETE-d8 | Up                          |
| 28  | 8-hydroxy-docosahexaenoic acid         | 8-HDoHE      | 343 109         | −70 | −20 | 17.5    | 5(S)HETE-d8 | –                           |
| 29  | 7-hydroxy-docosahexaenoic acid         | 7-HDoHE      | 343 141         | −60 | −18 | 17.3    | 5(S)HETE-d8 | –                           |
| 30  | 4-hydroxy-docosahexaenoic acid         | 4-HDoHE      | 343 101         | −70 | −17 | 18.2    | 5(S)HETE-d8 | –                           |
| 31  | 10-hydroxy-docosahexaenoic acid        | 10-HDoHE     | 343 151         | −60 | −17 | 16.9    | 5(S)HETE-d8 | –                           |
| 32  | 11-hydroxy-docosahexaenoic acid        | 11-HDoHE     | 343 149         | −60 | −19 | 17.0    | 5(S)HETE-d8 | Up                          |
| 33  | 13-hydroxy-docosahexaenoic acid        | 13-HDoHE     | 343 221         | −60 | −17 | 16.7    | 5(S)HETE-d8 | Up                          |
| 34  | 16-hydroxy-docosahexaenoic acid        | 16-HDoHE     | 343 233         | −75 | −19 | 16.5    | 5(S)HETE-d8 | Up                          |
| 35  | 20-hydroxy-docosahexaenoic acid        | 20-HDoHE     | 343 241         | −60 | −20 | 16.3    | 5(S)HETE-d8 | –                           |
| 36  | 17-hydroxy-docosahexaenoic acid        | 17-HDoHE     | 343 245         | −60 | −20 | 16.5    | 5(S)HETE-d8 | Up                          |
| 37  | 14-hydroxy-docosahexaenoic acid        | 14-HDoHE     | 343 205         | −60 | −18 | 16.7    | 5(S)HETE-d8 | –                           |
| 38  | Arachidonic acid                      | AA           | 303 259         | −80 | −20 | 21.6    | AA-d8       | Down                       |
| 39  | Eicosapentaenoic acid                 | EPA          | 301 257         | −65 | −15 | 20.4    | AA-d8       | –                           |
Table 3. Cont.

