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CONTEMPORARY REVIEW

Tipping the Balance: Hepatotoxicity and the 4 Apical Key Events of Hepatic Steatosis

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ABSTRACT

Hepatic steatosis is a condition were fat accumulates in the liver and it is associated with extra-hepatic diseases related to metabolic syndrome and systemic energy metabolism. If not reversed, steatosis can progress to steatohepatitis and irreversible stages of liver disease including fibrosis, cirrhosis, hepatocellular carcinoma, and death. From a public health standpoint, identifying chemical exposures that may be factors in steatosis etiology are important for preventing hepatotoxicity and liver disease progression. It is therefore important to identify the biological events that are key for steatosis pathology mediated by chemical exposure. In this review, we give a current overview of the complex biological cascades that can disrupt lipid homeostasis in hepatocytes in the context of 4 apical key events central to hepatic lipid retention: hepatic fatty acid (FA) uptake, *de novo* FA and lipid synthesis, FA oxidation, and lipid efflux. Our goal is to review these key cellular events and visually summarize them using a network for application in pathway-based toxicity testing. This effort provides a foundation to improve next-generation chemical screening efforts that may be used to prevent and ultimately reverse the growing incidence of fatty liver disease in our population.

Key words: non-alcoholic fatty liver disease; steatosis; adverse outcome pathway; mechanistic toxicology; high-throughput screening assays; chemical risk assessment.

FATTY LIVER AND TOXICITY

Fatty liver disease is a growing epidemic and currently affects 20%–30% of the U.S. population, making it the most common liver disease in the United States (Noureddin *et al.*, 2015). The 2 primary types of fatty liver disease are alcoholic liver disease (ALD) and nonalcoholic fatty liver disease (NAFLD). ALD is directly linked to excess alcohol consumption (>10 g/day). However, a variety of exposures can induce NAFLD, such as high-calorie high-fat diets, inactivity, therapeutic drugs, and environmental chemicals such as pesticides, solvents, polychlorinated biophenyls, dioxins, fungicides, herbicides, and insecticides (Al-Eryani *et al.*, 2015; Savolainen *et al.*, 1993). The main pathological feature of hepatic steatosis is excessive lipid accumulation, primarily triglyceride, within the cytosol of the hepatocyte. Histological features may appear as macrovesicular fatty change, distinguished by large cytoplasmic lipid droplets that displace the nucleus. A less common observation is microvesicular fatty change, marked by small diffuse lipid droplets that may not be visible, but may induce hepatocyte ballooning (Lee, 1994). Microvesicular fatty change is more often associated with toxicity leading to broad metabolic disruption that impacts mitochondrial and lipid metabolic pathways (Fromenty *et al.*,

1997). As an example, steatogenic and hepatotoxic contaminants (carbon tetrachloride and vinyl chloride [Cave *et al.*, 2010; Wahlang *et al.*, 2013]) and drugs (tetracycline, amiodarone, and valproic acid [Fromenty et al., 1997]) can not only impair mitochondrial function, but also increase proinflammatory cytokine production (Antherieu *et al.*, 2011; Kaiser *et al.*, 2012; Szalowska *et al.*, 2014). These events ultimately disrupt lipid synthesis, uptake, efflux, and metabolism, cumulatively leading to hepatic steatosis.

Data linking chemicals to steatosis etiology have been used by the U.S. EPA's Integrated Risk Information System database to derive reference values that help characterize public health risks and support risk management decisions (Kaiser *et al.*, 2012). The challenge is that NAFLD often goes undiagnosed before progressive and likely permanent liver injury occurs (Figure 1). From a clinicopathologic perspective, steatosis is often asymptomatic. Clinical biomarkers alanine and aspartate aminotransferases (ALT/AST) lack the sensitivity and specificity to distinguish steatosis from progressive liver disease, and diagnosis can only be made by liver biopsy. Furthermore, steatosis is recognized as the "hepatic manifestation" of metabolic syndrome (Marchesini *et al.*, 2003) and closely associated with obesity, diabetes, cardiovascular disease, and certain types of cancer (Noureddin *et al.*, 2015).

