Dioxin and related chemicals alter the expression of a number of genes by activating the aryl hydrocarbon receptors (AHR) to produce a variety of disorders including hepatotoxicity. However, it remains largely unknown how these changes in gene expression are linked to toxicity. To address this issue, we initially examined the effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a most toxic dioxin, on the hepatic and serum metabolome in male pubertal rats and found that TCDD causes many changes in the level of fatty acids, bile acids, amino acids, and their metabolites. Among these findings was the discovery that TCDD increases the content of leukotriene B4 (LTB4), an inducer of inflammation due to the activation of leukocytes, in the liver of rats and mice. Further analyses suggested that an increase in LTB4 comes from a dual mechanism consisting of an induction of arachidonate lipooxygenase-5, a rate-limiting enzyme in LTB4 synthesis, and the down-regulation of LTC4 synthase, an enzyme that converts LTA4 to LTC4. The above observations focusing on metabolomic changes provide novel evidence that TCDD accumulates LTB4 in the liver by an AHR-dependent induction of LTB4 biosynthesis to cause hepatotoxicity through neutrophil infiltration. Dioxin and related compounds are a class of pollutants that are spread widely throughout the environment (1) and have a number of toxic effects on living organisms. For example, treating laboratory animals with dioxins causes many forms of toxicity such as wasting syndrome, hepatotoxicity, and immunosuppression. These disorders caused by dioxins are of serious concern because of their persistence in humans as well as wildlife owing to their high lipophilicity and resistance to metabolic biotransformation (1, 2). Several cases involving human exposure to dioxins, such as the Yusho, Seveso, and Agent Orange incidents, have occurred in the past, and many of the victims of these incidents still suffer from chronic symptoms (3–5). Early studies by Nebert et al. (6) suggest that a genetic factor contributes to the induction of mouse aryl hydrocarbon hydroxylase by dioxin-like substances. Furthermore, Poland et al. (7) characterize this factor as the aryl hydrocarbon receptor (AHR), which exhibits a high affinity for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the most toxic dioxin, as well as related substances. These pioneer studies and subsequent investigations have convinced researchers that dioxin produces many toxic effects by activating the AHR (8–11). Upon activation by binding to dioxins, AHR migrates to the nucleus and
then associates with its cognitive sequence, the xenobiotic responsive element (XRE), present in the 5′-upstream region of the target genes to change their expression (12). More than 200 genes are governed by AHR and probably are synergistically associated with toxicity (12). However, the gene, or genes, that make the major contribution to toxicity has not yet been fully identified and understood.

Alternative approaches using metabolomics, a comprehensive analysis of cellular components, have been carried out to address the above issue. For instance, dioxins change the levels of a number of cellular components in the liver and serum of mice and rats (13–15). One of these reports provides novel evidence that TCDD reduces the circulating level of azelaic acid by attenuating the expression of carboxylesterase 3 to cause steatohepatitis (15). Similarly, our previous metabolomic investigations have suggested that maternal treatment with TCDD disturbs the tricarboxylic acid (TCA) cycle in the fetal hypothalamus, resulting in the sexual immaturity of the pups as they develop (16). In relation to these findings, AHR is suggested to play a role in energy balance through a change in the levels of nutrition-related factors (17). Considering all these findings, it is expected that subsequent studies based on metabolic changes will lead to a better understanding of the mechanism of dioxin toxicity. However, such an approach has not yet provided sufficient information. With this in mind, the present study initially examined the effect of TCDD on the hepatic and serum metabolome in male pubertal rats, further expanding the investigation by focusing on an eicosanoid, which has been suggested by metabolomics to be a target candidate for toxicity. More specifically, using metabolomics and an ultra-performance liquid-chromatography (UPLC)-TOF-MS system, we found that TCDD increases the hepatic content of leukotriene B4 (LTB4), an arachidonic acid metabolite produced by the lipoxygenase pathway. LTB4, a representative chemoattractant of neutrophils, activates this type of cell upon binding to its own receptors (BLT1) to evoke an inflammatory response via the release of chemokines and cytokines such as TNF (18, 19).

Dioxin accumulates leukotriene B4 in hepatocytes

![Image](https://via.placeholder.com/150)

Part of the change in hepatic components, including leukotrienes, was also observed in the serum (supplemental Fig. 1 and supplemental Table 2). The determination of LTB4 confirmed that TCDD caused about a 1.6-fold increase in this component in the liver (Fig. 1, G and H). It should be noted that there is a discrepancy in the magnification of the LT4 levels increase by TCDD between the determination and metabolomics data demonstrating an elevation of more than 20-fold. As seen in Fig. 1G, UPLC-TOF-MS analysis in a selected ion-monitoring mode focusing on the molecular weight of LTB4 (m/z 335.2228) showed the presence of another isomer the retention time of which was a little earlier than LTB4. From its mass and consistent chromatographic behavior with the authentic specimen, this isomer was identified as 17,18-dihydroxyeicosatetraenoic acid (17,18-DETA). This isomer appears to be produced by several cytochrome P450s (CYPs) including TCDD-inducible CYP1A1 and -1A2 followed by epoxide-hydrolase-mediated hydrolysis (26, 27). Although 17,18-DETA was increased more than 4-fold by TCDD, metabolomics analysis could not distinguish it from an increase in LTB4. This is one of the reasons for the large gap between the precise determination and metabolomic data described above. The toxicological significance of an increase in 17,18-DETA by TCDD will be discussed below. These results suggest that a marked change in the metabolome occurs in the liver through mechanisms distinct from a reduction in food intake and that accumulation of LTB4 contributes to TCDD-produced acute toxicity.
Figure 1. A change in the metabolomic profile and LTB4 content in the liver produced by TCDD and food restriction. A–F, male pubertal rats were exposed to either TCDD (60 μg/kg per os) or vehicle alone (each n = 12), and their metabolomic profiles in the liver were analyzed by UPLC-TOF-MS. The data obtained were further analyzed by a principal component analysis. A and B show the Score plots, which indicate similarity or dissimilarity in the profile among the compared groups; and C–F show the S-plots, with information regarding mass fragment ions of cellular components the levels of which were altered by treatments. In the experiments for the Score plot, control, pair-fed-control, and TCDD groups were obtained in negative (A) and positive (B) ion modes. Similarly, the S-plot (C, control versus TCDD; E, control versus pair-fed control) was analyzed under TOF-MS conditions in negative ion mode, and the experiments shown in D (control versus TCDD) and F (control versus pair-fed control) were performed in positive ion mode. G and H, selected ion monitoring at m/z 335.2228 for the determination of the hepatic content of LTB4. The retention time for LTB4 and 17,18-DETA was 13.5 and 13.1 min, respectively. Each bar represents the mean ± S.E. of 6 rats. #, significantly different from the control (p < 0.001).
Mechanism underlying a TCDD-produced increase in hepatic LTB4

