New trick from an old dog: The toxic lipid and oxidative stress mediator 4-hydroxynonenal activates peroxisome proliferator-activated receptor-β/δ

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Chemical-induced hepatotoxicity often results from generation of reactive intermediates and lipid peroxidation, with the cytotoxic aldehyde 4-hydroxynonenal (4-HNE) being a model molecule of oxidative stress. In the absence of the nuclear receptor PPARβ/δ, the mouse liver becomes more susceptible to pro-oxidative toxicants. We hypothesized this resulted from decreased feed-back regulation of metabolism by reactive lipids via PPARβ/δ. In support, oxidized VLDL and constituents including 4-HNE are PPARβ/δ agonists. Hepatocytes with diminished PPARβ/δ activity were more susceptible to 4-HNE-induced gene expression and cytotoxicity. This suggests PPARβ/δ agonists may be utilized to prevent or treat liver disease associated with oxidative damage.

Liver insufficiency and damage is a major cause of death and disease worldwide and may result from a variety of stimuli, including exposure to environmental toxicants, specific combinations or dosages of pharmaceuticals, and microbial metabolites. Greater than 2.2 million hospitalized Americans suffer adverse drug reactions each year, with liver toxicity presenting as the most common adverse effect, and approximately 100,000 individuals die unintentionally from administration of medications (1-3). Many additional cases of liver failure occur due to acute, chronic and degenerative disease processes, including those related to acetaminophen overdose, alcohol consumption and solvent exposures. Reactive oxygen intermediates (ROI) and other pro-oxidant agents elicit oxidative decomposition of polyunsaturated fatty acids (i.e, lipid peroxidation), an initiating event of many hepatotoxicants. Lipid peroxidation leads to the formation of a complex mixture of aldehydic end-products, including malonyldialdehyde (MDA), 4-hydroxy-2,3-nonenal (4-HNE), and other alkenals. These aldehydic molecules have been considered the ultimate mediators of toxic effects elicited by oxidative stress but may also affect cellular function at non-toxic levels via signal transduction, gene expression and cell proliferation. Although the overt toxicity caused by aldehydic end-products is due primarily to covalent binding to cellular macromolecules, the effects on signal transduction are less well known.

The peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that exist as three subtypes (α, β and γ), which exhibit tissue-specific expression, preferential ligand recognition, and distinct biological functions (4-9). Although important as targets of pharmaceutical intervention, there is increasing evidence that the biological niche occupied by the PPARs is that of receptor for fatty acid and their metabolites. Of the different PPARs, the PPARb/d isoform is the least well studied in terms of its biological role and endogenous ligands. PPARβ/δ plays an important role in differentiation of epithelial tissues, fatty acid catabolism in skeletal muscle, improvement of insulin sensitivity, attenuated weight gain and elevated HDL levels (10). Emerging evidence suggests the presence of this receptor is important in ameliorating the effects on hepatotoxicants. For example, histological examination of liver and analysis of markers of overt damage to this organ (serum GPT) with xenobiotics azoxymethane (AOM), carbon tetrachloride, arsenic and acetaminophen (APAP) demonstrated that the extent of liver toxicity in PPARβ/β-null mice was more severe as compared to wild-type mice. While necrotic cells were found in AOM-treated wild-type mice, bile duct hyperplasia, areas of regenerative hyperplasia and early adenomas were found exclusively in PPARβ/δ-null mice and markers of liver damage were seen earlier than in wildtype animals. Subchronic administration of CCl4 caused central lobular granular degeneration with necrosis in both genotypes, however lipid deposition surrounding lobules was only observed in PPARβ/δ-null mouse liver and overt damage to the liver was preferentially found in the mice lacking PPARβ/δ. Exacerbated overt toxicity with bile duct hyperplasia is found in PPARβ/δ-null mouse liver in response to exposure to both APAP and arsenic. While it is remotely possible that the metabolic fate of some of these hepatotoxicants could be influenced by PPARβ/δ, it is more likely that regulation of oxidative stress/inflammation could underlie the protective role of this receptor in liver. These chemicals share a common mechanism of overt toxicity via production of ROI and oxidized lipid intermediates. For example, CCl4 affects eicosanoid pathways (11, 12) and increase circulating prostaglandin E2 (PGE2) levels (13)