THE 4 APICAL KEY EVENTS OF FATTY LIVER

Lipid metabolism is modulated by broad chemical signals, such as nutrients, hormones, and environmental toxicants. *In vivo*, these autocrine, paracrine, and endocrine chemical signals mediate feedback and feed-forward loops by binding to cognate cell surface, cytosolic, and nuclear receptors. In essence, chemical contact at any point along this central nervous system-autonomic-cellular-molecular loop can modulate a complex network of signal transduction cascades and receptor crosstalk that manage responses including altered cellular permeability, protein activation, and gene transcription (Boron *et al.* 2008). Furthermore, endocrine signaling between the brain, gut, and adipose tissue plays an important role in regulating whole body metabolism. Therefore, endocrine disrupting chemicals such as obesogens, defined as "chemicals that inappropriately regulate and promote lipid accumulation and adipogenesis," have the potential to disrupt endocrine signaling and promote dyslipidemia. Several excellent articles review the topic of obesogens elsewhere (Grun *et al.*, 2009a; Grun *et al.*, 2009a,b, 2006).

Obesogenic chemicals initiate complex molecular signals at all levels of biological hierarchy, particularly through changes in gene expression, protein activity, mitochondrial dysfunction, and endoplasmic reticulum stress that may lead to NAFLD. However, these steatogenic effects are ultimately revealed by 4 apical key events (KEs) in the hepatocyte: hepatic fatty acid (FA) uptake, *de novo* FA and lipid synthesis, FA oxidation, and lipid efflux (Figure 2). Current understandings of the biologic underpinning of these 4 apical KEs are described below with the notion that chemical perturbation of these pathways may tip the balance toward hepatic steatosis.

Increased FA Uptake

FA uptake is dependent on the concentration of FA in the circulating blood as well as transporter activity and stoichiometry in the sinusoidal hepatocyte membrane. Nutritional status (fed vs fasted) and hormonal control of appetite satiety signals (orexins, ghrelin, insulin, and leptin) dictate the source (gut vs adipose tissue), and amounts of FA. Specifically, dietary FAs are hydrolyzed from triacylglycerol (TAG) by lipases, colipases, and bile salts in the intestinal lumen. FAs are then transported into enterocytes where they are resynthesized into TAG that are incorporated into chylomicron lipoproteins. Mature chylomicrons are distributed into the lymphatic system and enter the circulation via the thoracic duct and subclavian vein. Endothelial lipases release FA from the TAG rich chylomicron core to deliver FA to peripheral cardiac, muscle, and adipose tissues, or liver via portal circulation.

During insulin resistance or under fasting conditions, hormone signals (glucagon, catecholamines, and natriuretic peptides) activate G-protein coupled receptors (GPCR), such as β-adrenergic receptors, to induce adipose tissue lipolysis mediated by hormone sensitive lipase, adipose triglyceride lipase, αβ-hydrolase domain containing 5, and perilipin 1 (Granneman *et al.*, 2009). Lipolysis releases FA into the bloodstream, where they are bound by albumin before uptake by peripheral tissues. Certain pathophysiological conditions (such as obesity and diabetes) and chemical exposures (carbon tetrachloride [Weber *et al.*, 2003] and vinyl chloride [Cave *et al.*, 2010]) induce insulin resistance leading to adipose tissue lipolysis, independent of feeding. Furthermore, several of the aforementioned hormones controlling appetite and adipose biology are sensitive to chemical exposures (bisphenol A, organotins, persistent organic pollutants [POP], polybrominated flame retardants) that modulate their cognate receptors (estrogen receptor [ER], glucocorticoid receptor, GPCR, peroxisome proliferated activated receptor alpha and gamma [PPARα and γ], and aryl hydrocarbon receptor [AhR]) (Swedenborg *et al.*, 2009).