| No. | Compound Name                              | Abbreviation | MRM Transitions | DP  | CE | RT (min) | IS | Alteration by LPS Treatment |
|-----|--------------------------------------------|--------------|-----------------|-----|----|----------|----|--------------------------|
| 40  | Adrenic acid                               | ADA          | Q1: 331 Q3: 287 | −80 | −20| 22.3     | AA-d8 | –                        |
| 41  | Dihexacosaenoic acid                       | DHA          | Q1: 327 Q3: 283 | −95 | −20| 21.3     | AA-d8 | –                        |
| 42  | 13-o xo-octadecadienoic acid               | 13-o xoODE | Q1: 293 Q3: 113 | −70 | −30| 16.6     | 14,15 EET-d11 | –          |
| 43  | 9,10-epoxy-octadecenoic acid               | 9,10-EPOME | Q1: 295 Q3: 171 | −60 | −25| 18.4     | 14,15 EET-d11 | –          |
| 44  | 12,13-epoxy-octadecenoic acid              | 12,13-EpOME | Q1: 295 Q3: 195 | −60 | −25| 18.1     | 14,15 EET-d11 | –          |
| 45  | 5,5-o xo-eicosatetraenoic acid             | 5-o xoEET | Q1: 317 Q3: 203 | −40 | −25| 18.3     | 14,15 EET-d11 | –          |
| 46  | 15-5-o xo-eicosatetraenoic acid            | 15-o xoEET | Q1: 317 Q3: 113 | −40 | −25| 16.7     | 14,15 EET-d11 | Down       |
| 47  | 11,12-epoxy-eicosatrienoic acid            | 11,12-EET | Q1: 319 Q3: 167 | −60 | −20| 18.6     | 14,15 EET-d11 | –          |
| 48  | 14,15-epoxy-eicosatrienoic acid            | 14,15-EET | Q1: 319 Q3: 113 | −60 | −20| 18.8     | 14,15 EET-d11 | Down       |
| 49  | 5,6-epoxy-eicosatrienoic acid              | 5,6-EET | Q1: 319 Q3: 191 | −30 | −20| 18.8     | 14,15 EET-d11 | Up          |
| 50  | 15-o xo-eicosadienoic acid                 | 15-o xoDE | Q1: 321 Q3: 113 | −100 | −32| 18.1     | 14,15 EET-d11 | Up          |
| 51  | Heparitin B1                               | HXB          | Q1: 335 Q3: 183 | −40 | −20| 15.5     | 14,15 EET-d11 | –          |
| 52  | 19,20-epoxy-Doconapentaenoic acid          | 19,20-EpOPE | Q1: 343 Q3: 241 | −60 | −20| 17.6     | 14,15 EET-d11 | –          |
| 53  | Lipoxin Bγ                                | LXBγ | Q1: 351 Q3: 221 | −80 | −25| 8.4      | LTBγ-d4  | Up          |
| 54  | 20-carboxy- Leukotriene B4                 | 20-coo LTB4 | Q1: 365 Q3: 195 | −60 | −25| 6.4      | LTBγ-d4  | –          |
| 55  | 15-deoxy-Prostaglandin A3 or 15-deoxy-Δ12,14-PGJ2 | 15d-PGJ2 or 15d-PGJ2 | Q1: 315 Q3: 271 | −50 | −15| 15.2     | PGE-d4  | Up          |
| 56  | Tetranor-Prostaglandin F Metabolite        | tetranor-PGF | Q1: 329 Q3: 293 | −40 | −25| 3.1      | PGE-d4  | –          |
| 57  | Prostaglandin A2 or Prostaglandin B2 or Prostaglandin J2 | PGA2 or PGB2 or PGJ2 | Q1: 333 Q3: 271 | −30 | −20| 10.5     | PGE-d4  | Up          |
| 58  | 15-deoxy-Δ12,14-PGJ2                      | 15d-PGJ2 | Q1: 333 Q3: 271 | −30 | −20| 12.5     | PGE-d4  | Up          |
| 59  | Prostaglandin D3                           | PGE3 | Q1: 349 Q3: 269 | −55 | −25| 7.4      | PGE-d4  | Up          |
| 60  | Prostaglandin E3                           | PGE3 | Q1: 349 Q3: 269 | −55 | −25| 7.1      | PGE-d4  | Up          |