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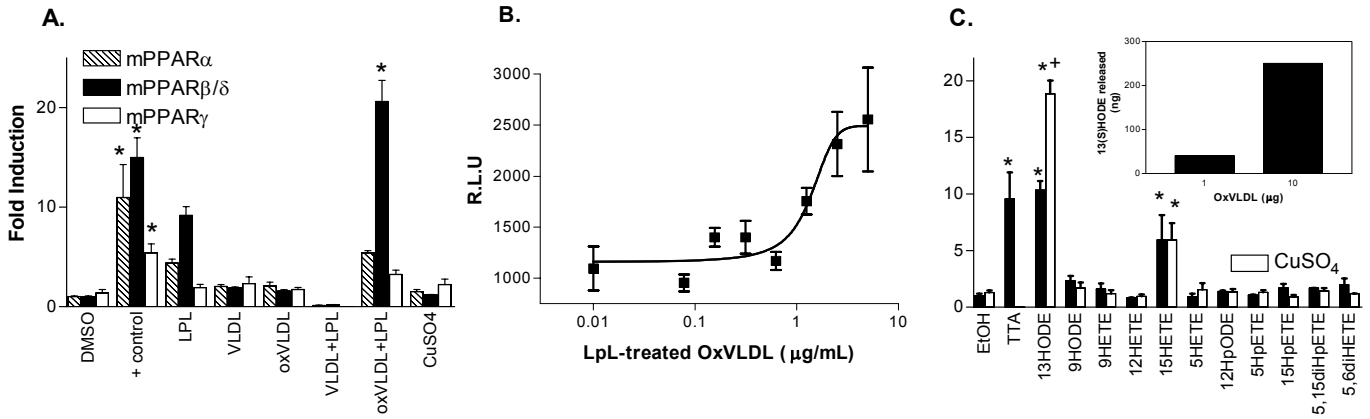

Figure 1. A. Oxidation of VLDL leads to increased activation of PPARβ. VLDL (10µg/ml) was oxidized with CuSO4 (10µM) for 72 h, or pretreated with LpL for 1 h at 35 oC, and the mixture was added to 3T3-L1 cells transfected with GAL4-LBD of each of the PPAR isoforms. Following treatment the cells were lysed and relative luciferase activity was determined. Results were corrected for DMSO. Figure 1B. Dose-dependent activation of PPARβ by LPL-treated oxVLDL. 3T3-L1 preadipocytes were transfected with Gal4-LBD-mPPARβ and treated overnight with oxidized VLDL incubated for 1 h with LpL. The cells were lysed and relative luciferase activity was determined. N = 8. Figure 1C. Oxidized 13(S)-HODE is a strong PPARβ agonist. Chemicals were obtained as described in Methods. UV-Visible spectroscopy was used to determine their respective concentrations, and the original solvent was evaporated under a stream of N2 and the chemical reconstituted in ethanol to 50 µM. Oxidation products were obtained via incubation with CuSO4 (10 µM) for 72 h. 3T3-L1 preadipocytes were transfected with Gal4-LBD-mPPARβ and treated with compound overnight. The cells were lysed and relative luciferase activity was determined. Results were normalized to EtOH/H2O. N = 3. *Significantly different than the EtOH control; +Significantly different than the non-CuSO4-treated counterpart. Inset: Oxidation of VLDL releases 13(S)-HODE. VLDL was oxidized using 10 µM CuSO4 for 72 h. Oxidized VLDL was then incubated with LpL for 1 h. An ELISA specific for 13(S)-HODE (Assay Designs) was performed to quantify the amount hydrolyzed from oxVLDL. Samples were prepared by diluting 1, and 10 µL of oxVLDL with standard diluent to a final volume of 100 µL (N = 2).

resulting in an enhanced production of 4-HNE and 4-HNE-protein adducts (14-16). Similarly, APAP-treated rats also produced greater quantities of PGE2, thromboxane A2 and leukotriene C4 from normal liver (17) accompanied by an increased 4-HNE and MDA formation in rat liver (18). One possible explanation for the increased susceptibility of PPARβ/δ null mice to hepatotoxicity is that agents such as CCl4 and APAP increase the production of an endogenous ligand for PPARβ/δ, which would in turn stimulate lipid metabolism and degradation of lipid peroxidation intermediates. In the absence of this receptor, the signaling cascade would be disrupted and accumulation of toxic lipids such as 4-HNE would result.

