Lipid Metabolism as a Therapeutic Target

Guest Editors: Terry K. Smith, Todd B. Reynolds, and Paul W. Denny
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Targeting lipid metabolism (biosynthesis and catabolism) associated with human diseases and pathogens with therapeutics has gained much momentum in recent years. This has come about in part due to the wide availability of sequenced genomes, the advancement in analytical techniques such as mass spectrometry and deep sequencing, and the increased understanding of signaling molecules. Collectively, this has advanced the knowledge of lipid metabolism for translational purposes, that is, diagnosis or treatment, and the papers and reviews in this special issue highlight some of these aspects.

One review in this issue by S. Young et al. outlines the major pathways in eukaryotic sphingolipid metabolism and catabolism and discusses these in relation to the possibilities of their therapeutic intervention against cancers, Alzheimer’s disease, inherited diseases, and numerous important human pathogens.

Such a pathogenic disease is leishmaniases, which is the subject of the paper by H. Ali et al., who investigate the reliance of old and new world *Leishmania* species on host sphingolipids and how an infection may or may not influence host sphingolipid biosynthesis.

Another review in this issue addresses the link between serum triglyceride levels caused by a dysregulation of lipoprotein lipase and the risk of development of various cancers, atherosclerosis, chylomicronemia, obesity, and type 2 diabetes. S. Takasu et al. conclude that as lipoprotein lipase plays important roles in many of these conditions and as such it is appropriate to treat it as a general target for chemo-preventive and chemotherapeutic agents.

This approach is nicely highlighted by the paper by R. Noriega-Cisneros et al., who investigate the effect of chronic administration of ethanolic extract of *Eryngium carlineae* in serum of streptozotocin-induced diabetic rats. They clearly show reduced levels of creatinine, uric acid, total cholesterol and triglycerides, thus as a general approach this could be used to reduce hyperlipidemia related to cardiovascular risk in diabetes mellitus.

In a related area, Saldanha et al. look at the behavior of human erythrocyte aggregation in presence of autologous lipoprotein, finding that human blood aliquots enriched with their own LDL-C and HDL-C showed higher levels of erythrocyte aggregation compared to controls.

N. Nikolić et al. show in their paper that overexpression of peroxisome proliferator-activated receptor γ coactivator-1α increases oxidative capacity of human skeletal muscle cells by improving lipid metabolism, thus increasing expression of genes involved in regulation of mitochondrial function and biogenesis and decreasing expression of the fast fiber-type gene marker MHCIIa. They conclude that obesity and obesity-related diseases could be therapeutically targeted by increasing expression of peroxisome proliferator-activated receptor γ coactivator-1α.

While M. Cahova et al., in their study on liver lysosomal lipase activity, conclude that overproduction of diacylglycerol may represent the causal link between high-fat die-induced hepatic triacylglycerol accumulation and hepatic insulin resistance via PKCe activation.

On a personal note, I would like to highlight that through my own research on protozoan parasites and that with various collaborators studying a wide variety of human pathogens and model systems for human diseases, mitochondrial dysfunction as a result of not maintaining its lipid homeostasis seems to be a leading cause for many of downstream
affects. Thus, I would suggest a better understanding of mitochondrial lipid metabolism (both biosynthetic and catabolic) will aid the development of effective novel therapeutics and diagnostics in the future.

Terry K. Smith
Todd B. Reynolds
Paul W. Denny
**Review Article**

**Sphingolipid and Ceramide Homeostasis: Potential Therapeutic Targets**

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**Abstract**

Sphingolipids are ubiquitous and essential structural components of eukaryotic membranes [3] as well as some prokaryotic organisms and viruses [2]. They are found predominantly in the outer leaflet of the plasma membrane [4], the lumen of intracellular organelles [5], and lipoproteins [2]. Sphingolipids (most notably ceramide) are also bioactive signalling molecules that control a plethora of cellular events including signal transduction, cell growth, differentiation, and apoptosis [6–10]. In addition, their role in protein kinase C regulation has more recently been elucidated [11]. It is noteworthy that some sphingolipid metabolites can exhibit both structural and signalling functionalities. For example, glycosphingolipids have been reported to be involved in cellular recognition complexes, for example, blood group antigens, cell adhesion, and the regulation of cell growth [4].

Over the last decade, there has been an exponential increase in the study of sphingolipids. However, the investigation and deciphering of the functions of each specific sphingolipid remains challenging due to the complexity in sphingolipid metabolic interconnection, their varied biophysical properties (neutral or charged), the hydrophobic nature of the enzymes involved, and the presence of multiple pathways that can operate in parallel [12]. The interaction of sphingolipid biosynthesis with other cellular metabolic pathways, for example glycerolipid metabolism, introduces another...
layer of complexity and the cellular role of an individual sphingolipid could be defined as a multidimensional in terms of subcellular localisation, regulation and mechanism of action(s) [12].

Whilst the scientific literature has been enriched by articles focused on structural diversity [13–18] and cellular metabolism [2, 8–10, 12, 19–21], this paper focuses on the key enzymes involved in the regulation of ceramide, a central sphingolipid and a key bioactive molecule [12]. To this end we discuss the roles of these enzymes in the regulation of biosynthesis, and in the recycling, salvage, and degradation of complex sphingolipids, in mammalian, fungal and protozoan systems. In addition, we relate these observations to disease and potential therapies.

2. Sphingolipid Metabolism

Sphingolipid metabolism is a critical cell process [22] constituting a highly complex network of interconnected pathways, with ceramide (and to a lesser degree dihydroceramide) occupying a central position in both biosynthesis and catabolism. Therefore, this simple but highly bioactive sphingolipid represents a metabolic hub [12]. In terms of ceramide, the routes of formation can be grouped into either de novo synthesis; or recycling, salvage, and degradation (Figure 2). Sphingolipid metabolism has been extensively studied in mammalian and fungal systems, where many of the enzymes involved have been identified and characterised. Consequently, the mammalian pathways will be used as the reference model in the following discussion.