Hepatic FA uptake (long chain, >16 carbons) is facilitated primarily by FA transporter proteins 2 and 5 (FATP2/5), caveolins, FA translocase (FAT)/CD36, and plasma membrane-associated FA-binding protein (Glatz *et al.*, 2010). FAT/CD36 expression and translocation to the plasma membrane is highly inducible, plays an important role in the regulation of hepatic FA uptake, is positively correlated with TAG levels in humans with steatosis (Greco *et al.*, 2008), and upregulated in mice exposed to POP (Angrish *et al.*, 2012; Lee *et al.*, 2010). The overall rate and amount of hepatic FA uptake is dependent on hepatic transporter abundance and activity. This activity is regulated by key lipogenic transcription factors, such as the liver X receptor (LXR), that are modulated by a number of natural (hydroxycholesterols, polyunsaturated FAs, prostaglandins, genistein, and diterpenes) and synthetic (GW3965 and T0901317) ligands (Huang, 2014; Zhao *et al.*, 2010).

Increased De Novo FA and Lipid Synthesis

The biological synthesis of FA is another mechanism that can lead to increased lipid levels. Under normal prandial conditions, glucose is taken up into the liver by the insulin independent transporter GLUT2 and enters into the glycolytic pathway. Insulin signaling promotes glucose breakdown to pyruvate by increasing the activity of glucokinase, phosphofructokinase, and pyruvate kinase. Insulin also stimulates mitochondrial pyruvate decarboxylation to acetyl-CoA and NADH regeneration by activating pyruvate dehydrogenase (Boron *et al.*, 2008). Furthermore, acetyl-CoA enters the tricarboxylic cycle (TCA) or is transported across the mitochondrial membrane and into the cytosol under conditions of high oxaloacetate production via the citrate shuttle. Once cytosolic, acetyl-CoA is resynthesized from citrate by acetyl-CoA citrate lyase. Acetyl-CoA carboxylase performs the rate-limiting step in FA β-oxidation and FA