To clarify the mechanism whereby TCDD causes an accumulation of LTB4 in the liver, we examined the hepatic expression of mRNAs coding for LTB4-synthesizing and -metabolizing enzymes. TCDD increased the mRNA level of arachidonate lipoxygenase-5 (ALOX5), which mediates the conversion of arachidonic acid to LTA4, a precursor of LTB4 (Fig. 2A). On the other hand, LTC4 synthase, which catalyzes conversion of LTA4 to another metabolite, LTC4, was reduced following TCDD treatment (Fig. 2A). Although pair-feeding did not influence ALOX5 mRNA, this treatment markedly reduced LTC4 synthase mRNA in the same manner as TCDD treatment (Fig. 2A). The mRNAs coding for cytosolic phospholipase A2, LTA4 hydrolase, and CYP4F1 were all insensitive to both TCDD and pair-feeding treatments (Fig. 2A). A TCDD-produced change in the expression of ALOX5 and LTC4 synthase mRNAs occurred in a dose-dependent manner, and the dose required was comparable with that for the induction of CYP1A1, one of the genes most sensitive toward TCDD (Fig. 2B). The dose of TCDD needed for these changes was markedly lower than that for the increased expression of prostaglandin-endoperoxide

Figure 2. Effect of TCDD and food restriction on the expression of hepatic enzymes involved in arachidonic acid utilization, LTB4 synthesis/inactivation, and related enzymes. Male pubertal rats were exposed to either TCDD (1–60 μg/kg po) or vehicle alone, and their livers were collected 7 days after treatment. A and B, the relative levels of mRNAs indicated were analyzed by real-time RT-PCR and normalized by β-actin mRNA. C and D, immunoblotting for ALOX5 and LTC4 synthase. The representative blotting image for each group is shown at a position above the bar graph. The expression levels of cytosolic ALOX5 and microsomal LTC4 synthase were normalized by β-actin and β-tubulin, respectively. Each bar represents the mean ± S.E. of 4–6 rats. Statistical significance in A and B–D was determined by one-way analysis of variance with Tukey’s test (**, p < 0.01) and Williams’s test (*, p < 0.05), respectively. Cont, control; Pair, pair-fed control.
**Discussion**

Although the synthesis and degradation of eicosanoids, which are governed by AHR and other nuclear receptors, probably provoke a number of beneficial and malignant responses, it is still largely unknown how the downstream mechanisms and their interplay are linked to the outcome (31). The present study provides novel evidence that TCDD increases the hepatic content of LTB4 through the simultaneous mechanisms of ALOX5 induction and LTC4 synthase down-regulation in an AHR-dependent manner (Fig. 6). In agreement with the evidence that LTB4 activates neutrophils to facilitate an inflammatory response, including the release of cytokines (18), TCDD causes infiltration of neutrophils into the liver and induces the hepatic expression of TNF and PTGS2; the deletion of the BLT1 gene blocked these effects. In addition, an increase by TCDD in ALT and AST was suppressed by knocking out BLT1 (Fig. 5E). In support of this, co-treatment with zileuton, an ALOX5 inhibitor, of TCDD-exposed rats also restored an induction of both PTGS2 mRNA and ALT activity without affecting the expression of CYP1A1 mRNA (supplemental Fig. 6). These results demonstrate that neutrophil infiltration due to an increase in LTB4 is one of the causal factors for TCDD-produced hepatotoxicity.

**Role of an increase in LTB4 in TCDD-induced hepatotoxicity**

If neutrophil infiltration caused by LTB4 contributes significantly to TCDD-induced hepatotoxicity, blocking the infiltration should reverse or alleviate the disorder. To examine this hypothesis, we conducted an experiment using mice lacking BLT1, which is expressed on the surface of neutrophils (29). As expected, although BLT1-KO mice (30) did not express any BLT1 (Fig. 5A), induction of CYP1A1 and ALOX5 by TCDD was observed both in WT and BLT1-KO mice (Fig. 5, A and B). In accordance with neutrophil infiltration into the liver, TCDD concomitantly increased the hepatic level of BLT1 mRNA in T-mice (Fig. 5A). The same was not observed in BLT1-KO mice (Fig. 5A). Even deleting the BLT1 gene failed to restore body-weight loss, hepatomegaly, and thymic atrophy caused by TCDD (supplemental Fig. 4). However, the TCDD-induced neutrophil infiltration in the liver of WT mice was completely abolished by knocking out the BLT1 gene (Fig. 5, B and C). The localization of macrophages in the liver remained unchanged even following BLT1-KO and TCDD treatments (supplemental Fig. 5). In agreement with these observations, although TCDD induced the hepatic expression of the inflammatory markers TNF and PTGS2, these changes were also reduced in BLT1-KO mice (Fig. 5D). In addition, TCDD-produced induction of all anticardinase transferase (ALT) and aspartate aminotransferase (AST), serum markers of hepatotoxicity, was suppressed by knocking out BLT1 (Fig. 5E).

**Cells responding to TCDD to induce 5-lipoxygenase: role of hepatocytes other than infiltrated leukocytes and macrophages including Kupffer cells**

To identify the cells which respond to stimulation by TCDD to release LTB4, we analyzed the localization of ALOX5 in the liver. In agreement with the role of LTB4 in neutrophil activation, TCDD dramatically increased the fluorescent foci caused by ly-6g, a neutrophil marker, as well as ALOX5 in the liver (Fig. 4, A and B). However, the ALOX5-positive foci increased by TCDD were localized in a different area from that where the ly-6g signal was observed (Fig. 4A and supplemental Table 3). In addition, neither the expression of CD68, a macrophage marker, nor the percentage of its co-localization with ALOX5 was affected by TCDD treatment (Fig. 4, C and D, and supplemental Table 3). Treating primary hepatocytes prepared from control male pubertal rats with TCDD induced mRNAs coding for ALOX5 as well as CYP1A1 in a dose-dependent manner (Fig. 4E). These results suggest that TCDD acts directly on hepatocytes, rather than neutrophils and macrophages, to induce the expression of ALOX5, resulting in the infiltration of neutrophils into the liver due to the increased release of a drawing factor, LTB4.