2.1. De Novo Synthesis. The first step in the de novo biosynthesis of sphingolipids is the condensation of serine and palmitoyl CoA, a reaction catalysed by the normally rate-limiting serine palmitoyltransferase (SPT, EC 2.3.1.50) to produce 3-ketodihydrosphingosine [23]. SPTs are members of the pyridoxal 5’-phosphate-dependent α-oxoamine synthase family who share a conserved motif (T[FL][GTS][K][SAG][FLV][G]) around the PLP-binding lysine (in bold). The mammalian SPTs [23] (and those of other eukaryotes [24, 25]) are membrane bound in the endoplasmic reticulum as a heterodimer of subunits LCB1 and LCB2 (~53 and ~63 kDa); these are both type I integral membrane proteins sharing ~20% identity. The bacterial SPT is ~30% identical to both mammalian LCB1 and LCB2 at the amino acid level and has the conserved lysine residue in the PLP-binding motif, however the soluble 45 kDa protein forms active homodimers [26]. Palmitoyl-CoA functions as the best substrate of mammalian SPT in vitro, while it is also the dominant acyl-CoA in vivo, and thus the sphingoid bases from mammalian cells are predominantly C16 [23]. In contrast, the enzyme from the bacteria Sphingomonas wittichii utilises stearoyl-CoA most efficiently [27]. A third subunit increasing enzyme activity has been identified in Saccharomyces cerevisiae [28] and more recently 2 nonhomologous but functionally related proteins have been characterised in a mammalian system [29]. Furthermore, these additional subunits confer distinct acyl-CoA substrate specificities to the mammalian SPT thus explaining the diversity of long chain bases found in mammals [30]. As the “gatekeeper” of sphingolipid biosynthesis, loss of SPT has a catastrophic effect on mammalian cell viability with a partial loss of SPT function seen in the inherited progressive disorder, Hereditary Sensory Neuropathy type I (HSN1) [23]. The molecular basis of this condition is discussed later in this paper.

Following sphinganine (dihydrosphingosine) formation, metabolic differences are encountered. Whilst in fungi and higher plants sphinganine is hydroxylated to phytosphingosine then acylated to produce phytoceramide, in animal cells sphinganine is acylated to dihydroceramide which is later desaturated to form ceramide [31]. Ceramide (or phytoceramide), a central sphingolipid, is then transported from the ER to the Golgi apparatus where further synthesis of complex sphingolipids takes place [7, 19, 20, 32]. Ceramide can be phosphorylated by ceramide kinase [33], glycosylated by glucosyl or galactosyl ceramide synthases [34], or acquire a variety of neutral or charged head groups to form various

![Figure 1: (a) The chemical structures of sphingosine and C18-ceramide; (b) the lipids isolated by Thudichum.](image-url)
complex phosphosphingolipids depending on the host organism. For example, in animal cells ceramide is a substrate for sphingomyelin (SM) synthase to produce SM [35]. In contrast, fungi and higher plants utilise phytoceramide to produce inositol phosphorylceramide (IPC) as their principal phosphosphingolipid, a reaction catalysed by IPC synthase [36, 37]. In these organisms IPC is later glycosylated to produce more complex phosphosphingolipids, for example, mannose-IPC (MIPC), in yeast [38, 39]. Finally, the protozoa (exemplified by the Kinetoplastidae) represent a distinct third group in which ceramide [21] acquires a phosphorylinositol head group from phosphatidylinositol (PI) to produce IPC via IPC synthase [40] (Figure 3).

The synthesis of complex sphingolipids such as SM are key regulatory synthetic steps, as the rate of synthesis not only decreases the amount of ceramide, but also indirectly increases the total amount of ceramide-containing molecules that potentially could be degraded/catabolised to form ceramide.

Importantly, in such biosynthetic steps the evolutionarily divergent SM and IPC synthases are central in controlling the delicate balance of glycerolipids (PI/PC in and diacylglycerol-DAG out) on one hand, and sphingolipids (phytoceramide/ceramide in and IPC/SM out) on the other. Therefore, these enzymes have an important role as regulators of proapoptotic ceramide and promitogenic DAG [41]. In addition to a mitogenic role, DAG has also been attributed to play a role in several enzyme activation and regulatory functions [1, 2]. Notably, IPC synthase inhibitors are acutely fungicidal, with the accumulation of ceramide proposed to induce apoptosis [42]. Thus this enzyme represents an attractive target for antifungals and more recently this has been extended to the kinetoplastid protozoa [43, 44].

2.2. Recycling, Salvage, and Degradation. In addition to the de novo synthesis, the recycling, salvage, and degradation pathways modulate cellular levels of sphingolipids. These pathways operate in the direction of ceramide regeneration from

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**Figure 2:** A simplified diagram of sphingolipid metabolism in mammals. The key regulatory synthetic steps are indicated by the "traffic light" symbols. SPT: Serine Palmitoyltransferase; 3-KSR: 3-Ketosphingosine Reductase; CerS: Ceramide Synthase; CDase: Ceramidase; CDes: Ceramide Desaturase; SMS: Sphingomyelin Synthase; SMase: Sphingomyelinase; CK: Ceramide Kinase; GCS: Glucosylceramide Synthase; GCase: Glycosidases; SK: Sphingosine Kinase; SPP: Sphingosine-1-Phosphate Phosphatase; S1P: Sphingosine-1-Phosphate.
complex sphingolipid reservoirs, for example glycosphingolipids (GSLs) and (SMs), through the action of specific hydrolases and phosphodiesterases.