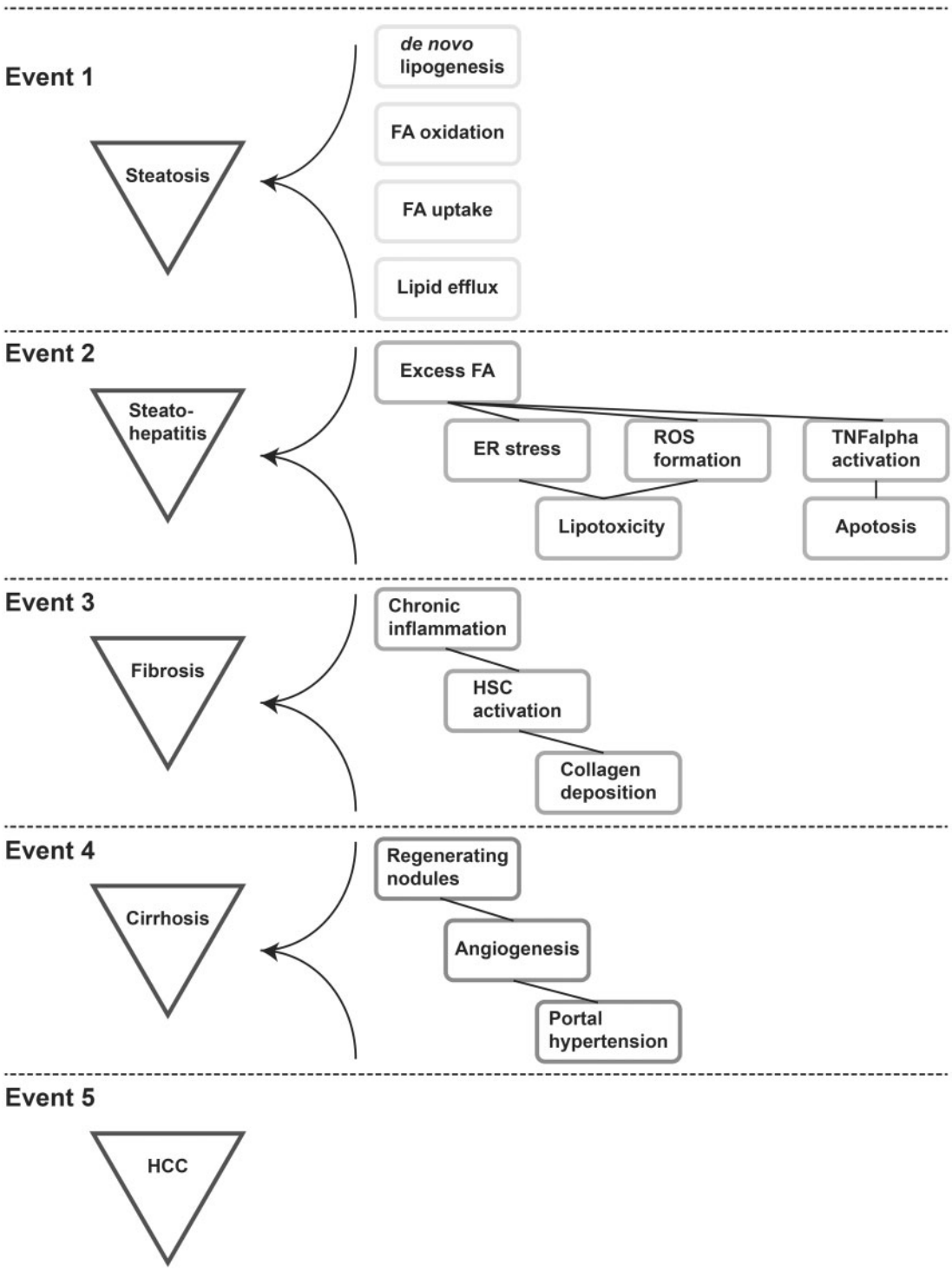

FIG. 1. Events of progressive fatty liver injury. Progressive liver disease (triangles) and pathways (rectangles) involved in their pathology.

synthesis by carboxylating acetyl-CoA producing malonyl-CoA (Brownsey *et al.*, 2006). Malonyl-CoA, along with acetyl-CoA and NADPH, are condensed in a series of decarboxylation reactions catalyzed by FA synthase (FAS) to yield palmitate, a 16-carbon saturated FA (Chan *et al.* 2010). FA synthesis is intimately dependent on glycolysis and mitochondrial function (electron transport, mitochondrial permeability transition, mitochondrial membrane potential, etc.) for acetyl-CoA precursor and NADPH cofactor generation. Similarly, insufficient NAD^+ is known to impair mitochondrial FA β-oxidation (discussed below). The implication is that chemicals targeting mitochondrial and glycolytic function (as is the case for many organochloride insecticides and herbicides) may have steatogenic potential (Kaiser *et al.*, 2012).

Palmitate is the precursor for all *de novo* synthesized long chain FAs and is modified by a series of elongases and desaturases to yield long chain mono- and poly-unsaturated FAs (MUFA and PUFA, respectively) (Guillou *et al.*, 2010). In humans,