| 61  | 15-keto-Prostaglandin E2                  | 15k PGE2 | Q1: 349 Q3: 113 | −35 | −30| 8.3      | PGE-d4  | –          |
| 62  | Prostaglandin K2                           | PGE2 | Q1: 349 Q3: 205 | −50 | −30| 8.3      | PGE-d4  | Up          |
| 63  | 15-keto-Prostaglandin F2                   | 15k PGE2 | Q1: 351 Q3: 113 | −40 | −35| 8.6      | PGE-d4  | Up          |
| 64  | Prostaglandin E2                           | PGE2 | Q1: 351 Q3: 271 | −50 | −25| 8.1      | PGE-d4  | Up          |
| 65  | Prostaglandin D2                           | PGE2 | Q1: 351 Q3: 271 | −50 | −25| 8.4      | PGE-d4  | Up          |
| 66  | 13,14-dihydro-15-keto Prostaglandin E2     | dhk PGE2 | Q1: 351 Q3: 207 | −40 | −25| 8.4      | PGE-d4  | Up          |
| 67  | 13,14-dihydro-15-keto Prostaglandin D2     | dhk PGE2 | Q1: 351 Q3: 207 | −40 | −25| 9.3      | PGE-d4  | Up          |
| 68  | Prostaglandin F2α                          | PGF2α | Q1: 353 Q3: 193 | −50 | −30| 8.5      | PGE-d4  | Up          |
| 69  | 15-keto-Prostaglandin F1α                  | 15k PGF1α | Q1: 353 Q3: 113 | −50 | −35| 3.1      | PGE-d4  | –          |
| 70  | 11β,13,14-dihydro-15-keto Prostaglandin F2α | 11β dhk PGF2α | Q1: 353 Q3: 113 | −50 | −35| 9.3      | PGE-d4  | Up          |
| 71  | Prostaglandin E1                           | PGE1 | Q1: 353 Q3: 273 | −55 | −25| 8.1      | PGE-d4  | Up          |
| 72  | Prostaglandin D1                           | PGE1 | Q1: 353 Q3: 273 | −55 | −25| 8.5      | PGE-d4  | Up          |
| 73  | Prostaglandin F1α                          | PGF1α | Q1: 355 Q3: 293 | −75 | −30| 8.2      | PGE-d4  | Up          |
| 74  | 13,14-dihydro-Prostaglandin F2α            | dh PGF2α | Q1: 355 Q3: 275 | −40 | −25| 8.9      | PGE-d4  | –          |
| 75  | Dihomo-Prostaglandin J2                   | Dihomo-PGJ2 | Q1: 361 Q3: 299 | −55 | −25| 13.0     | PGE-d4  | Up          |
| 76  | Dihomo-15-deoxy-Prostaglandin J2           | Dihomo-15d-PGJ2 | Q1: 361 Q3: 299 | −55 | −25| 14.4     | PGE-d4  | Up          |
| 77  | Thromboxane B1                            | TXB1 | Q1: 367 Q3: 169 | −50 | −25| 6.4      | PGE-d4  | –          |
| 78  | Dihomo-Prostaglandin F2α                  | Dihomo-PGF2α | Q1: 381 Q3: 221 | −75 | −35| 10.0     | PGE-d4  | Up          |
| 79  | Dihomo-Prostaglandin D2                   | Dihomo-PGD2 | Q1: 379 Q3: 299 | −65 | −30| 10.4     | PGE-d4  | Up          |
First, we applied principal component analysis (PCA) [33,34] to differentiate the RAW264.7 cells treated with various LPS concentrations. This resulted in effective separation in the corresponding PCA score plot (Figure 5). Each point represents an individual sample and the scatter of samples indicates similarities or differences in eicosanoid composition. Samples treated with 0, 1, and 10 ng/mL LPS were scattered on the lower side of the plot and those treated with 100 and 1000 ng/mL LPS were scattered on the upper side. The degree of inflammation increased with increasing LPS concentration. This demonstrated that eicosanoid contents differ depending on the degree of inflammation.