If our hypothesis were correct, endogenous ligands of PPARβ/δ should include oxidized lipids, in particular those derived from fatty acids. The VLDL particle, which is naturally generated in the liver, is a mixture of free and esterified cholesterols, triglycerides (formed from various fatty acids) and apolipoproteins. As seen in Fig. 1A, the oxidation of VLDL with CuSO4 (oxVLDL) failed to activate any of the PPAR isoforms, including PPARβ/δ. However, incubation of oxVLDL with Lipoprotein Lipase (LpL) greatly increased PPARβ/δ activity specifically, with minimal increases in activity of the other two PPAR subtypes. Furthermore, in an effort to establish the concentration at which PPARβ activity is maximally stimulated by oxVLDL, a dose-response relationship was established (Fig. 1B) with a half maximal concentration (EC50) of approximately 20 µg/ml. To determine which, if any, fatty acids released by oxVLDL are playing a role in the enhancement of PPARβ/δ activity, we conducted a screen study involving 11 fatty acids and their CuSO4 oxidation products. In Fig. 1C we show that, while 13(S)-HODE alone activates PPARβ/δ, its oxidation product increases activity roughly two-fold higher. No significant enhancement of PPARβ/δ was observed with any of the other fatty acids tested, except 15-HETE, which also activated the PPARβ/δ isoforms. Furthermore, we hypothesized that the polyunsaturated fatty acid 13(S)-HODE, an oxidation product of the 15-lipoxygenase metabolic pathway, is hydrolyzed upon incubation with LpL to the free fatty acid which, ultimately, induces PPARβ/δ activity. Both 13(S)-HODE and its hydroperoxy derivative, 13(S)-HpODE, were equally potent in their ability to activate PPARβ/δ (data not shown). To examine if 13(S)-HODE was released from oxidized VLDL treated with LpL, we used an enzyme-linked immunosorbent assay specific for the S-enantiomer of 13-HODE. Our results show that the release of 13(S)-HODE increased correspondingly with the amount of oxVLDL used (Fig. 1C, Inset). Based on the EIA, we estimated that 1 µg of LpL-treated oxidized VLDL contains approximately 37.5 ng of 13(S)-HODE. Interestingly, while CuSO4 oxidation of 13(S)-HODE also generated a PPARβ/δ agonist with greater affinity for the subtype than the fatty acid itself, oxidation of 15-HETE in the same manner did not have any effect on its ability to increase receptor activity in our reporter assay. Together, these results indicated that receptor activation by products of lipid peroxidation were likely to be endogenous ligands for the PPARβ/δ protein.