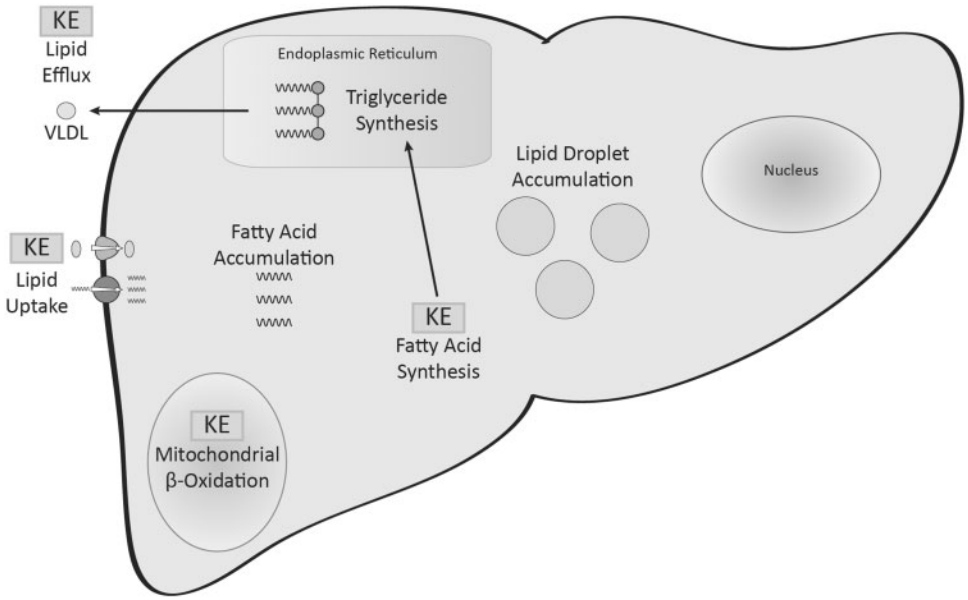

FIG. 2. Hepatic lipid flux and 4 apical key events that define fatty liver. Fatty liver is represented by an imbalance between 4 apical key events (KE) which result in fatty acid (FA) and lipid droplet accumulation: hepatic FA uptake, FA and lipid synthesis, decreased hepatic FA oxidation, and/or decreased hepatic secretion of very low-density lipoproteins (VLDL).

the ER-bound enzyme, stearoyl-CoA desaturase (SCD, also regulated by LXR), catalyzes the rate-limiting step in MUFA synthesis by desaturating palmitate and stearate into palmitoleate and oleate, the predominant FA in TAG (Wood *et al.*, 1969). In mice, *Scd1* deficiency protects from steatosis (Miyazaki *et al.*, 2007) and *Scd1* expression is induced by dioxin exposure (Angrish *et al.*, 2011).

TAG are the dominant form of stored energy in mammals and relatively benign compared to their bioactive FA constituents. However, excessive TAG accumulation in tissues leads not only to steatosis, but also obesity, insulin resistance, Type II diabetes, and atherosclerosis. In the liver, TAGs are synthesized from FA and glycerol esters in the ER where the final step is catalyzed by either diacylglycerol acyl transferase (DGAT) 1 or 2. *DGAT2* mRNA levels are highly expressed in human liver (Yen *et al.*, 2008) and induced, along with *SCD* and *ACACA*, by XBP1, a key regulator of the ER stress response (Lee *et al.*, 2008). This suggests that chemical-mediated induction of ROS either via mitochondrial toxicity or hepatic microsomal cytochrome P450 (CYP) activity (discussed below) may increase TAG synthesis and hepatic lipid levels (Trauner *et al.*, 2010).

Decreased FA Oxidation

Mitochondrial function is critical to maintain lipid balance. Hepatotoxicants (eg, Mirex and ethylene glycol) that induce mitochondrial dysfunction have the potential to alter lipid balance by inhibiting mitochondrial β-oxidation leading to accumulation of intracellular FA and TAG (Kaiser *et al.*, 2012). During periods of energy need or lipid imbalance, mitochondrial FA β-oxidation generates ATP, acetyl-CoA that is catabolized by the TCA cycle, and the cofactors $FADH_2$ and NADH that are required by the mitochondrial electron transport chain. A caveat is that cytosolic long chain FAs must traverse both the outer and inner mitochondrial membranes before the mitochondrial β-oxidative machinery can access them, whereas short chain FAs simply diffuse into the mitochondrial matrix. In the case of long chain FAs, they are first activated by acyl-CoA synthetase to acyl-CoA. Carnitine palmitoyl transferase 1 is regulated by PPARα and performs the rate-limiting step in long chain mitochondrial FA β-oxidation by replacing CoA with a carnitine moiety in preparation for transport via the carnitine pathway. Carnitine transferase is directly inhibited by malonyl-CoA, a precursor in FA synthesis, so when energy levels are high, β-oxidation is inhibited. When energy levels are low and β-oxidation is active, acyl-carnitines pass through a porin into the mitochondrial inner membrane space and across the inner mitochondrial membrane by the carnitine/acyl carnitine transporter. Once in the mitochondrion, carnitine palmitoyl transferase 2 regenerates acyl-CoA from the acyl-carnitine for β-oxidation cycling (Boron *et al.*, 2008).