Second, changes in the levels of 79 eicosanoids can be described in a heatmap (Figure 6). This hierarchical clustering enabled effective differentiation of the five groups. Several of the 79 eicosanoid species were upregulated by treatments with 100 and 1000 ng/mL LPS. In contrast, the levels of other eicosanoids decreased with increasing LPS concentration. The levels of still other species were not correlated with LPS concentration. Therefore, up- and down-regulation of eicosanoids were associated with LPS-induced inflammation.

Figure 5. Principal component analysis (PCA) score plot of RAW264.7 cells treated with 0, 1, 10, 100, and 1000 ng/mL LPS.

Figure 6. Hierarchical clustering of 79 eicosanoid datasets from RAW264.7 cells treated with 0, 1, 10, 100, and 1000 ng/mL LPS.
3. Discussion

To evaluate altered eicosanoid metabolism in RAW264.7 cells, we focused on the pathway of eicosanoid synthesis from AA (Figure 7). Several PGs, such as PGD$_2$, PGE$_2$, and PGF$_{2\alpha}$, are synthesized from AA by cyclooxygenase (COX)-1 and COX-2. Furthermore, 15-HETE is synthesized from AA by 15-lipoxygenase (LOX). In addition, 12-LOX and 5-LOX also synthesize 12-HETE and 5-HETE, respectively, from AA. Our results showed that AA was down-regulated following treatment with high concentrations of LPS. This indicated that AA was used as the substrate to synthesize several eicosanoids. PGE$_2$, which plays a pro-inflammatory role [35,36], was upregulated following treatment with high concentrations of LPS. In contrast, two other PGs (PGE$_2$ and PGF$_{2\alpha}$) and 15-HETE were upregulated. In addition, 12-HETE was upregulated slightly and 5-HETE was down-regulated following treatments with high concentrations of LPS. Therefore, COX-1, COX-2, and 15-LOX were highly activated by LPS-induced inflammation, whereas 12-LOX was only slightly activated and 5-LOX was suppressed.

Figure 7. The eicosanoid synthesis pathway from arachidonic acid (AA) and eicosanoid levels in RAW264.7 cells as a function of LPS concentration (0, 1, 10, 100, and 1000 ng/mL). COX, cyclooxygenase; LOX, lipoxygenase; PG, prostaglandin; HETE, hydroxyeicosatetraenoic acid.

Many previous studies have reported the utility of various eicosanoid species as biomarkers for diseases and pathophysiological conditions [11,37–39]. For example, the endogenous levels of AA, PGE$_2$, and 12-HETE were significantly altered in cancerous mucosa [40]. This indicates that inflammation is correlated with tumorigenesis. Rago et al. [41] also reported the quantities of several eicosanoids in human plasma to develop biomarkers to distinguish three groups: healthy individuals, hypertensive patients, and severe atherosclerotic patients. The results showed that lower levels of 8-HETE, LTB$_4$, 9-HODE, and 13-HODE are potential biomarkers for severe heart disease. Eicosanoid metabolism may differ depending on biological samples and pathophysiological events. However, the detailed metabolism of major and minor eicosanoids in human diseases that involve inflammation has yet to be studied.

To characterize eicosanoid metabolism in inflammatory cells, comprehensive profiling of various eicosanoids is required. In this study, we evaluated changes in the levels of not only known eicosanoids related to inflammation but also other, less well-known species. The following 39 eicosanoids were upregulated following treatment with high concentrations of LPS: (1) eicosanoids derived from AA: 9-HETE, 11-HETE, 12-HETE, 15-HETE, 11,12-DHET, 12-HHT, PGA$_2$, PGD$_2$, PGE$_2$, PGF$_{2\alpha}$, dihomo PGD$_2$, dihomo PGF$_{2\alpha}$, dihomo PGJ$_2$, dihomo 15d PGJ$_2$, 15d-PGA$_2$, 15d-PGD$_2$, 11β dhk PGF$_{2\alpha}$, dhk PGD$_2$, dhk PGE$_2$, PGK$_2$, 15k PGF$_2$, and LXB$_4$; (2) eicosanoids derived from
linoleic acid: 9-HODE, and 13-HODE; (3) eicosanoids derived from eicosapentaenoic acid: 9-HEPE, 11-HEPE, 15-HEPE, 15-oxoEDF, PGD₂, and PGE₂; (4) eicosanoids derived from docohexaenoic acid: 11-HDoHE, 13-HDoHE, 16-HDoHE, and 17-HDoHE; (5) eicosanoids derived from gamma-linoleic acid: 13-HOTre-g; and (6) eicosanoids derived from dihomo-gamma-linoleic acid: 15-HETrE, PGD₁, PGE₁, and PGF₁α. The following seven eicosanoids were down-regulated following treatments with high concentrations of LPS: (1) eicosanoids derived from AA: 5-HETE, 5,6-DHET, 5,6-EET, and 11,12-EET; (2) eicosanoids derived from eicosapentaenoic acid: 5-HEPE; and (3) eicosanoids derived from dihomo-gamma-linoleic acid: 5-HETrE. Other eicosanoid species were not influenced by LPA treatment in a concentration-dependent manner.