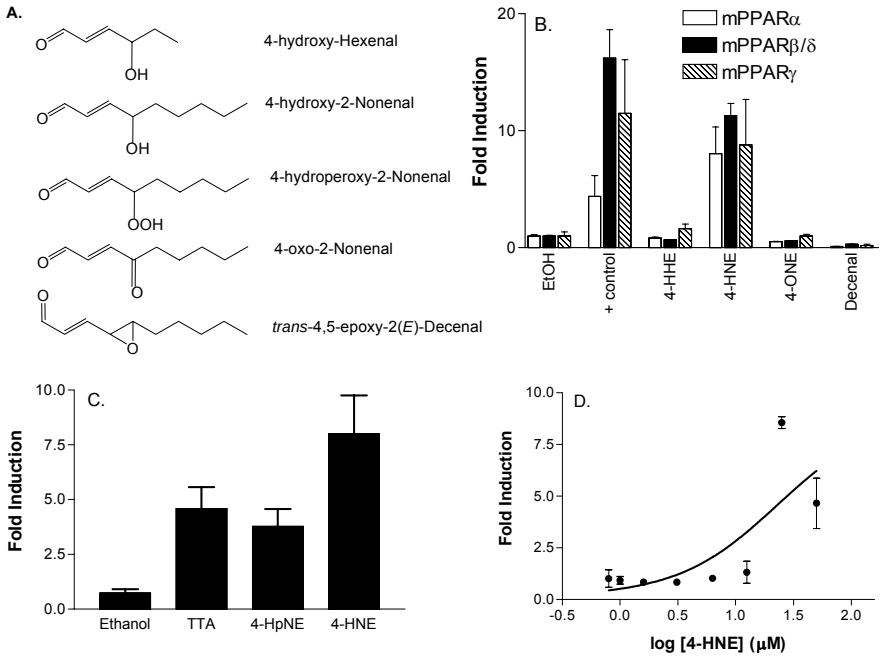

Figure 2. A. Structures of the test molecules. Figure 2B. PPARβ/δ is activated in a dose-by 4-HNE in 3T3-L1 preadipocytes. The cells were transfected with Gal4-LBD-mPPARβ/δ followed by treatment with each of the lipid peroxidation products or vehicle control for 12 h. Following the treatment, cells were lysed and luciferase activity was determined and normalized to EtOH. Treatment was with with 25 μM 4-HHE, 4-HNE, 4-ONE and trans-4,5-epoxy-2E-decenal (Decenal; Cayman) overnight. C. Cells were treated with 25 μM 4-HpNE or 4-HNE for 12 h. D. Dose-response of PPARβ/δ activation by 4-HNE. PPARb activity is increased with addition of 25 μM 4-HNE, while cellular toxicity is observed at higher concentrations. N = 8.

In order to determine which specific type of aldehydic product induces PPARβ/δ activity, a structure-activity relationship was established using a representative compound from each family of lipid peroxidation product, 2-alkenals, 4-hydroxy-2-alkenals, and ketoaldehydes (Fig. 2). 2-Alkenals were represented by trans-4,5-epoxy-2(E)-decenal, ketoaldehydes were represented by 4-ONE, and 4-hydroxy-2-alkenals were represented by both 4-HHE and 4-HNE, aldehydes which differ only in their carbon chain length (Fig. 2A). Reporter assays clearly showed that 4-HNE alone enhanced the activity of all three PPAR subtypes, with the greatest augmentation afforded to the PPARβ/δ subtype (Figure 2B). On the other hand, 4-HHE, 4-ONE, and trans-4,5-epoxy-2(E)-decenal did not activate any of the PPARs. Periods of oxidative stress initiate free-radical mediated oxidative damage of lipids, such as 13(S)-HODE. The breakdown pathway from lipid to 4-HNE includes a hydroperoxy-derivative, 4-hydroperoxynonenal (4-HpNE), which is rapidly reduced to 4-HNE by cellular peroxidases. In fact, 4-HNE, the resultant hydroxy-derivative formed from 4-HpNE, activated PPARβ/δ to a greater extent than its precursor (Fig. 2C). Furthermore, a dose-dependent activation of PPARβ/δ was also seen with 4-HNE (Fig. 3D), where maximal receptor activation was observed around 25 μM. Cellular toxicity was observed at concentrations higher than 30 μM and biological concentration of 4-HNE are known to be within this range.

In order to analyze if 4-HNE affected gene expression or toxicity in a PPARβ/δ-dependent manner, studies were performed on murine SV-40 immortalized hepatocytes prepared from wild-type (MuSH WT) and PPARβ/δ-/- (MuSHβ/δ-/-) mice. Such hepatocytes were treated with vehicle control (DMSO), a known PPARβ/δ agonist tetradecylthioacetic acid (TTA), 13(S)-HODE and 4-HNE. The expression of a PPARβ/δ-regulated gene, adipose differentiation related protein (ADRP), was up-regulated in a PPARβ/δ-dependent manner in cells treated with 25μM each of 13 (S)-HODE and 4-HNE (Fig. 3A).