FA catabolism is intrinsically dependent on mitochondrial function and, under conditions leading to mitochondrial toxicity and/or FA overload, peroxisomal β- and microsomal ω-oxidation pathways are activated. Very long chain FA peroxisomal β-oxidation occurs similarly to mitochondrial β-oxidation, except cycling generates ROS in the form of hydrogen peroxide that must be neutralized by antioxidant enzymes to prevent damage (Hardwick *et al.*, 2009). The CYP450 gene family metabolizes endogenous and exogenous chemicals through redox cycling to generate a hydroxylated compound (R-OH) and water. However, ω-oxidation of FAs by some CYP family members (eg, CYP4) decouples catalytic cycling, releasing ROS, and lipid peroxides (Berlanga *et al.*, 2014; Hardwick *et al.*, 2009). Thus, activation of these oxidative pathways may lead to ER stress and hepatic lipid accumulation. PPARα transcriptionally regulates many mitochondrial and peroxisomal FA genes (Rakhshandehroo *et al.*, 2010) and, if activated, may reduce steatosis. However, if antagonized, PPARα may inhibit FA oxidation pathways and induce steatosis (Wahlang *et al.*, 2013).

Decreased Lipid Efflux

Very low density lipoprotein (VLDL) secretion provides a means to transfer fats from the liver to adipose and other tissue for storage during feeding or during postprandial periods (Gibbons, 1990). If secretion is impaired, as shown in mice exposed to dioxin (Angrish *et al.*, 2013; Lee *et al.*, 2010), lipids may accumulate in the liver.