In conclusion, a profiling method based on UPLC-MS/MS was used to characterize the effect of LPS treatment on eicosanoid metabolism in RAW264.7 cells. The degree of inflammation increased with increasing LPS concentration. A total of 79 eicosanoids were identified in RAW264.7 cells. PCA and heatmap generation were used to differentiate RAW264.7 cells treated with different concentrations of LPS. The five groups were well separated in the PCA score plot, and the heatmap was used to identify the up- or downregulation of eicosanoids according to LPS concentration. To our knowledge, this study is the first attempt to assess the levels of cellular eicosanoids altered by the degree of inflammation. A total of 39 eicosanoids were upregulated, and seven were down-regulated by LPS treatment in a concentration-dependent manner. Our novel UPLC-MS/MS technique has the potential for eicosanoid profiling and evaluating correlations between inflammation and eicosanoid metabolism. The eicosanoids up- or down-regulated by LPS can be applied as typical biomarkers for inflammation. In the future, the levels of inflammation-related eicosanoids in biological samples are needed to estimate their roles in cell function and pathophysiological events.

4. Materials and Methods

4.1. Reagents

HPLC-grade water, methanol, acetonitrile, and isopropyl alcohol were purchased from J.T. Baker (Avantor Performance Material, Inc., Center Valley, PA, USA). Acetic acid and ammonium acetate were obtained from Sigma-Aldrich (St. Louis, MO, USA). The eicosanoid standards were as follows: 14,15-EET-d₁₁, 5(S)HETE-d₈, LTB₄-d₄, PGE₂-d₄, PGD₂-d₄, and AA-d₈ (Cayman Chemical, Ann Arbor, MI, USA). A Strata-x 33-µm polymerized solid reverse-phase extraction column (cat # 8B-S100-UBJ) was purchased from Phenomenex (Torrance, CA, USA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from HyClone Laboratories Inc. (Logan, UT, USA). Escherichia coli LPS and Griess reagent were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

4.2. Cell Culture and LPS Treatment

Murine macrophage RAW264.7 cells (KCLB 40071; Korean Cell Line Bank, Seoul, Korea) were cultured in DMEM containing 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin in a humidified atmosphere with 5% CO₂ at 37 °C. Cells were seeded in a 100-mm Petri dish, cultured for 24 h, and incubated for a further 18 h after treatment with 100 µL LPS (0, 1, 10, 100, and 1000 ng/mL). Cell pellets were collected from each culture dish and the cells were enumerated using a hemocytometer. Finally, 1 × 10⁷ cells were used in the analyses.

4.3. Nitric Oxide (NO) and Enzyme-Linked Immunosorbent Assays (ELISAs) Analyses

NO concentrations in the culture supernatants were determined using a spectrophotometric assay based on the Griess reaction. A calibration curve was constructed using known concentrations (0–100 µM) of sodium nitrite. Levels of proinflammatory cytokines such as TNF-α and IL-6 were analyzed using commercial ELISA kits (BD Biosciences, San Diego, CA, USA) according to the manufacturer’s instructions.
4.4. Sample Preparation

A simple solid-phase extraction method was applied for the extraction of eicosanoids [25]. Cells were resuspended in 1 mL of 10% methanol in water (v/v) and sonicated for 5 min. Samples were then spiked with 10 ng of deuterated internal standards. Eicosanoids were extracted using Strata-x 33-µm polymerized solid reverse-phase extraction columns. Briefly, the columns were activated with 3.5 mL of 100% methanol followed by 3.5 mL of water for equilibration. After loading the samples, the columns were washed with 3.5 mL of 10% methanol in water to remove non-specific-binding metabolites. Eicosanoids were eluted into 1 mL methanol. The eluted samples were dried using a Speed-Vac concentrator (Labconco, Kansas City, MO, USA) and resuspended in 90 µL of Solvent A, as described below. The samples were stored at −80 °C until analysis.