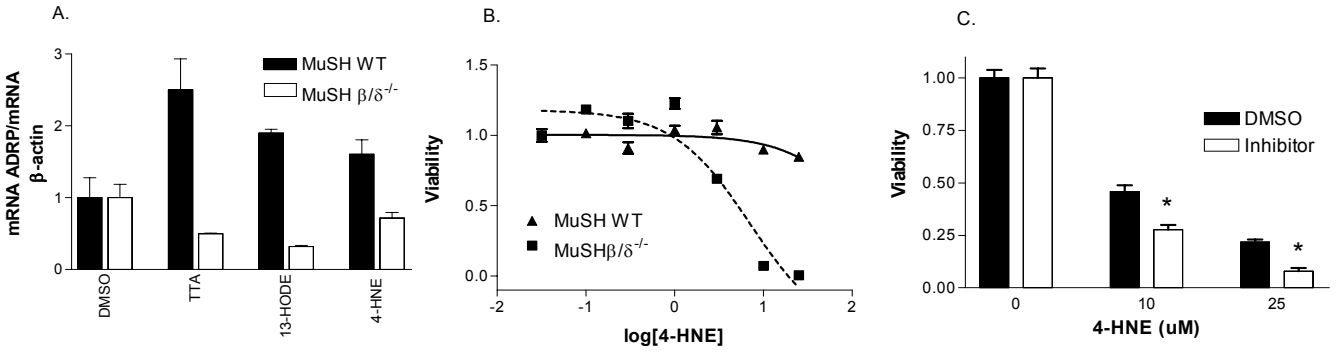

Figure 3. A. 4-HNE regulates gene expression in a PPARβ/δ -dependent manner. MuSH WT and MuSHβ/δ -/- cells were treated with 13(S)-HODE and 4-HNE overnight and the cells were lysed. Total RNA was extracted using Tri-Reagent and RT-PCR performed for ADRP and corrected for b-actin expression. N = 3. Figure 3B. PPARβ is crucial in ameliorating the toxic effects of 4-HNE. MuSHWT and MuSHβ-/- hepatocytes were treated with 0, 0.1, 0.3, 1, 3, 10 or 25 μM 4-HNE. The data is normalized for % viable cells, shown on the y-axis. N = 8. Representative of six independent experiments. Figure 3C. Inhibition of PPARβ/δ leads to increased susceptibility to oxidative damage. MuSHWT cells were pre-treated with vehicle or GW9662 for 6 h prior to treatment with 4-HNE. * and ** Significantly different than the DMSO counterpart. N = 5.