Sphingolipid recycling can be categorised as either lysosomal or nonlysosomal degradation. In lysosomal degradation, catabolism of GSLs occurs as sugar residues are cleaved leading to the formation of glucosylceramide and galactosylceramide. In turn, specific β-glucosidases and galactosidases hydrolyse these lipids to form ceramide which can then be subsequently deacylated by an acid ceramidase to form sphingosine [12, 45], which can then be salvaged to form ceramide by reacylation. Defects in the function of these enzymes lead to a variety of lysosomal storage disorders such as Gaucher, Sandhoff, and Tay-Sachs diseases, resulting from the impairment of membrane degradation [46]. This will be discussed in more detail later.

Degradation of sphingolipids is a necessary part of maintaining lipid homeostasis, thus SM levels are maintained by catabolic action of sphingomyelinases (SMases), either neutral or acidic, releasing ceramide and the corresponding headgroup, phosphorylcholine in the case of SM. Acid sphingomyelinase (aSMase) was the first cloned human SMase and was initially assumed to be not much more than a housekeeping gene with a prominent role in the turnover of sphingomyelin in the lysosome. However, unusually aSMase has revealed itself to encode two unique enzymes through the differential trafficking of a single-protein precursor [47]. In addition to the commonly studied lysosomal aSMase, an alternative form is secreted extracellularly and may have a role in the nonlysosomal hydrolysis of SM both in the outer leaflet of the plasma membrane and in lipoproteins in the bloodstream [48]. These studies indicate that ceramide production by sphingolipid hydrolysis in different cellular or extracellular locations may provide different metabolic effects and biological impacts. Indeed, the nonlysosomal degradation of SM is catalysed by neutral and alkaline SMases in a variety of intracellular and extracellular locations. The least studied of these SMases, the alkaline SMase (Alk-SMase), is highly tissue specific with trypsin resistance and bile salt dependency and has a key role in the dietary acquisition of ceramide by digesting SM in the gut [49]. Notably, animal studies have shown Alk-SMase is specifically downregulated in colon cancer, while membrane SM accumulates. The supplementation of dietary SM can prevent the promotion of further colonic tumors [50].

SMases are commonly activated by growth factors, cytokines, chemotherapeutic agents, irradiation and nutrient removal [51], and though they can differ in their subcellular localisation and tissue specificity, all are thought to regulate the local ceramide concentration and any corresponding stress-induced responses [52]. Ceramide and associated metabolites, such as sphingosine-1-phosphate, are known to function as second messengers, stimulating various biological activities in mammalian cells, including the activation of protein-kinases and/or protein-phosphatases 2A [53]. Increased levels of ceramides can exert antiproliferation effects, induce apoptosis, and play major roles in mitogenesis and endocytosis. There is a growing body of evidence that suggests Mg2+-dependent neutral sphingomyelinases (nSMases) are the major source for stress-induced ceramide

Figure 3: Divergence in the postceramide biosynthetic steps highlighting the conserved intersection with phospholipid metabolism as a regulator of the balance between promitogenic diacylglycerol (DAG) and proapoptotic ceramide. The key regulatory synthetic steps are indicated by the “traffic light” symbols. CS: Ceramide Synthase; CDes: Ceramide Desaturase; SMS: Sphingomyelin Synthase; SL: Sphingolipid; IPC: Inositol Phosphorylceramide; PC: Phosphatidylcholine.
production [51]. nSMases are ubiquitously expressed in mammalian cells, predominately membrane bound on the outer leaflet of the plasma membrane where most of the SM is located [52]. Other mammalian nSMases localise to the ER, where the predicted low abundance of SM has led to speculation that they may have additional lipid substrates such as lyso-platelet-activating factor [54]. In all cases of sphingolipid catabolism, the released ceramide can be either recycled into sphingolipid synthesis or degraded to sphingosine [12, 45]. The resultant sphingosine, produced from either pathway, is either recycled into sphingolipid biosynthesis or phosphorylated by a cytosolic sphingosine kinase (SK) yielding sphingosine-1-phosphate (S1P). S1P can itself be dephosphorylated back to sphingosine or irreversibly degraded by S1P lyase into the nonsphingolipid species ethan-olamine phosphate and hexadecenal, representing a unique exit point from the sphingolipid metabolic pathway [12, 45]. In fact this is the mechanism by which the kinetoplastid Leishmania obtain ethanolamine [21].

Another kinetoplastid, Trypanosoma brucei, has shown that an ER nSMase directly involved in sphingolipid catabolism is essential because its formation of ceramide is required for post-Golgi sorting and deposition of the essential glycosylphosphatidylinositol-anchored variant surface glycoprotein on the cell surface [55]. Similarly, the Leishmania nSMase is essential for virulence and, whilst able to catabolise inositol phosphorylceramide (IPC), demonstrated greater activity with SM [56].

The corresponding yeast nSMase homologue (Isc1) is also capable of IPC catabolism, generating ceramide. During early growth Isc1p resides in the ER, but in late logarithmic growth it is found in the outer leaflet of the mitochondria, where the resulting ceramide formation plays a crucial role in the reprogramming of mitochondrial gene expression during the transition from anaerobic to aerobic metabolism, coupled with a change in carbon source, that is, glucose to ethanol [57, 58].

3. Ceramide Homeostasis

As discussed, ceramides are central intermediates of sphingolipid metabolism. In addition to forming the basis of complex sphingolipids, ceramide is a bioactive molecule that regulates a myriad of cellular pathways including apoptosis, cell senescence, the cell cycle, and differentiation [59]. In addition, this lipid species is involved in the cell response to stress challenge. Notably, several anticancer drugs, for example, etoposide and daunorubicin, have been found to function by elevating the level of cellular ceramide triggering apoptosis [60–63]. The apoptotic role of ceramide [64, 65] contrasts with that of the mitogenic agonist DAG. Whilst the former stimulates signal transduction pathways associated with cell death or growth inhibition, DAG activates the various isoforms of protein kinase C associated with cell growth and survival. Thus, ceramide and DAG generation may regulate cellular homeostasis by inducing death and growth, respectively. Given that ceramide and DAG are a substrate and a byproduct, respectively, of SM and IPC syntheses, these enzymes are hypothesized to play a central role in homeostasis [9].