**Role of AHR in the TCDD-produced accumulation of LTB4 in the liver**

To discover whether AHR contributes to the alteration in the level of LT-synthesizing enzymes and LTB4 content, we generated rats lacking AHR (AHR-KO rats) by deleting a part of the AHR gene (Fig. 3A). As shown in Fig. 3B, the rats we produced did not express any AHR. The body weight loss, hepatomegaly, and thymic atrophy caused by exposing WT rats to TCDD disappeared by knocking out the AHR gene (Fig. 3, C and D). In addition, neither an increase in the expression of ALOX5 and CYP1A1 nor a decrease in LTC4 synthase expression was observed in AHR-KO rats (Fig. 3E). In accordance with this finding, the hepatic level of LTB4 in AHR-KO rats remained unchanged even after TCDD treatment (Fig. 3F). An increase in the ALOX5 mRNA and LTB4 content in the liver was also observed in mice (supplemental Fig. 3). The above changes occurred after fewer doses in C57BL/6 mice, a strain that has Ahr with a high affinity for ligands, than in DBA/2J mice that express low-affinity Ahr (supplemental Fig. 3A). These results demonstrate that TCDD facilitates LTB4 synthesis in an AHR-dependent fashion, and also in mice, TCDD causes an accumulation of LTB4 in the liver through the induction of its biosynthesis governed by AHR.

**Cells responding to TCDD to induce 5-lipoxygenase: role of hepatocytes other than infiltrated leukocytes and macrophages including Kupffer cells**

To identify the cells which respond to stimulation by TCDD to release LTB4, we analyzed the localization of ALOX5 in the liver. In agreement with the role of LTB4 in neutrophil activation, TCDD dramatically increased the fluorescent foci caused by ly-6g, a neutrophil marker, as well as ALOX5 in the liver (Fig. 4, A and B). However, the ALOX5-positive foci increased by TCDD were localized in a different area from that where the ly-6g signal was observed (Fig. 4A and supplemental Table 3). In addition, neither the expression of CD68, a macrophage marker, nor the percentage of its co-localization with ALOX5 was affected by TCDD treatment (Fig. 4, C and D, and supplemental Table 3). Treating primary hepatocytes prepared from control male pubertal rats with TCDD induced mRNAs coding for ALOX5 as well as CYP1A1 in a dose-dependent manner (Fig. 4E). These results suggest that TCDD acts directly on hepatocytes, rather than neutrophils and macrophages, to induce the expression of ALOX5, resulting in the infiltration of neutrophils into the liver due to the increased release of a drawing factor, LTB4.
Therefore, it should be noted that prostaglandins and/or thromboxanes produced via the phospholipase A2-PTGS2 pathway may play a substantial role in the cellular toxicity of TCDD (32). We confirmed that the TCDD dose needed to induce PTGS2 mRNA is more than 20 μg/kg. However, a significant change in the expression of ALOX5 and LTC4 synthase mRNAs was caused by TCDD starting at a dose of 5 μg/kg. Thus, the accumulation of LTB4 appears to occur after distinctly lower doses of TCDD compared with PTGS2 induction. Furthermore, knocking out the BLT1 gene drastically sup-
pressed the hepatic induction of PTGS2 produced by TCDD. Based on these observations, it seems likely that TCDD initially draws neutrophils into the hepatocytes by facilitating the ALOX5/LTB4 pathway, and the increased inflammation in the tissues then activates PTGS2 and its downstream cascade. These different pathways for inflammation are thought to act in concert to cause hepatotoxicity and/or exacerbate it, although the ALOX5/LTB4 pathway plays a more important role.

ALOX5 catalyzes a two-step reaction from arachidonic acid to LTA4 via 5-hydroperoxyeicosatetraenoate (5-HpETE) (33). Because 5-HpETE is also converted to 5-hydroxyeicosatetraenoate (5-HETE) by glutathione peroxidase (34), the regulation of the ALOX pathway leads to parallel alterations in 5-HETE and LTB4 biosynthesis. However, unlike with LTB4, our metabolomics suggested that TCDD attenuates the level of 5-HETE in the liver (supplemental Table 1). Such inconsistency can be explained by the effect of TCDD on reductive activity, because we confirmed that TCDD strongly suppresses the conversion from 5-HpETE to 5-HETE in the cytosol of the liver (supplemental Fig. 7). Taken together, it is suggested that an anti-parallel alteration between 5-HETE and LTB4 biosynthesis comes from the TCDD-produced inhibition of the reductive activity for 5-HpETE. In addition to inducting ALOX5, down-regulating the reductive capacity for 5-HpETE also may contribute to an up-regulation of LTB4 biosynthesis.

Figure 4. Effects of TCDD on the accumulation and co-localization of ALOX5 and neutrophils/macrophages in liver sections prepared from male pubertal rats and TCDD-induced expression of ALOX5 mRNA in primary hepatocytes. A, shows the hepatic section stained simultaneously with antibodies against ALOX5 (red) and ly-6g (green), a neutrophil marker. Likewise, in C, the results of the double staining of ALOX5 (red) and CD68 (green), a macrophage marker, are shown. B and D, the antibody-positive foci were counted using ZEN software (Carl Zeiss) (B) and were calculated as the ratio relative to the control (D). Each bar represents the mean ± S.E. of 4 rats. NS, not significant. E, the relative levels of CYP1A1 and ALOX5 mRNAs in primary cultured hepatocytes were analyzed by real-time RT-PCR. Each bar represents the mean ± S.E. of 4 – 6 cultures. *, p < 0.05; #, p < 0.001.

Dioxin accumulates leukotriene B4 in hepatocytes
Although the increased production of LTB4 takes place in leukocytes and other cells including macrophages (35), little co-localization was observed between ALOX5 and neutrophils/macrophages in the liver sections from TCDD-treated as well as untreated rats. However, a TCDD dose-dependent induction of ALOX5 mRNA was reproduced in primary cultured rat hepatocytes. Based on these observations, it is strongly suggested that the place where TCDD initially increases LTB4 production to attract neutrophils is in the hepatocytes. In this context, it has been reported that neutrophil granulocytes lack AHR expression (36). Although monocytes including Kupffer cells modestly express the AHR (36, 37), TCDD-dependent induction of the expression of CYP1A1 in these cells is far less than found in hepatocytes (37). Taken together, it is conceivable that AHR-dependent regulation of gene expression is more sensitive in hepatocytes than inflammatory cells because of the higher expression of the AHR. These pieces of evidences support the possibility that TCDD preferably targets the expression of ALOX5 in hepatocytes rather than inflammatory cells through AHR activation to increase the production of LTB4.