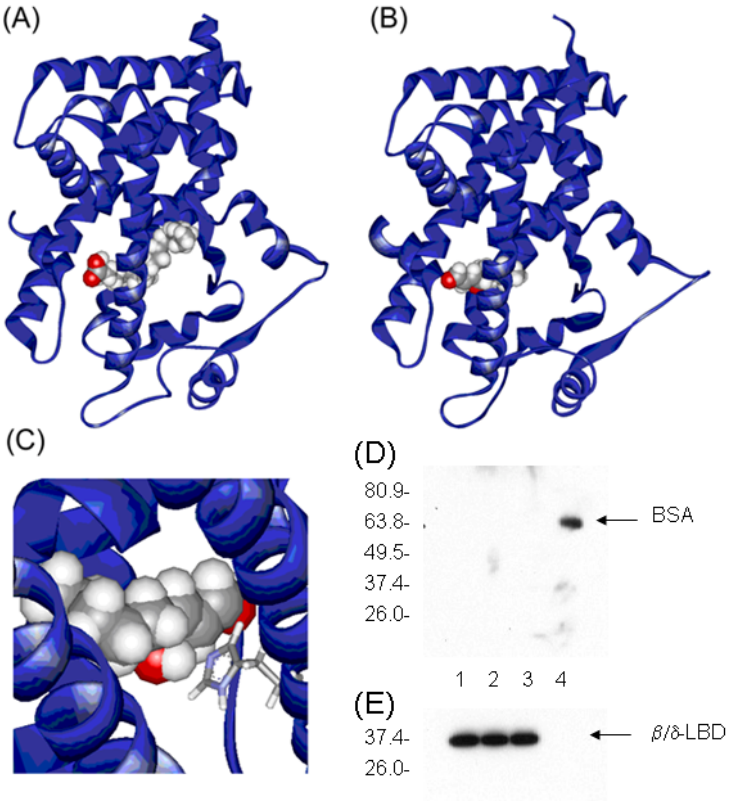

Figure 4.A: X-ray structure of PPARβ/δ bound to eicosapentaenoic acid. Figure 4B: A molecular model of PPARβ/δ bound to (S)-4-hydroxynonenal (CPK model) in a conformation similar to the natural ligand. Figure 4C: Visualization of the putative hydrogen-bonding interaction between His413 (stick model) and the 4-hydroxyl group of the ligand (CPK model). Figure 4D. 4-HNE activates PPARβ/δ via a direct and non-covalent interaction. Mouse PPARβ/δ LBD was expressed using an in vitro transcription-translation system (Roche) and incubated with 4-HNE. Immunoblot analysis was used to determine 4-HNE-protein adducts. Lane 1 PPARβ/δ-LBD alone; Lane 2, PPARβ/δ-LBD plus EtOH; Lane 3, PPARβ/δ-LBD plus 4-HNE (100 μM); Lane 4, BAS plus 4-HNE (100 μM). Figure 4E. Identical incubation as performed above with immunoblot analysis of PPARβ/δ.

Importantly, the cells lacking PPARβ/δ were more susceptible to 4-HNE cytotoxicity than the wildtype cells (Fig. 3B) and treating MuSH WT cells with a chemical antagonist of PPARβ/δ increased sensitivity to this toxic lipid (Fig. 3C).

Molecular modeling was employed using the coordinates of PPARβ/δ bound to eicosapentaenoic acid (PDB ID: 3GWX, Figure 4A) and was examined for the putative association with 4-HNE. PPARβ/δ bound to (S)-4-hydroxynonenal (CPK model) in a conformation similar to the natural ligand (Figure 4B). However, a putative hydrogen-bonding interaction between His413 (stick model) and the 4-hydroxyl group of the 4-HNE ligand (CPK model) occurs (Figure 4C), suggesting the association is in some ways unique. Knowing that 4-HNE interacts with a wide variety of proteins via the formation of covalent adducts with cysteinyl, lysyl, and histidyl residues, we used a mixed polyclonal antibody towards 4-HNE protein adducts to detect the presence of such covalent products with the mouse PPARβ/δ isoform (Fig. 4D). However, we were unable to show covalent interactions between 4-HNE and PPARβ/δ strongly suggesting that the association with PPARβ/δ is reversible.

The last nine carbons of arachidonic and linoleic acid are represented in 4-HNE and the precursor to 13(S)-HODE, 13(S)-HOPDE may be further oxidized by a Hock Cleavage mechanism to yield 4-HNE. The arachidonic and linoleic acid oxidation leads to the formation of a highly unstable hydroperoxide and racemic derivative, 4-HpNE, which can then be metabolized by cellular peroxidases to 4-HNE and its isomer, 4-ONE. Consistent with this idea, treatment of 4-HpNE also activated PPARβ/δ in the reporter while treatment of cells with its reduced product, 4-HNE, led to a greater increase in the PPARβ/δ reporter activity. In order to rule out the possibility that 4-HNE-treatment resulted in an oxidative-stress mediated increase in other endogenous ligands, we used 4-ONE as a control to show that a related lipid peroxidation product, which differs from 4-HNE in having a carbonyl rather than a hydroxyl group at 4-C, was ineffective in activating the receptor. The results unequivocally confirm that PPARβ/δ activation by this class of molecule is specifically effected by 4-HNE treatment. Furthermore, even other lipid peroxidation products such as 4-HHE and decenal were not capable of activating PPARβ/δ isoform. In addition to being controls, these assays also shed some light on the possible structural and spatial requirements of an endogenous ligand for PPARβ/δ in that a nine carbon chain length with a hydroxyl group at 4-C seems essential for ligand-dependent activation. Based on the molecular modeling information, it is possible that an association between the hydroxyl group and Histidine-413 is necessary for optimal receptor activation.

PPARβ/δ is the least understood of the three PPAR subtypes in many respects, including the identification of endogenous ligands. This receptor is ubiquitously expressed and is often found in higher abundance than PPARα or γ in brain, adipose tissue and skin. Examination of the PPARβ/δ null mice has shown a role for PPARβ/δ in development, myelination of the corpus callosum, lipid metabolism, and epidermal cell proliferation. Few high affinity ligands for PPARβ/δ are known, either xenobiotic or endogenous. However, fatty acids are weak activators of this receptor with roughly the same preference as PPARα. Interestingly, similar to PPARα and γ, incubation of triglyceride rich lipoproteins with LPL results in the production of PPARβ ligands. One of the potential products from the oxVLDL particular that affects PPARβ/δ is 13-S-hydroperoxyoctadecadienoic acid (13(S)-HODE). In addition to being the primary product of 15-lipoxygenase-1 with linoleic acid as the substrate, this molecule may be an indicator of potential lipid peroxidation produced from VLDL.