Recently, a human SM synthase-related protein has been shown to function as a ceramide sensor [66] with a crucial role in protecting cells against ceramide-induced cell death. Disruption of this sensor leads to ceramide accumulation in the endoplasmic reticulum and mitochondrial-mediated apoptosis. This process is suppressed by targeting a ceramide to mitochondria indicating that transfer of ceramide from the endoplasmic reticulum to mitochondria, via an unknown mechanism, is a key step in committing cells to death. The presence of a mitochondrial ceramide synthase has also been reported and hypothesized to play a role in this apoptotic process [67]. Together, these findings provide mechanistic evidence for the proapoptotic accumulation of ceramide in the mitochondria and demonstrate that the regulation of ceramide homeostasis is a vital cellular function.

4. Defects in Sphingolipid Metabolism

Despite sphingolipids being minor components in some cells, their accumulation in certain cells and tissues forms the basis of many human diseases.

4.1. Sphingolipidoses. Defects in sphingolipid catabolism, that is, lipid hydrolases, form the basis of a wide variety of human diseases. These diseases, collectively known as sphingolipidoses (Figure 4), belong to the lysosomal storage diseases and are inherited disorders characterised by accumulations in specific lipids in certain tissues and/or organs.

The most common is Gaucher disease, in which glucosylceramide accumulates due to a deficiency of glucosylceramide-β-glucosidase, causing changes in the specialised membrane microdomains termed lipid rafts. This in turn seems to impair lipid and protein sorting and consequently causes the patholgy characterising this disorder. For example, lipid rafts are necessary for correct insulin signalling, and a perturbed lipid raft composition impairs insulin signalling leading to the insulin-resistance observed in patients with Gaucher disease [68, 69].

Fabry disease is an X-chromosomal-linked inherited deficiency of lysosomal α-galactosidase A, causing deposition of globotriaosylceramide in the lysosomes of endothelial, perithelial and smooth-muscle cells of blood vessels. This leads to renal, cardiac and/or cerebral complications and, most commonly, death before the age of 50 [70, 71].

Tay-Sachs disease or GM2-gangliosidosis comes in various forms, the most extreme, infantile form being caused by defects in β-hexosaminidase A and has a high heterozygote frequency (1:27) among Ashkenazi Jews. This condition leads to death between the second and fourth years of life [72, 73].

Sandhoff disease is characterized by storage of negatively charged glycolipids and elevation of uncharged glycolipids. This disease has various clinical forms, infantile, juvenile, and adult, all with varying pathological manifestations, including a chronic variant similar to Tay-Sachs disease [74, 75].
The inherited Niemann-Pick disease (types A and B) is characterised by a deficiency in the lysosomal acidic SMase, causing an accumulation not only of sphingomyelin but also of glycosphingolipids, sphingosine and others in multilamellar storage bodies [76].

There are many other related and associated genetic diseases, Metachromatic Leukodystrophy caused by a deficiency of arylsulfatase A, Krabbe disease or globoid cell leukodystrophy caused by a deficiency of galactosylceramide-β-galactosidase and Farber disease caused by a deficiency of lysosomal acid ceramidase causing storage of excess ceramide in the lysosomes. For further details of these and other sphingolipid metabolic diseases, refer to an excellent review by Kolter and Sandhoff [77].

4.2. Hereditary Sensory Neuropathy. Clinical disorders are also associated with alterations in SPT activity, although a complete lack of SPT activity is predicted to be embryonically lethal. The inherited disease hereditary sensory neuropathy type I (HSN1) is a progressive degeneration of lower limb sensory and autonomic neurons and has been associated with mutations in the human LCB1 gene [78, 79]. LCB1 (SPTLC1) mutations confer dominant negative effects on SPT, thus substantially reducing SPT activity and hence the rate of de novo synthesis of sphingolipids [80]. Recently, point mutations have been found in the catalytic SPT subunit (LCB2 or SPTLC2) in patients suffering from HSN1. These were confirmed to affect SPT activity using an in vitro system. No mutations were observed in the third (SPTLC3) subunit in these patients [81].

Point mutations in the SPT complex also affect SPT activity in terms of substrate specificity and alanine can be used instead of serine in the condensation with palmitoyl-CoA, resulting in the formation and accumulation of 1-deoxy-sphinganine in the serum of these patients [82].

4.3. Alzheimer’s Disease. A relatively recent discovery was the highly altered sphingolipid metabolism in brain cells in Alzheimer’s disease [83]. The latest study has highlighted the key role of an nSMase in the disease by promoting the damaging effects of fibrillar amyloid-β 1–42 peptide-activated astroglia through ceramide production and thus apoptosis in neuronal cells [84].

**Figure 4:** Defects in mammalian sphingolipid catabolism and their associated sphingolipidoses. The enzymes responsible are indicated at the glycosidic linkages on which they act.
In addition, SPT has been shown to be downregulated by the amyloid precursor protein [85]. This novel physiological function of the amyloid precursor protein suggests that SPT and sphingolipid metabolism is involved in Alzheimer’s disease pathology.

4.4. Other Diseases. Obesity and its established association with insulin resistance, type 2 diabetes, and cardiovascular disease are directly and/or indirectly involved in the overaccumulation of long-chain fatty acids. The resulting surplus to the storage capacity of adipose tissue results in deposition in nonadipose tissues, such as the liver, muscle, heart, and pancreatic islets. This leads to deleterious effects, not only as atherosclerosis, but excess lipids are also forced into alternative nonoxidative pathways resulting in the formation of reactive lipid moieties, such as sphingolipids, that promote metabolically relevant cellular dysfunction (lipotoxicity) and programmed cell death (lipoapoptosis) [86–88].