In contrast to neutrophils, treatment with TCDD and knocking out BLT1 scarcely affected the localization of macrophages. Therefore, TCDD seems not to influence macrophages to produce inflammatory disorders in the liver. However, it should be noted that macrophages may play a central role in chronic inflammation, whereas neutrophils are mobilized for the acute

Figure 5. Suppression by knocking out BLT1 on TCDD-induced hepatotoxicity. A, the relative levels of mRNA indicated were analyzed by real-time RT-PCR. ND, not detectable. B and C, immunofluorescent staining of ALOX5 and ly-6g. Hepatic section was stained by anti-ALOX5 (red), anti-ly-6g (green), and 4′,6′-diamino-2-phenylindole (blue). In C, antibody-positive foci were counted, and the data obtained were processed to obtain bar graphs. D, the relative levels of TNF and PTGS2 mRNA were analyzed by real-time RT-PCR. E, the serum activity of ALT and AST was measured using a commercial kit. Each bar represents the mean ± S.E. of 3–5 mice. Significant differences between the pairs are indicated: *, p < 0.05; **, p < 0.01; and #, p < 0.001.
Dioxin accumulates leukotriene B4 in hepatocytes

**Figure 6. A proposed mechanism underlying dioxin-produced hepatotoxicity to show the role of LTB4 accumulation in the liver.** TCDD induces the expression of ALOX5 in hepatocytes via the activation of AHR to produce the accumulation of LTB4 in the liver. It causes neutrophil infiltration from the blood vessel into the liver and facilitates inflammation by releasing inflammatory cytokines including TNF. Thus, LTB4 accumulation causes and/or aggravates acute hepatotoxicity. AA, arachidonic acid; cPLA2, cytosolic phospholipase A2; PG, prostaglandin; TX, thromboxane; LTA4H, LTA4 hydrolase; LTC4H, LTC4 synthase.

effects of inflammation (38). Indeed, it has been suggested that LTB4 induces the expression of monocyte chemoattractant protein-1 to differentiate monocytes to macrophages, leading to the gathering of macrophages at inflammatory sites (18). This LTB4 effect is believed to produce some chronic diseases associated with inflammation (39). In this context, it has been reported that TCDD activates TNF/IL-1 signaling to cause chronic hepatotoxicity including liver tumor (23, 24). Because the present study focused on the LTB4 targets linked to the acute toxicity of dioxins, it remains to be seen whether macrophages are actually involved in chronic inflammation-related disorders produced by dioxins. In addition to hepatotoxicity (40), there are a number of dioxin-induced chronic disorders, such as cardiovascular diseases and arteriosclerosis, which are assumed to arise from increased inflammation especially when caused by an increase in cytokines and chemokines (41). Thus, for a better understanding of dioxin-produced chronic disorders, further research focusing on the activation of macrophages is urgently required.

Although restricting food intake attenuated the hepatic expression of LTC4 synthase, ALOX5 was insensitive to this treatment. Accordingly, our metabolomic analysis indicated that a modest increase and decrease in LTB4 and LTC4, respectively, was observed in the pair-fed group. Previous reports have demonstrated that pair-feeding alone fails to cause any induction of ALT, although body weight loss occurs by restricting food intake (42). The knocking out of LTC4 synthase does not produce any significant increase in the circulation of LTB4 (43). It is, therefore, reasonable to consider that the AHR-dependent induction of ALOX5 makes a greater contribution to TCDD-produced hepatotoxicity than the reduced expression of LTC4 synthase. In agreement with this, co-treatment of zileuton, an ALOX5 inhibitor, blocked the TCDD-produced induction of PTGS2 mRNA and ALT activity. Because there are several core motifs of XRE in the 5’-upstream region of ALOX5 and its intronic regions, a TCDD-AHR complex is possibly associated directly with these sites to induce the expression. Alternatively, TGFβ and calcitriol, regulators of ALOX5 expression (44), may be involved in its induction, because TCDD induces the expression of both TGFβ (15) and calcitriol (45). More studies are needed to obtain a complete understanding of the molecular mechanism for LTB4 accumulation caused by TCDD-AHR.

Although the TCDD-induced infiltration of neutrophils was abolished by knocking out BLT1, this treatment could not completely reverse TCDD-produced inflammation and hepatic damage. For this reason, other factors having a role distinct from the BLT1-linked pathway are suggested to contribute to dioxin-produced hepatotoxicity. Regarding this possibility, BLT2, a low-affinity receptor of LTB4 (39, 46), activates NF-κB, an inflammation-inducing factor, through an increase in reactive oxygen species in various tissues including the liver (47). Thus, it is conceivable that LTB4 binds to this receptor as well as BLT1 to increase the inflammatory response. As expected, regarding the dioxin-produced increase in active oxygen species, a number of reports have demonstrated it in various tissues including the liver (48). Our metabolomic analysis indicated that TCDD increased the hepatic level of oleic acid, and free fatty acids including oleic acid are reported to activate NF-κB signaling by reducing the stability of lysosomes (49). Thus, more studies from the above viewpoint will also be necessary to clarify other possible mechanisms in terms of hepatic disorders caused by dioxins.

Selected ion monitoring for LTB4 in UPLC-TOF-MS analysis revealed that TCDD increases not only LTB4 but also another isomer, 17,18-DETA (see Fig. 1G), whereas metabolomics could not distinguish them. In addition, fine determination did not support an extensive increase in the LTB4 content as predicted by metabolomics. Such discrepancy seems to reflect the following technical limitations of comprehensive metabolomic analysis. 1) All of these changes are predicted by referring only the retention time and mass information of the detected ions to online databases; and 2) most metabolomic analyses put a high value on detecting a greater number of metabolites without considering extraction efficiency. Therefore, we should be aware that, until a fine determination has been conducted, we cannot conclude an alteration in the level of metabolites using only the metabolomic data. Our precise determination discovered the overlap between LTB4 and 17,18-DETA, supporting the above-mentioned issues. Alternatively, we used Tris-HCl and methanol to extract as many metabolites as possible. However, it is likely that the preferred method is to extract hydrophilic compounds rather than arachidonic metabolites including LTB4. In addition to the overlap with 17,18-DETA, the lower extraction efficiency in metabolomics may possibly also contribute to the estimation of much greater changes in the level of LTB4. In agreement with this conclusion, among the raw metabolomic data gained from 12 control rats, the predicted LTB4 peaks could be detected in only three of them.