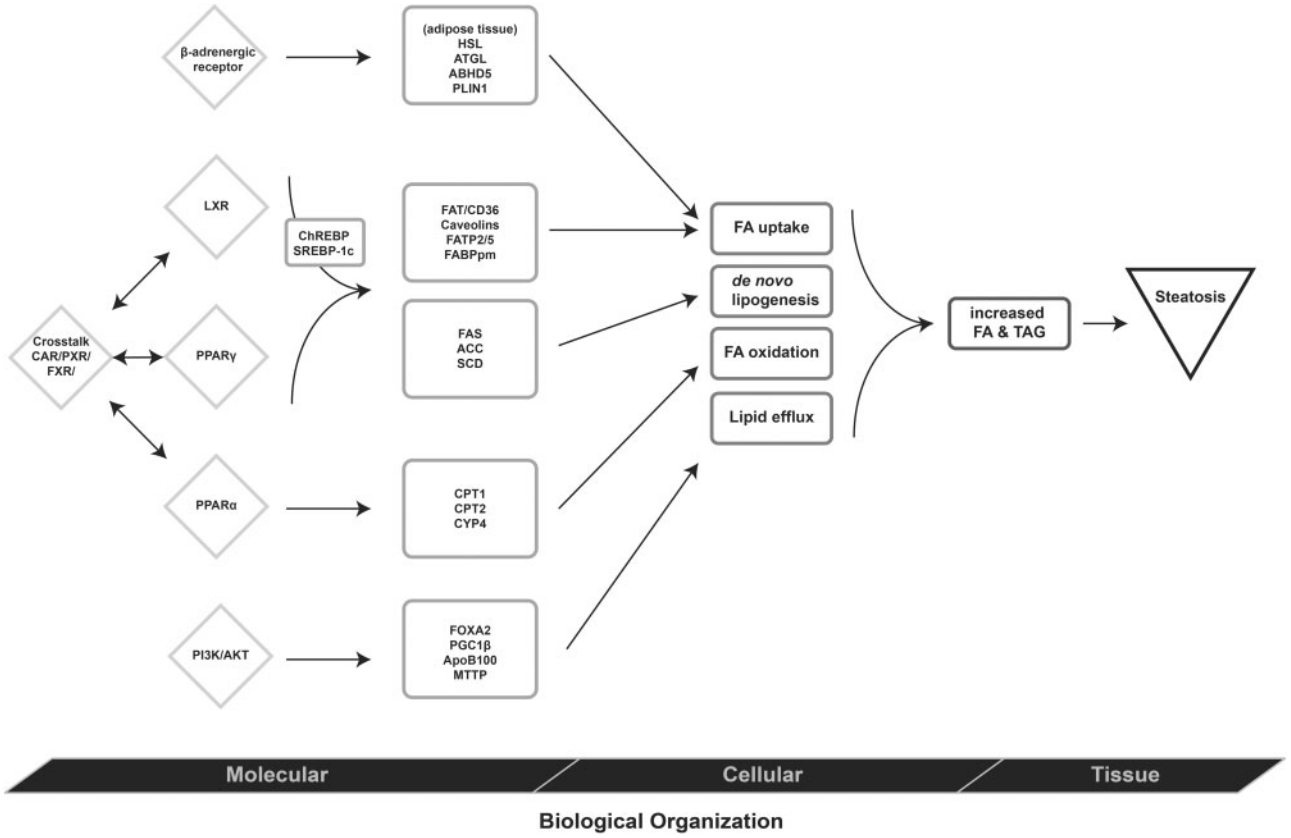

FIG. 3. Biological pathways leading to a steatosis adverse outcome. A network for hepatic steatosis is presented as molecular initiating events (diamonds) that differentially regulate molecular targets leading to alterations in key events (boxes) including fatty acid (FA) uptake, *de novo* lipogenesis, FA oxidation, and lipid efflux. An imbalance between these key events may ultimately lead to increased hepatic FA and triglyceride levels and a steatosis adverse outcome (triangle).

VLDLs are exclusively synthesized in the hepatic ER and are made up of TAGs, cholesterol, phospholipids, and ApoB100. In humans, ApoB100 is only synthesized by the liver and is the scaffold for lipid attachment. The ApoB100 provides a scaffold for the VLDL precursor as it accumulates a larger bulk of TAG (transferred by microsomal triglyceride transfer protein [MTTP] [Gibbons *et al.*, 2004]), cholesterol, and a phospholipid coat during maturation in the ER lumen and Golgi apparatus before export (Gibbons, 1990).

Components of the VLDL secretory apparatus (in particular, ApoB100 synthesis, DGAT2 activity, and MTTP-mediated TAG transfer to the maturing ApoB100 protein) are regulated by a complex interplay between hormonal/nutritional status (Li *et al.*, 2015; Pereira *et al.*, 2011). For example, during times of energy excess, the liver directs excessive TAG via VLDL to adipose tissue for storage, but VLDLs are also secreted by the liver in response to fasting to provide TAG energy sources to muscle tissue (Boron *et al.*, 2008). Transcriptional lipid efflux regulators are less clear, but evidence suggests that activation of the PI3K/AKT signaling pathway inhibits forkhead box A2 (FOXA2)/PPAR coactivator 1 beta (PGC-1β) that are required for *MTTP* expression and VLDL secretion (Wolfrum *et al.*, 2006). Furthermore, ER stress inhibits ApoB100 secretion, potentially leading to hepatic lipid accumulation (Ota *et al.*, 2008).