5. Sphingolipid Biosynthesis: An Attractive Drug Target

Due to the complexities of sphingolipid metabolism and associated defects in a variety of tissue types and cell compartments, there is a significant challenge in the understanding, diagnosis, and treatment of genetic diseases such as those discussed above. However, clinical manipulation sphingolipid metabolism will prove key in the treatment of these conditions; in addition it is becoming clear that by inducing the accumulation of proapoptotic ceramide, therapies for cancer and infectious disease may be developed.

5.1. Sphingolipidoses. The general strategy to treating the inherited human diseases involving sphingolipid metabolism, the sphingolipidoses is the restoration of the defective lysosomal degradation. These include enzyme replacement, heterologous bone marrow transplantation as a form of cell-mediated therapy, gene therapy and the use of chemical chaperones for enzyme-enhancement therapy. An alternative strategy is the reduction of substrate influx into the lysosomes using substrate reduction (substrate deprivation) therapy ([77] and references therein).

5.2. Cancer. The roles of ceramide in diverse cellular responses to stress, particularly apoptosis, has been discussed above and as mentioned elevated ceramide often result from treatment with anticancer drugs and also irradiation. Therefore the manipulation of sphingolipid biosynthesis and homeostasis to elevate ceramide levels and induce programmed cell death is a viable strategy for anticancer therapies [89]. Conversely, dysregulation of ceramide metabolism affects the cellular response to chemotherapy or other anticancer regimens by rendering the cells more resistant to killing; in these cases therapeutic manipulation of ceramide metabolism could overcome this resistance [90, 91]. Further developments in the manipulation of sphingolipid metabolism as an anticancer strategy will undoubtedly follow with the breakthrough discovery that FTY720, a water-soluble sphingosine analogue effective in many cancer models which acts by downregulating nutrient transporter proteins in cancer cells at least partially via ceramide generation [92]. The resulting starvation induces a homeostatic autophagy selectively in cancer cells sensitive to nutrient limitation, while normal cells have the ability to adapt and survive by becoming quiescent. Notably AAL-149, an FTY720 analogue, similarly kills patient-derived leukemic cells, but not cells of healthy donors, without the dose-limiting toxicity of FTY720. Thus, by targeting the sphingolipid metabolism of cancer cells rather than any specific oncogenic defect, such compounds should have potent activity against a range of tumours, particularly if applied in combination with inhibitors of autophagy.

5.3. Pathogens. The essential functions of sphingolipids, coupled with the divergence of the biosynthetic pathway between mammals and eukaryotic pathogens have resulted in the investigation of the biosynthetic enzymes as possible drug targets for antifungal and antiprotozoal. Consequently, inhibitors of many of the steps in sphingolipid biosynthesis have been described [2, 43]. Before the synthesis of ceramide/phosphoceramide the sphingolipid biosynthetic pathway is largely conserved across evolution. At least in part because of this, all the inhibitors identified as targeting fungal enzymes in this part of the biosynthetic pathway are nonselective and inhibit the mammalian orthologues [43]. This has curtailed their clinical application as anti-fungal agents, for example fumonisin B1 which inhibits the fungal phytoceramide synthase demonstrated mammalian toxicity [93]. In contrast the post-ceramide divergence represented by the absence of IPC synthase and inositol-based sphingolipids in mammalian cells, highlights the therapeutic potential of inhibitors targeting fungal IPC synthases. Such inhibitors could result in selective antifungal drugs with minimal host toxicity. Additionally, the identification and isolation of functional orthologues of the fungal enzyme in the kinetoplastid protozoan parasites (Leishmania spp., Trypanosoma brucei and Trypanosoma cruzi) indicated that IPC synthase is a valid target for antiprotozoal compounds [3]. One of the T. brucei sphingolipid synthases, a novel bifunctional enzyme catalysing the synthesis of both IPC and SM, is essential for parasite growth and can be inhibited in vitro by the antifungal aureobasidin A at low nanomolar concentrations [43]. As the causative agent of Chagas’ disease, Trypanosoma cruzi has a complex lifecycle with an essential intracellular stage in vertebrate hosts, in addition to an extracellular existence in an insect vector. Necessary to persistence of the lifecycle is the synthesis of surface glycosylphosphatidylinositol (GPI) anchored glycoconjugates, meaning that the biosynthesis of GPI anchors is attractive target for new therapies against Chagas’ disease [93]. As many T. cruzi GPI anchors contain IPC as the lipid portion, the sole IPC synthase is highlighted as a new therapeutic target for Chagas disease.

To date only the natural compounds—aureobasidin A [33, 94, 95], Khafrefungin [94], and Rustmicin [96, 97] have been reported as potent inhibitors of the fungal IPC synthase. As discussed, there is an inhibitory effect of aureobasidin A against the Kinetoplastid enzyme orthologues [43, 98, 99], although the specificity of this remains unclear [3, 100, 101].
Unfortunately, further development of all the three known inhibitors of IPC synthase has stalled either due to lack of physical properties required for an acceptable pharmacokinetic profile [42, 102], or because their highly complex structures render chemical synthesis challenging. Moreover, the few synthetic efforts to modify or synthesise analogues that have been reported, resulted in compounds with either reduced or no activity [103, 104]. What has proven more successful however is the development of substrate (ceramide) analogues, with targeted inhibition against the protozoal IPC synthases in cellulo [105].