17,18-DETA is a major metabolite of eicosapentaenoic acid, and as described previously, several CYPs including TCDD-inducible CYP1A1/2 are known to catalyze this reaction (26,
In agreement with this result, the increase in 17,18-DETA formation caused by TCDD disappeared in AHR-KO rats. It has been shown that 17,18-DETA may be used as a biomarker for Alzheimer’s disease (50). In this regard, a cohort study concerning the Agent Orange episode has suggested that the exposure to dioxin that occurred several decades ago is associated with neurotoxic disorders including Alzheimer’s disease (4). Thus, studies focusing on eicosanoids such as 17,18-DETA could help us better understand dioxin-produced neurotoxicity. 17,18-Epoxyeicosatetraenoic acid, a precursor of 17,18-DETA, is reported to have potential as a potassium-channel activator and a calcium-sensitivity modifier (51, 52). Based on these mechanisms, 17,18-epoxyeicosatetraenoic acid seems to affect the function of the pulmonary arteries, airway smooth muscle, and cardiovascular system including the contractile apparatus (51, 52). In addition, 17,18-DETA inhibits the TNF-dependent inflammatory response in the lung (53). In this context, other eicosapentaenoic acid metabolites, such as resolvins E1, are possible to bind weakly to BLT1 to antagonize the effect of LTB4 in leukocytes, resulting in the suppression of inflammation (54).

As far as we know, there is no information available about the effect of 17,18-DETA or its precursor epoxy derivative on the liver. However, alternative approaches focusing on their beneficial effects may contribute to novel therapeutics for LTB4-induced tissue damage.

In summary, this study focusing on metabolomic changes provides the novel hypothesis that the accumulation of LTB4 through an AHR-dependent increase in its biosynthesis is a mechanism whereby TCDD causes acute hepatotoxicity. In a similar manner, this could be linked not only to hepatotoxicity but also to damage to other tissues. In fact, the present study found that the induction of ALOX5 produced by dioxin occurs in the thymus, spleen, and kidney. Further research focusing on LTB4-produced inflammation and other changes in metabolome are needed to clarify dioxin-produced disorders. Our findings have led us to propose that subsequent studies based on cellular metabolomic changes will be useful for obtaining a better understanding of the molecular mechanisms underlying the biological responses and disorders generated by endogenous/exogenous factors.

**Experimental procedures**

**Materials**

TCDD was purchased from AccuStandard Inc. (New Haven, CT). Authentic standards of LTB4, 5-HETE, 5-HpETE, and 17,18-DETA were obtained from Cayman Chemical (Ann Arbor, MI). Rabbit polyclonal antibodies against rat ALOX5 and LTC4 synthase were obtained from Cayman Chemical and Arbor, MI). Rabbit polyclonal antibodies against rat ALOX5 were obtained from Cayman Chemical (Ann Arbor, MI). Authentic standards of LTB4, 5-HETE, 5-HpETE, and DIOXIN were purchased from Cell Signaling Technology (Danvers, MA). The other reagents were of the best quality available commercially.

**Generation of AHR-KO rats**

Heterozygous rats carrying a defective AHR gene were produced by Transposagen Biopharmaceuticals (Lexington, KY) with Kud:WI rats as the background strain using XTNTM TALE nuclease (TALEN) technology (Transposagen Biopharmaceuticals). This methodology was designed to delete part of exon 2 of the AHR gene. The targeted exon 2, which encodes a DNA-binding motif with a basic helix-loop-helix structure, is also used in other models of AHR-null animals generated previously in mice (9) and Sprague-Dawley rats (11). The sequence for gene modification was set at 5'-TTCTAAACGACACAGAG-ACCGGGCTGAACACAGAGTTAGAAGCCGCTGCTA-3'. The XTNTM TALEN mRNA, which contained the above sequence and spanned a region 130–178 bp from the initiation codon of AHR mRNA, was transcribed, capped, and tailed with poly-A. After purification, this preparation was microinjected at a dose of 10 ng/μl into the pronuclei of a total of 290 embryos. Of these, 168 were implanted into the dams of pseudo-pregnant rats. A total of 25 pups were born, and their genomic DNA, extracted from the ear notches, was used as the template to diagnose the presence of the modified sequence in the second exon by PCR. The mutant alleles of AHR were detected in seven pups, and each DNA sample extracted was then sequenced to confirm that there was no mutation other than the second exon. Deletion of the same 17 bp (del-17) was observed in five of the seven animals, and the two different alleles were identified as del-5- and del-2-type mutations. Founder animals (F0) carrying the del-17 allele were back-crossed with Kud:WI rats to generate F1 heterozygotes. Litters born from AHR heterozygous dams were examined for their genotypes according to the method reported previously (55). The primer sequences used for genotyping were as follows: forward, 5'-CAAACCCTTCTTAAACGACACAGAG-3'; and reverse, 5'-CCTTAGAACGGAGATTGTCCAG-3'.

**Animals and treatments**

All animal experiments were preapproved by the Institutional Animal Care and Experiment Committee of Kyushu University. Male Kud:WI rats (4 weeks old) were purchased from Kyudo Co. Ltd. (Tosu, Japan). Male C57BL/6J mice and DBA/2J mice (both 4 weeks old) were obtained from CLEA Japan, Inc. (Tokyo). The details for the generation of BLT1-KO mice used in this study have been described elsewhere (30). For the metabolomic analyses, the 4-week-old male Kud:WI rats were housed individually in metabolism cages and treated with TCDD at a single oral dose of 60 μg/kg/2 ml of corn oil. Pair-fed control rats were prepared and treated according to our current report (56), i.e. in this group, rats were restricted in the amount of food they had to the level consumed by the TCDD-treated group, whereas the control group was allowed free access to food. Blood and tissue samples were collected 7 days after TCDD treatment for metabolomics using a UPLC-TOF-MS method. In other experiments, 2–4 animals were kept in the same cage and treated with the following doses of TCDD: rats,
**Dioxin accumulates leukotriene B4 in hepatocytes**

1, 5, 10, 20, or 60 μg/kg; C57BL/6J and BLT1-KO mice, 1, 5, 10, 20, 50 or 100 μg/kg; and DBA/2J mice, 5, 10, 20, 100, 500 or 1000 μg/kg. To examine the effect of ALOX5 inhibition, the rats exposed to TCDD (60 μg/kg) were co-treated orally with zileuton, an ALOX5 inhibitor. Based on reports that the dose of 40 mg/kg zileuton suppresses LTB4 production (57), we set the dose to 10 or 50 mg/kg/day. Tissues were removed from these animals after 7 days of treatment.