There is a wealth of information on 4-HNE as being a marker of lipid peroxidation as well as a mediator of hepatotoxicity and a variety of other pathophysiological

conditions including cancer, Alzheimer's disease and atherosclerosis (19). Being a lipophilic molecule, 4-HNE can cross subcellular compartments and ultimately react with the variety of different cell proteins. Target molecules that are inhibited by 4-HNE include the glucose transporter GLUT3, tau, the proteasome and IκB kinase (19). While 4-HNE formed covalent adducts with all the three above mentioned proteins, results from our studies show that the interaction of 4-HNE with PPARβ/δ did not proceed through such a Michael addition mechanism.

In summary, we have shown that the lipid peroxidation product 4-HNE, which was regarded to be a mutagen for all these years, has now been demonstrated to have a nuclear receptor binding activity. This receptor, PPARβ/δ is thereby activated and provides a feed-back regulation of gene expression that ameliorates toxicity to this tissue. The discovery of 4-HNE as an endogenous nuclear receptor ligand also rescues PPARβ/δ from the orphanage as well as reveals a crosstalk of PPARβ/δ in cellular events involved in inflammation and energy homeostasis. Given the large number of individuals hospitalized due to chemically-induced liver damage, it is attractive to speculate that this knowledge may be exploited for more effective means of protecting or treating hepatotoxicity as well as other diseases in which 4-HNE plays a causative role.

1. W. M. Lee, J. R. Senior, Toxicol Pathol 33, 155 (2005).
2. W. M. Lee, N Engl J Med 349, 474 (Jul 31, 2003).
3. W. M. Lee, Semin Liver Dis 23, 217 (Aug, 2003).
4. J. P. Vanden Heuvel, Curr Atheroscler Rep 6, 432 (Nov, 2004).
5. S. A. Khan, J. P. Vanden Heuvel, J Nutr Biochem 14, 554 (Oct, 2003).
6. J. M. Peters, J. P. Vanden Heuvel, in Cellular and Molecular Toxicology J. P. Vanden Heuvel, G. H. Perdew, W. B. Mattes, W. F. Greenlee, Eds. (Elsevier Science, Amsterdam, The Netherlands, 2002), vol. 14, pp. 133-158.
7. J. P. Vanden Heuvel, J. M. Peters, Comments Toxicol. 7, 233 (2001).
8. J. P. Vanden Heuvel, Toxicol Sci 47, 1 (1999).
9. J. P. Vanden Heuvel, J Nutr 129, 575S (1999).
10. A. D. Burdick, D. J. Kim, M. A. Peraza, F. J. Gonzalez, J. M. Peters, Cell Signal (Aug 15, 2005).
11. S. Basu, Toxicology 189, 113 (Jul 15, 2003).
12. N. Kawada, Y. Mizoguchi, K. Kobayashi, S. Yamamoto, S. Morisawa, Gastroenterol Jpn 25, 363 (Jun, 1990).
13. M. Lopez-Parra et al., Br J Pharmacol 135, 891 (Feb, 2002).
14. D. P. Hartley, K. L. Kolaja, J. Reichard, D. R. Petersen, Toxicol Appl Pharmacol 161, 23 (Nov 15, 1999).
15. M. Comporti, Free Radic Res 28, 623 (Jun, 1998).
16. D. P. Hartley, D. J. Kroll, D. R. Petersen, Chem Res Toxicol 10, 895 (Aug, 1997).
17. F. Culo et al., Eur J Gastroenterol Hepatol 7, 757 (Aug, 1995).
18. J. Liu et al., Hepatology 37, 324 (Feb, 2003).
19. K. Uchida, Prog Lipid Res 42, 318 (Jul, 2003).

Atheroscler Rep **6**, 432-40 (Nov,