In addition to sphingolipid synthesis as a therapeutic target against parasitic protozoa, degradation of sphingolipids similarly is an area providing new opportunities for anti-protozoal compounds. *Leishmania* spp. use sphingolipid biosynthesis to generate ethanolamine (Etn), essential for the survival and differentiation from procycltics to virulent metacyclics [21]. A likely starting point for Etn production is the degradation of IPC, and a putative neutral SMase and/or IPC hydrolyase (IPCase), designated *ISCL* was identified in the *L. major* genome [106]. *ISCL* showed much greater activity against non-self SM over IPC, suggesting a role in host SL degradation confirmed by *ISCL* null mutants failing to induce lesions in susceptible BALB/c mice. Further investigation revealed that host SL catabolism by *Leishmania* was essential to resist the harsh acidic environment in the phagolysosomes of macrophages [107]. These findings reveal that SL catabolism, as well as anabolism, by *Leishmania* is necessary for proliferation of the parasite in the mammalian host, making the ISCL enzyme an equally attractive target for inhibition studies. *Trypanosoma cruzi* invades mammalian cells by attaching and mimicking injury to the host plasma membrane, inducing a repair process that involves the Ca2+-dependent exocytosis of lysosomes [108]. As host acid sphingomyelinase (aSMase) is delivered by lysosomes to the plasma membrane, its ceramide-generating activity promotes rapid endocytosis to internalise the seemingly damaged membrane and the attached parasites. Consequently any inhibition or reduction of this lysosomal aSMase blocks *T. cruzi* invasion, though subsequent treatment with an extracellular sphingomyelinase can restore the infection to normal levels. In a similar approach it has been demonstrated that inhibition of host cell SPT, and so sphingolipid biosynthesis, by myriocin, suppresses hepatitis C virus replication [109]. In addition, it has emerged that ceramide induces activation of double-stranded RNA-dependent protein kinase-mediated antiviral response [110]. These recent findings suggest that manipulation of host sphingolipid metabolism may provide a new combined therapeutic strategy for treatment of both protozoal and viral infections.

6. Perspective

Recent studies of the genetic, biochemical and cell biology of sphingolipids have provided exciting new insights into their function, regulation and control, allowing the consideration of future manipulations to aid the fight against human diseases including cancer and major fungal and parasitic infections. In addition, this information may ultimately aid the treatment of several rare genetic disorders (e.g. the sphingolipidoses) and, perhaps, Alzheimer’s disease. However, in most cases these studies are at an early stage and further work is required to establish proof of concept. This will undoubtedly be achieved as progress towards a fuller understanding of the complex and multilayered metabolic pathways of sphingolipid metabolism is realized, and as inhibitors of the enzymes involved become available.

**Abbreviations**

- **SM:** Sphingomyelin
- **IPC:** Inositol phosphorylceramide
- **MIPC:** Mannose-IPC
- **PE:** Phosphatidyl ethanolamine
- **DAG:** Diacylglycerol
- **CS:** Ceramide synthase
- **CDase:** Ceramidase
- **SMases:** Sphingomyelinases
- **aSMase:** Acid sphingomyelinase
- **Alk-SMase:** Alkaline SMase
- **SPT:** Serine palmitoyltransferase
- **3-KSR:** 3-Ketosphingosine reductase
- **CerS:** Ceramide synthase
- **CDase:** Ceramidase
- **CX:** Ceramide desaturase
- **SMS:** Sphingomyelin synthase
- **SMase:** Sphingomyelinase
- **CK:** Ceramide kinase
- **GCS:** Glucosylceramide synthase
- **GCase:** Glycosidases
- **SK:** Sphingosine kinase
- **SPP:** Sphingosine-1-phosphate phosphatase
- **SIP:** Sphingosine-1-phosphate
- **HSN1:** Hereditary sensory neuropathy type I

**Author Contribution**

Both Simon A. Young and John G. Mina contributed equally to this paper.

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Research Article

Hypolipidemic Activity of Eryngium carlinae on Streptozotocin-Induced Diabetic Rats

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Diabetes mellitus (DM) is a significant risk factor for the development of cardiovascular complications. This study was undertaken to investigate the effect of chronic administration of ethanolic extract of Eryngium carlinae on glucose, creatinine, uric acid, total cholesterol, and triglycerides levels in serum of streptozotocin- (STZ-) induced diabetic rats. Triglycerides, total cholesterol, and uric acid levels increased in serum from diabetic rats. The treatment with E. carlinae prevented these changes. The administration of E. carlinae extract reduced the levels of creatinine, uric acid, total cholesterol, and triglycerides. Thus administration of E. carlinae is able to reduce hyperlipidemia related to the cardiovascular risk in diabetes mellitus.

1. Introduction

Diabetes mellitus (DM) describes a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both [1]. The effects of DM include long-term damage, dysfunction, and failure of various organs. Diabetic people present a 2–4-fold higher risk of developing cardiovascular disease than normoglycemic population of similar age and sex. The cardiovascular complications attributable to atherosclerosis are responsible for 70–80% of all causes of death in patients with diabetes and represent more than 75% of all hospitalizations for diabetic complications [2]. The primary goal of diabetes treatment is the prevention of macrovascular complications (myocardial infarction, heart failure, ischemic stroke), as well as the microvascular complications (retinopathy, neuropathy, and nephropathy); for that reason, most patients require not only a good glycemic control but also treatment for dyslipidemia [3]. Dyslipidemia (disruption in the normal levels of lipids, mainly cholesterol and triglycerides) is considered a major cardiovascular risk factor in diabetes [2]. Therefore, the detection of dyslipidemia and its treatment to reduce the cardiovascular risk and its consequences are required in diabetic patients.

The growing need to find alternatives for the treatment of diabetes justifies the study of medicinal plants used in traditional medicine. There are several reports about the beneficial effects of a wide range of plants to treat diabetes. Hypoglycemic effect was observed in extracts of plants such as Amaranthus viridis [4] and Psidium guajava [5] whose effects are almost comparable with the synthetic drug tolbutamide, while Zaleya decandra [6] has effects equivalent to glibenclamide. Other plants also appear to exert their beneficial effects by improving the dyslipidemia and oxidative stress that characterize diabetes. For example, Morus indica L. [7] decreases LDL-cholesterol and VLDL-cholesterol level and increases HDL-cholesterol levels, while Phyllanthus amarus [8] improves antioxidant status and reduces the risk of oxidative stress.