**Metabolomics**

The liver was homogenized in 4 volumes of 50 mM Tris-HCl (pH 7.4), which was centrifuged at 2000 × g for 10 min. The supernatant or serum (each 30 μl) obtained was mixed vigorously for 5 min with 3 volumes of methanol. After centrifugation at 1000 × g for 10 min, the pellet was again extracted with 3 volumes of methanol. Both supernatants were mixed, and the solvent was evaporated using a vacuum concentrator (CVE-3100, Tokyo Rikakikai Co., Ltd., Tokyo). The extract obtained was dissolved in 10% methanol at a concentration of 2.5 μl/mg of liver or 0.5 μl/μl of serum. A portion (10 μl) of these samples was subjected to UPLC-TOF-MS (LCT-Premier XE, Waters, Milford, MA) for metabolomic analysis. The operating conditions for UPLC and TOF-MS and the procedures for multivariate analysis were selected according to the methods described elsewhere (16, 56).

**Determination of LTB4**

The content of hepatic LTB4 was determined by UPLC-TOF-MS using the methods reported previously (58) with some modifications. Liver (0.85 g) was homogenized with 4 volumes (relative to the tissue weight) of 1 mM HCl immediately after removal and mixed with 3 volumes of cold methanol containing 0.1% (w/v) butylated hydroxyanisole and 0.1% (w/v) acetic acid. After centrifugation at 2000 × g for 15 min, the supernatant was loaded onto a SepPack cartridge (Waters). After washing the cartridge with 5 ml of water and then 5 ml of hexane, LTB4 was eluted with 2 ml of methyl formate, and the solvent of the elute was evaporated under a stream of nitrogen. The residue obtained was reconstituted with 150 μl of methanol/acetonitrile (1:1, v/v), and an aliquot (10 μl) was subjected to UPLC-TOF-MS analysis. The TOF-MS instrument was operated in negative-ion mode under the same conditions as those used in the metabolomic analysis (56). The operating conditions for liquid chromatography were as follows: column, ACQUITY UPLC® BEH C18 column (1.7 μm particle size, 2.1 × 100 mm, Waters); column temperature, 4 °C; mobile phase, A = 0.1% acetic acid and B = acetonitrile/2-propanol (90/10, v/v); elution program, (% B in A (min)), 25% (0–3), 25–95% (3–24), 95% (24–25.5), 95–25% (25.5–25.53), and 25% (25.53–30); and flow rate, 0.2 ml/min. Under these conditions, LTB4 was detected at a retention time of 13.5 min by monitoring its molecular ion (base peak, m/z 335.2228 [M + H]–). A calibration curve was prepared by adding 50 ng of LTB4-d4 (Cayman Chemical) to the 2000 × g supernatant. The linearity of the calibration curve was confirmed over a range of 0.9 to 59 ng/g of liver.

**RT-PCR**

The expression of mRNAs was quantified by real-time RT-PCR according to the method described previously (59). The primer sequences are shown in [supplemental Table 4](#). The relative level of mRNA was determined using the 2−ΔCT method. The amount of target mRNA was normalized by β-actin mRNA and is shown as a ratio to the control.

**Immunoblotting**

Immunoblotting was carried out according to the procedures reported previously (60) with some modifications. The liver was homogenized in 4 volumes (relative to the tissue weight) of 25 mM Tris-HCl (pH 7.4) containing 1 mM EGTA, 1 mM EDTA, 1 mM sodium fluoride, and a protease inhibitor mixture (Roche Diagnostics) and centrifuged at 9000 × g for 20 min. The supernatant was then centrifuged at 105,000 × g for 60 min to obtain the cytosolic fraction. The microsomal residue was suspended in 25 mM Tris-HCl (pH 7.4), and β-actin and β-tubulin were used as loading controls for ALOX5 (cytosolic protein) and LTC4 synthase (microsomal protein), respectively. Samples were electrophoresed on a 7.5% SDS-PAGE except for LTC4 synthase (15%), and the separated proteins were transferred to a PVDF membrane (Merck-Millipore, Billerica, MA). The target proteins were then treated with their respective primary antibodies and incubated with secondary antibodies conjugating HRP for 30 min at room temperature. After washing six times with TBS containing 0.05% Triton X-100, the membrane was immersed in a Clarity Western ECL substrate (Bio-Rad) for 5 min at room temperature. The relative levels of the target proteins were determined using a ChemiDoc™ MP system operated by Image Lab™ software (Bio-Rad) in an auto-exposure mode, and they were normalized either by β-actin or β-tubulin. The amounts of the proteins used for electrophoresis were as follows: ALOX5, 30 μg; β-actin, 3 μg; LTC4 synthase, 80 μg; and β-tubulin, 6 μg.

**Immunohistopathological examination**

Liver samples were embedded in Tissue-Tek® OCT compound (Sakura FineTechnical Co., Ltd., Tokyo), and frozen in 2-methylbutane at −80 °C. The frozen liver was sliced into 5-μm-thick sections using a microtome (CM3050S, Leica Microsystems, Wetzlar, Germany). Each section was then mounted on a glass slide, fixed in 10 ml of cold acetone for 10 min, and washed three times with 10 ml of PBS. The sections were pretreated in 0.1 ml of PBS containing 3% BSA for 60 min at room temperature and then washed twice with 10 ml of PBS. Cryosections were incubated overnight with rabbit polyclonal antibody against human ALOX5 (Sigma-Aldrich, 500-fold dilution) and mouse monoclonal antibody against rat CD68, a marker for macrophages (Bio-Rad, 500-fold dilution). After washing six times with 10 ml of PBS containing 0.05% Tween 20, the sections were treated with DAPI as well as secondary antibodies conjugated with fluorescent dyes (Alexa Fluor® 488 and Alexa Fluor® 647) for 60 min at room temperature. For co-staining of ly-6g, a neutrophil marker, and ALOX5, liver sections were incubated overnight with anti-ALOX5 antibody and rat anti-mouse ly-6g antibody conjugated with a fluorescent dye (FITC, Abcam, 100-fold dilution). For visualization of...
AOLX5 and cell nuclei, sections were treated with Alexa Fluor® 647-conjugated secondary antibody and DAPI for 60 min at room temperature (37°C). After washing six times with 10 ml of 0.05% (w/v) Tween 20 in PBS, the sections were covered with 0.1 ml of mounting medium (90% glycerol containing 5% (w/v) n-propyl gallate, 0.25% (w/v) 1,4-diazabicyclo[2.2.2]octane, and 0.0025% (w/v) p-phenylenediamine) and examined by confocal microscopy (LSM700, Carl Zeiss, Jena, Germany). The area and intensity of the signals were measured using ZEN software, which controlled the microscopy.