Molecular Initiating Events

Environmental and pharmaceutical exposures can induce NAFLD by many different mechanisms (Al-Eryani *et al.*, 2015), including receptor-ligand interactions. The nuclear receptors LXR and PPARα are key hepatic regulators of lipid homeostasis and typically heterodimerize with retinoid X receptor (RXR) before binding their cognate DNA recognition sequences to control gene transcription. LXR directly regulates lipogenic pathways that include carbohydrate response-element binding protein (Iizuka *et al.*, 2004; Mitro *et al.*, 2007), the sterol regulatory element binding protein-1c (Biddinger *et al.*, 2005; Miyazaki *et al.*, 2004), the hepatic FA uptake transporter CD36 (Zhou *et al.*, 2008), FAS, and SCD (Ntambi *et al.*, 2004). PPARα is activated by FAs and fibrate drugs and regulates FA oxidation, mitochondrial, peroxisomal, and microsomal ω-oxidation pathways (Hardwick *et al.*, 2009; Rakhshandehroo *et al.*, 2010). PPARγ is not highly expressed in liver tissue, but is induced by unsaturated FAs and members of the antidiabetic compounds, thiazolidinedione, and its activation may elicit steatosis (Al Sharif *et al.*, 2014).

Constitutive androstane receptor (CAR), pregnane X receptor (PXR), and farnesoid X receptor (FXR) share some characteristics with LXR and PPARs; they are also nuclear receptor subfamily 1 members that heterodimerize with RXR and have been implicated in NAFLD (Handschin *et al.*, 2005; Konno *et al.*, 2008). Importantly, crosstalk between CAR, PXR, FXR, LXR, and PPAR (potentially through direct heterodimerization or RXR competition) has been reported to play a functional role in lipid metabolism (Handschin *et al.*, 2005; Ide *et al.*, 2003; Xiao *et al.*, 2010; Yoshikawa *et al.*, 2003). Other extra nuclear receptors, including (but not limited to) hepatocyte nuclear factor alpha (Hardwick *et al.*, 2009) and the AhR (Angrish *et al.*, 2012, 2013), have also been implicated in in NAFLD etiology. This myriad of chemical-molecular interactions initiate complex pleiotropic effects along the CNS-autonomic-organ-cellular-molecular network that ultimately perturb key lipid metabolism pathways.

FUTURE DIRECTIONS

This review describes the pathologic, mechanistic, and toxicological underpinnings of the 4 apical KEs of hepatic steatosis. The future challenge is to apply this information to next generation toxicity testing strategies that are predictive of complex disease, such as steatosis. Application of adverse outcome pathway (AOP)-based information may assist in this endeavor. AOPs connect initial biological response to an adverse outcome through defined pathways that broadly incorporate molecular, cellular, organ, individual, and population-based information (Villeneuve *et al.*, 2014a,b). These pathways can be causal, inferential, mechanistic, or correlative to the adverse outcome (Ankley *et al.*, 2010). We summarized the key biological events leading to steatosis outlined in the review using this framework (Figure 3). The overall purpose of this framework is to identify molecular initiating events (eg, receptor activation) or KEs (eg, hepatic FA uptake, *de novo* FA and lipid synthesis, FA oxidation, and lipid efflux) that are indicative and predictive of a downstream adverse outcome. Testing paradigms can then be designed to measure these events in various biological and computational models. Importantly, such testing may be achieved using high throughput methodologies that can rapidly screen and prioritize thousands of chemicals of concern (Judson *et al.*, 2010, 2014). In addition to developing new assays, we also anticipate that AOPs can ground current *in vitro* receptor-based assays with complimentary, and quantitative, steatosis key event pathway information (Kavlock *et al.*, 2010Kavlock *et al.*, 2009, 2010). We have made steatosis AOP information freely available as "living documents" at the Collaborative AOP-Wiki (https://aopkb.org/aopwiki, accessed February 9, 2016) with the intent that as more is understood about the etiology of steatosis, these AOPs can be further developed. Ultimately, these efforts will help improve chemical screening efforts to prevent and reverse the growing incidence of NAFLD in our population.

DISCLAIMER

The research described in this article has been reviewed by the National Health and Environmental Effects Research Laboratory of U.S. Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflect the views and the policies of the Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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