In México, the use of plants for diabetes treatment is generally carried out in an empirical basis. In this regard, it seems...
that many plants for diabetes treatment were originally used for a variety of kidney disorders and most notably for their diuretic effect [9]. This study was conducted to analyze the effect of chronic administration of ethanolic extract of *E. carlinae* in diabetic rats. This plant has often used in traditional medicine to treat diabetes; however there are no studies indicating the effects of its consumption. *Eryngium carlinae* F. Delaroche is a perennial herb plant considered a weed belonging to the family Umbelliferae. It is distributed in forests of fir, pine and pine-oak hillsides, and canyons, deep soils rich in organic matter. It is distributed from 2020–2590 meters above sea level. It is commonly known as “Frog herb”. Decoctions of the aerial parts of the plant are used to treat coughs, indigestion, diseases of the prostate, lipid disorders, and diabetes [10]. It has been attributed healing and diuretic properties to extracts from the plant. Other species of *Eryngium* (*Eryngium columnare*) have been used to treat kidney disease, diarrhea, allergy, cough, and cancer [10]. Therefore, the aim of this research was to determine the effect of chronic oral administration of ethanolic extract of *E. carlinae* on some biochemical parameters in STZ-induced diabetic rats.

**2. Materials and Methods**

2.1. Vegetal Material. Plant samples of *Eryngium carlinae* F. Delaroche were collected in September and October, 2008, in the region of Nuevo San Juan Parangaricutiro, Michoacán, México. The plant was identified by Miguel Angel Bello-González PhD (Faculty of Agrobiology, Universidad Michoacana de San Nicolás de Hidalgo) genus and species preserving. A voucher specimen was deposited at the Biology Faculty Herbarium of the Universidad Michoacana de San Nicolás de Hidalgo (no. 15214). The aerial part of the plant was dried at room temperature and pulverized.

2.2. Extract Preparation. Ethanolic extract of the plant was prepared by adding 1,000 mL of absolute ethanol to 100 g of plant powder and kept at 5°C for five days. The extract was then filtered, concentrated in a rotary evaporator at vacuum and at a temperature lower than 50°C, evaporated at room temperature, suspended in ethanol 96%, and stored in the dark at 5°C. The percentage yield of the dry residue was 1.27% w/w.

2.3. Animals. Male Wistar rats weighing 280–360 g were used. They were housed and maintained at room temperature with day/night cycles of 12 h. They were fed with standard rodent diet and water *ad libitum*. We followed the recommendations of the regulatory standard for the use of animals issued by the Mexican Ministry of Agriculture in the paragraph of the Federal Regulations for the Use and Care of Animals (NOM-062-ZOO-1999). This research was also approved by the Institutional Committee for Use of Animals of the Universidad Michoacana de San Nicolás de Hidalgo.

2.4. Diabetes Induction. Diabetes was induced by intraperitoneal administration of STZ (45 mg/kg of body weight) dissolved in citrate buffer (pH 4.5). Control rats were injected with citrate buffer alone. Five days after streptozotocin administration, the glucose levels were determined to confirm diabetes. Rats exhibiting blood glucose levels >300 mg/dL were considered for the study.

2.5. Experimental Protocol. Rats were randomly divided into four groups of six rats. Group I, control (vehicle, ethanol 50%), Group II, control + *E. carlinae* (30 mg/kg of body weight), Group III, diabetic (vehicle, ethanol 50%), Group IV, diabetic + *E. carlinae* (30 mg/kg of body weight). The extract was given by oral administration using oral gavage. The treatment was continued daily for 40 days.

2.6. Effects of *E. Carlinae* on Glycemia and Body Weight. Glucose estimation was started just before extract administration and followed every 5 days using a commercial glucometer (Accu-Check Sensor III Glucometer) through a puncture in the tail tip, and animal weight was recorded at a time during the 40 days.

2.7. Effects of *E. Carlinae* on Hematological and Biochemical Parameters. At 40 days of treatment, the animals were fasted overnight and sacrificed by decapitation. The blood was collected, and serum was separated and used for biochemical estimations. The levels of glucose, creatinine, uric acid, total cholesterol, and triglycerides were estimated spectrophotometrically using a commercial assay kit (BioSystems, Spain). Glucose was determined measuring enzymatic oxidation catalyzed through the Trinder reaction [11]. Creatinine was determined by a kinetic method without deproteinization as reported by Junge et al. [12]. Uric acid was determined by an enzymatic photometric method using TBHBA (2, 4, 6-tri-bromide-3-hydroxybenzoic acid). Total cholesterol was determined measuring the enzymatic hydrolysis and oxidation with the Trinder reaction [13]. Triglycerides were determined by an enzymatic colorimetric method using glycerol-3-phosphate oxidase [14]. Whole blood samples were used for hemoglobin (Golden Bell, México) and glycosylated hemoglobin (DiaSys, Germany) determinations using commercial assay kits.

2.8. Statistical Analysis. The results were expressed as the mean ± Standard Error (SE) of at least six independent experiments. Statistical significances (*P* ≤ 0.05) were determined with Student’s *t*-test using GraphPad Prism 5 software.

**3. Results**

Changes in the body weight of the rats are presented in Table 1. In control groups, the extract did not alter the body weight gain as this parameter increased 31% and 29% at the end of the treatment in both control and control + *E. carlinae* groups, respectively, compared to their initial body weight. Similarly, no differences in body weight were detected between diabetic and diabetic + *E. carlinae* groups throughout the study.
With regard to blood glucose levels, Table 1 shows the records taken during the 40 days of treatment with *E. carlinae*. It can be observed a significant increase in glucose levels in diabetic groups compared with controls at the beginning of treatment. Oral administration of *E. carlinae* (30 mg/kg of body weight) for 40 days showed no reduction in blood glucose.