Reductive activity for 5-HpETE

5-HpETE reduction was determined according to the published method (34) with some modifications. The cytosolic proteins (50 ng) were incubated for 10 min at 37°C in 0.1 M Tris-HCl buffer (pH 7.5) containing 8.9 µM 5-HpETE, 5 mM EDTA, 0.5 mM glutathione, and 3% ethanol. After adding methanol to stop the reaction, the solution was subjected to a UPLC-TOF-MS system with a BEH C18 column; the instrument was operated in negative-ion mode under the same conditions as those used in metabolomics. The liquid-chromatography mobile phase was 0.1% formic acid (A) and acetonitrile (B) under an elution program involving a gradient from 30 to 80% B for 30 min. The flow rate was 0.4 ml/min. 5-HETE and 5-HpETE were detected at a retention time of 16.85 and 17.68 min, respectively, determined using QuanLynx software (Waters).

Culture of hepatocytes

Hepatocytes were isolated from male rats at 6 weeks of age according to an established method (61). The hepatocytes (1 × 10⁵ cells) obtained were seeded on a poly-L-lysine-coated 48-well plate and preincubated in 0.1 ml of DMEM containing 10% FBS, 100 units of penicillin, and 100 µg/ml streptomycin at 37°C with 5% CO₂. After 4 h, the cultured cells were treated with 0.01–1 nM TCDD for 24 h, and the expression levels of target mRNAs were determined by real-time RT-PCR.

Statistical analysis

The statistical difference between the two groups was compared by Student’s t test. In the comparison among multiple groups, statistical differences were calculated by one-way analysis of variance with a post-hoc test (Tukey’s test or Williams’s test) using Excel Statistics 2010 software (Social Survey Research Information Co. Ltd., Tokyo). The statistical significance was set at p < 0.05.

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Dioxin accumulates leukotriene B4 in hepatocytes

19. Serezani, C. H., Lewis, C., Jancar, S., and Peters-Golden, M. (2011) Leukotriene B4 amplifies NF-κB activation in mouse macrophages by reducing SOCS1 inhibition of MyD88 expression. J. Clin. Invest. 121, 671–682

20. Kim, N. D., Chou, R. C., Seung, E., Tager, A. M., and Luster, A. D. (2006) A unique requirement for the leukotriene B4 receptor BLT1 for neutrophil recruitment in inflammatory arthritis. J. Exp. Med. 203, 829–835

21. Sawai, H., Okawa, Y., Yamada, H., Kumamaru, H., Harada, A., Okano, H., Yokomizo, T., Iwamoto, Y., and Okada, S. (2010) The LTB4-BLT1 axis mediates neutrophil infiltration and secondary injury in experimental spinal cord injury. Am. J. Pathol. 176, 2352–2366

22. Oyosho, M. K., He, R., Li, Y., Mondal, S., Yoon, J., Afshar, R., Chen, M., Lee, D. M., Luo, H. R., Luster, A. D., Cho, J. S., Miller, I. S., Larson, A., Murphy, G. F., and Geha, R. S. (2012) Leukotriene B4-driven neutrophil recruitment to the skin is essential for allergic skin inflammation. Immunity 37, 474–758

23. Pande, K., Moran, S. M., and Bradfield, C. A. (2005) Aspects of dioxin toxicity are mediated by interleukin 1-like cytokines. Mol. Pharmacol. 67, 1393–1398

24. Kennedy, G. D., Nukaya, M., Moran, S. M., Glover, E., Weinberg, S., Balbo, S., Hecht, S. S., Pitot, H. C., Drinkwater, N. R., and Bradfield, C. A. (2014) Liver tumor promotion by 2,3,7,8-tetrachlorodibenzo-p-dioxin is dependent on the aryl hydrocarbon receptor and TNF/F-1 receptors. Toxicol. Sci. 140, 135–143

25. Harris, M. W., Moore, J. A., Vos, J. G., and Gupta, B. N. (1973) General biological effects of TCDD in laboratory animals. Environ. Health Perspect. 5, 101–109

26. Schwarz, D., Kisselev, P., Ericksen, S. S., Szklarz, G. D., Chernogolov, A., Honeck, H., Schunck, W. H., and Roots, I. (2004) Arachidonic and eicosapentaenoic acid metabolism by human CYP1A1: highly stereoselective formation of 17(18)-E(1)-epoxyeicosatrienoic acid. Biochem. Pharmacol. 67, 1445–1457

27. Konkel, A. and Schunck, W. H. (2011) Role of cytochrome P450 enzymes in the bioactivation of polycyclic saturated fatty acids. Biochem. Biophys. Acta 1814, 210–222

28. Puga, A., Hoffer, A., Zhou, S., Bohm, J. M., Leikauf, G. D., and Shertzer, G. H. (1997) Sustained increase in intracellular free calcium and activation of cyclooxygenase-2 expression in mouse hepatoma cells treated with dioxin. Biochem. Pharmacol. 54, 1287–1296

29. Yokomizo, T., Izumi, T., Chang, K., Takuwa, Y., and Shimizu, T. (1997) A lipoxygenase activities catalyzes leukotriene A4 synthesis from arachidonic acid in rabbit alveolar macrophages. Arch. Biochem. Biophys. 385, 231–241

30. Pfluger, P. T., Richter, S., Kneidinger, D., Waltenberger, D., Woisetschläger, M., and Strobl, H. (2009) Aryl hydrocarbon receptor activation inhibits in vitro differentiation of human monocytes and Langerhans dendritic cells. J. Immunol. 183, 66–74

31. Germorec, D. R., Adams, N. M., and Luster, M. I. (1995) Comparative assessment of metabolic enzyme levels in macrophage populations of the F344 rat. Biochem. Pharmacol. 50, 1495–1504

32. Kaplaniski, G., Marin, V., Montero-Julian, F., Mantovani, A., and Farnarier, C. (2003) IL-6 a regulator of the transition from neutrophil to monocyte recruitment during inflammation. Trends Immunol. 24, 25–29

33. Subbarao, K., Jala, V. R., Mathis, S., Suttles, J., Zacharias, W. A., Ahmed, I., Ali, H., Tseng, M. T., and Haribabu, B. (2004) Role of leukotriene B4 receptors in the development of atherosclerosis: potential mechanisms. Arterioscler. Thromb. Vasc. Biol. 24, 369–375

34. Pierre, S., Chevallier, A., Teixeira-Clerc, F., Ambolet-Camoit, A., Bui, L. C., Bats, A. S., Fournet, J. C., Fernandez-Salguero, P., Aggerbeck, M., Lotersztajn, S., Barouki, R., and Coumoul, X. (2014) Aryl hydrocarbon receptor-dependent induction of liver fibrosis by dioxin. Toxicol. Sci. 137, 114–124