4.5. UPLC-MS/MS Conditions

The UPLC analyses were performed on a Waters ACQUITY UPLC instrument (Waters Corp., Milford, MA, USA). The temperature of the column oven and autosampler were set at 40 and 4 °C, respectively. An Acquity BEH300 C18 column (2.1 × 150 mm ID; 1.7 µm; Waters Corp.) was used for the separation of eicosanoids. Solvent A consisted of water/acetonitrile/acetic acid (70:30:0.02; v/v/v) and solvent B of acetonitrile/isopropyl alcohol (50:50, v/v). The gradient elution program was as follows: 0–1 min, B 0%; 1–3 min, B 0%–25%; 3–11 min, B 25%–45%; 11–13 min, B 45%–60%; 13–18 min, B 60%–75%; 18–18.5 min, B 75%–90%; 18.5–20 min, B 90%; and 20–21 min, B 90%–0%. The column was equilibrated with 0% Solvent B for 4 min prior to analysis of the next sample. The total run time was 25 min for each analysis. The flow rate was 0.5 mL/min and the injection volume was 40 µL for each run.

For the MS analyses, an ABI/Sciex (Foster City, CA, USA) 5500 QTRAP hybrid, triple quadrupole, linear ion trap mass spectrometer equipped with a Turbo V ion source, together with the Analyst 1.5.1 software package (ABI/Sciex, Foster City, CA, USA), was used. Ultra-pure nitrogen gas was used as the collision gas for eicosanoids. The typical operating source conditions for the analysis of eicosanoids in negative ion ESI mode were optimized using deuterated standards as follows: curtain gas (CUR) = 10 psi, GS1 = 30 psi, GS2 = 30 psi, ionspray voltage (IS) = −4500 V, collision gas setting (CAD) = high, Temp = 525 °C, Ihe = on, entrance potential (EP) = −10 V, and collision cell exit potential (CXP) = −10 V.

4.6. Validation Study

External standard curves were established from the analysis of mixed eicosanoid standards at 16 different concentrations (0.1, 0.3, 0.6, 1, 3, 6, 10, 30, 60, 100, 300, 600, 1000, 3000, 6000, and 10,000 pg). In the case of 14,15 EET-d11, seven different concentrations (1000, 3000, 6000, 10,000, 30,000, 60,000, and 100,000 pg) were applied. The LOD, LOQ, linear ranges, and correlation (R²) values were determined by the external standard curve. The RSDs (%) of the relative RTs and the relative peak areas were estimated after six replicate analyses of 3000 pg of each standard.

4.7. Data Processing and Statistical Analysis

LC-MS data were obtained using the Analyst 1.5.1 software package (ABI/Sciex, Foster City, CA, USA). Eicosanoid peaks were assigned by comparisons of retention times with those of the internal standards. The Skyline software package (MacCoss Laboratory, University of Washington, Seattle, WA, USA) was used as an in-house database to determine the peak area of each assigned lipid from replicate raw data. The extracted peak areas of lipid peaks were normalized to the appropriate internal standard [42]. Hierarchical clustering of the quantified eicosanoids and PCA analyses was performed on the MetaboAnalyst web site [43].
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Abbreviations

LPS Lipopolysaccharide
UPLC Ultra performance liquid chromatography
MS Mass spectrometry
PG Prostaglandin
LT Leukotriene
ELISA Enzyme-linked immunosorbent assays
NO Nitric oxide
TNF-α Tumor necrosis factor-α
IL-6 Interleukin-6
MRM Multiple reaction monitoring
CE Collision energy
DP Declustering potential
RSD Relative standard deviation
LOD The limit of detection
LOQ The limit of quantification
IS Internal standard
PCA Principal component analysis
EDTA Ethylenediaminetetraacetic acid

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