35. Matsumura, F. (2009) The significance of the nongenomic pathway in mediating inflammatory signaling of the dioxin-activated Ah receptor to cause toxic effects. Biochem. Pharmacol. 77, 608–626

36. Augustine, P. C., and Thomas, O. P. (1979) Emerica meleagrimitis in young turkeys: effects on weight, blood, and organ parameters. Avian Dis. 23, 854–862

37. Kanaoka, Y., Maekawa, A., Penrose, J. F., Austen, K. F., and Lam, B. K. (2001) Attenuated zymosan-induced peritoneal vascular permeability and IgE-dependent passive cutaneous anaphylaxis in mice lacking leukotriene C4 synthase. J. Biol. Chem. 276, 22608–22613

38. Brungs, M., Rädmark, O., Samuelsson, B., and Steinshilper, D. (1995) Sequential induction of 5-lipoxygenase gene expression and activity in Mono Mac 6 cells by transforming growth factor β and 1,25-dihydroxyvitamin D3. Proc. Natl. Acad. Sci. U.S.A. 92, 107–111

39. Nishimura, N., Nishimura, H., Ito, T., Miyata, C., Izumi, K., Fujimaki, H., and Matsumura, F. (2009) Dioxin-induced up-regulation of the active form of vitamin D is the main cause for its inhibitory action on osteoblast activities, leading to developmental bone toxicity. Toxicol. Appl. Pharmacol. 236, 301–309

40. Yokomizo, T., Kato, K., Terawaki, K., Izumi, T., and Shimizu, T. (2000) A second leukotriene B(4) receptor, BLT2: a new therapeutic target in inflammation and immunological disorders. J. Exp. Med. 192, 421–432

41. Cho, K. J., See, J. M., and Kim, J. H. (2011) Bioactive lipoxigenase metabolites stimulation of NADPH oxidases and reactive oxygen species. Mol. Cells 32, 1–5

42. Stohs, S. J. (1990) Oxidative stress induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Free Radic. Biol. Med. 9, 79–90

43. Feldstein, A. E., Wernberg, N. W., Canbay, A., Guicciardi, M. E., Bronk, S. F., Rydzewski, R., Burgart, L. J., and Gores, G. J. (2004) Free fatty acids promote hepatic lipotoxicity by stimulating TNF-α expression via a lysosomal pathway. Hepatology 40, 185–194

44. Tajima, Y., Ishikawa, M., Maekawa, K., Murayama, M., Senoo, Y., Nishi-maki-Mogami, T., Nakanishi, H., Ikeda, K., Arita, M., Taguchi, R., Okuno, A., Mikawa, R., Niida, S., Takikawa, O., and Saito, Y. (2013) Lipidomic analysis of brain tissues and plasma in a mouse model expressing mutated human amyloid precursor protein/Tau for Alzheimer’s disease. Lipids Health Dis. 12, 68

45. Morin, C. S., Sirois, M., Echave, V., Rizcallah, E., and Rousseau, E. (2009) Relaxing effects of 17(18)-EpETE on arterial and airway smooth muscles in human lung. Am. J. Physiol. Lung Cell. Mol. Physiol. 296, L130–1139

46. Falck, J. R., Wallukat, G., Puli, N., Goli, M., Arnold, C., Konkel, A., Rothe, M., Fischer, R., Müller, D. N., and Schunck, W. H. (2011) 17(18),18(13)-Epoxycycloartenoic acid, a potent eicosapentaenoic acid (EPA)-derived regulator of cardiomyocyte contraction: structure-activity relationships and stable analogues. J. Med. Chem. 54, 4109–4118

47. Morin, C., Sirois, M., Echave, V., Albadine, R., and Rousseau, E. (2010) 17,18-Epoxycycloartenoic acid targets PPARα and p38 mitogen-activated protein kinase to mediate its anti-inflammatory effects in the lung: role of soluble epoxide hydrolase. Am. J. Respir. Cell Mol. Biol. 43, 564–575

48. Arita, M., Ohira, T., Sun, Y. P., Elangovan, S., Chiang, N., and Serhan, C. N. (2007) Resolvin E1 selectively interacts with leukotriene B4 receptor BLT1 and ChemR23 to regulate inflammation. J. Immunol. 178, 3912–3917

49. Tsujimoto, S., Ishida, T., Takeda, T., Ishii, Y., Onomura, Y., Tskimori, K., Takechi, S., Yamaguchi, T., Uchi, H., Suzuki, S. O., Yamamoto, M.,
Himeno, M., Furue, M., and Yamada, H. (2013) Selenium-binding protein 1: its physiological function, dependence on aryl hydrocarbon receptors, and role in wasting syndrome by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Biochim. Biophys. Acta* **1830**, 3616–3624

56. Kakizuka, S., Takeda, T., Komiya, Y., Koba, A., Uchi, H., Yamamoto, M., Furue, M., Ishii, Y., and Yamada, H. (2015) Dioxin-produced alteration in the profiles of fecal and urinary metabolomes: a change in bile acids and its relevance to toxicity. *Biol. Pharm. Bull.* **38**, 1484–1495

57. Bailie, M. B., Dahm, L. J., Peters-Golden, M., Harris, R. R., Carter, G. W., and Roth, R. A. (1995) Leukotrienes and 5-anaphthylisothiocyanate-induced liver injury. *Toxicology* **100**, 139–149

58. Strassburg, K., Molloy, B. J., Mallet, C., Duesterloh, A., Bendik, I., Hanke-meier, T., Langridge, J., Vreeken, R. J., and Astarita, G. (2015) Targeted lipidomics of oxylipins (oxygenated fatty acids), *Waters Application Notes*, Vol. 147, pp. 20–30, Waters, Milford, MA

59. Takeda, T., Fujii, M., Hattori, Y., Yamamoto, M., Shimazoe, T., Ishii, Y., Himeno, M., and Yamada, H. (2014) Maternal exposure to dioxin imprints sexual immaturity of the pups through fixing the status of the reduced expression of hypothalamic gonadotropin-releasing hormone. *Mol. Pharmacol.* **85**, 74–82

60. Ishida, T., Kan-o, S., Mutoh, J., Takeda, S., Ishii, Y., Hashiguchi, I., Akamine, A., and Yamada, H. (2005) 2,3,7,8-Tetrachlorodibenzo-p-dioxin-induced change in intestinal function and pathology: evidence for the involvement of aryl hydrocarbon receptor-mediated alteration of glucose transportation. *Toxicol. Appl. Pharmacol.* **205**, 89–97

61. Seglen, P. O. (1976) Preparation of isolated rat liver cells. *Methods Cell Biol.* **13**, 29–83