Table 2 shows the levels of hemoglobin and glycosylated hemoglobin in the different groups. The results show that hemoglobin levels are not affected by treatment with *E. carlinae*. Glycosylated hemoglobin determinations show a significant increase in the percentage of glycosylated hemoglobin in diabetic group compared to control group, and the treatment with *E. carlinae* did not change this behavior.

Table 3 shows the levels of glucose, creatinine, uric acid, total cholesterol, and triglycerides in serum of all groups. It is important to point out that these determinations were made with animals 12 h fast. The levels of glucose, uric acid, total cholesterol, and triglycerides in serum were significantly increased in the diabetic group when compared to control group. The treatment with *E. carlinae* decreased creatinine, uric acid, total cholesterol, and triglycerides levels in serum compared with the diabetic group.

### 4. Discussion and Conclusions

Oral administration of the ethanolic extract of *E. carlinae* was conducted for 40 days in a concentration of 30 mg/kg of body weight. During the treatment it was observed that the control group had a weight gain corresponding to age-appropriate growth (Table 1). In contrast, the diabetic group showed no weight gain, which is characteristic of this model of diabetes, and the treatment with *E. carlinae* did not change this tendency.

Glycemic control is a priority in diabetic patients because it has relationship with a decrease in microvascular complications in diabetes [15]. In the measurements made at the end of treatment (Table 1), there was no reduction in glucose level in the groups that received *E. carlinae*. The glycohemoglobin (HbA1c) is a general term used to describe hemoglobin that has been modified by the addition of glucose through a nonenzymatic mechanism, and the HbA1c is one of those glycosides compounds in particular that reflects average blood glucose in patient 2 or 3 months before blood collection [16, 17]. According to the United Kingdom Prospective Diabetes Study (UKPDS), each 1% reduction in glycosylated hemoglobin (HbA1c) was associated with a 37% reduction in microvascular complications, 18% fewer myocardial infarction, and 21% fewer diabetes-related deaths [15]. The results obtained from the measurement of HbA1c (Table 2) show a clear difference between control (HbA1c 3.32%) and diabetic (HbA1c 7.48%) group. The diabetic group that received *E. carlinae* did not show significant decrease in HbA1c (HbA1c 7.34%). Based on this and the results from Table 1, we can conclude that the ethanolic extract *E. carlinae* has not hypoglycemic effect. However, it is noteworthy that many of the plants that have been reported as hypoglycemic agents are less effective in severe diabetes [18], so it may be necessary to evaluate the extract in another model of diabetes to rule out completely its possible hypoglycemic action.

Diabetes leads to renal dysfunction [19]. Measurement of creatinine can be considered as a marker of renal dysfunction. Our results suggest that the administration of *E. carlinae* in the diabetic group decreased level of creatinine (Table 3), probably by improving renal function. The complex pathogenesis for the development of diabetic nephropathy is not well understood. One factor that has been associated with renal and cardiovascular disease is serum uric acid. It has been found that level of uric acid circulating in the upper end of normal range concentration is an independent predictor for development of diabetic nephropathy, which supports the concept that uric acid may be involved in the pathogenesis of diabetic microvascular complications [20]. Uric acid level in the diabetic group is elevated compared to control group.

### Table 1: Effect of *E. carlinae* on body weight and blood glucose.

| Groups       | Body weight (g) | Blood glucose (mg/dL) |
|--------------|-----------------|-----------------------|
|              | Initial (day 0) | Final (day 40)       |
|              | 0               | 5                     |
|              | 10              | 20                    |
|              | 25              | 30                    |
|              | 35              | 40                    |
| Control      | 349 ± 4         | 456 ± 10              |
|              | 102 ± 4         | 109 ± 13              |
|              | 92 ± 4          | 89 ± 1                |
|              | 93 ± 13         | 86 ± 4                |
|              | 90 ± 17         | 74 ± 0.0              |
|              | 82 ± 4          |                       |
| Control + *E. carlinae* | 317 ± 12     | 408 ± 17              |
|              | 11 ± 7          | 116 ± 10              |
|              | 97 ± 4          | 95 ± 2                |
|              | 105 ± 2         | 90 ± 4                |
|              | 94 ± 4          | 84 ± 3                |
|              | 85 ± 2          |                       |
| Diabetic     | 307 ± 15        | 305 ± 17              |
|              | 551 ± 20*       | 423 ± 16              |
|              | 411 ± 13        | 549 ± 21              |
|              | 535 ± 22        | 580 ± 15              |
|              | 538 ± 22        | 530 ± 30              |
|              | 571 ± 29        |                       |
| Diabetic + *E. carlinae* | 298 ± 12     | 302 ± 14              |
|              | 436 ± 16*       | 372 ± 14              |
|              | 365 ± 19        | 475 ± 33              |
|              | 373 ± 24        | 410 ± 29              |
|              | 405 ± 38        | 566 ± 15              |
|              | 537 ± 27        |                       |

Values are mean ± SE. *P ≤ 0.05 versus control group day 0.

### Table 2: Effect of *E. carlinae* on hemoglobin and glycosylated hemoglobin (HbA1c).

| Groups          | Hemoglobin (mg/dL) | Glycosylated hemoglobin (% HbA1c) |
|-----------------|--------------------|----------------------------------|
| Control         | 16 ± 0.7           | 3.32 ± 0.07                      |
| Control + *E. carlinae* | 16 ± 0.1     | ND                               |
| Diabetic        | 17 ± 0.5           | 7.48 ± 0.08*                     |
| Diabetic + *E. carlinae* | 16 ± 0.8     | 7.34 ± 0.04                      |

Values are mean ± SE. *P ≤ 0.05 versus control group.
(Table 3). Interestingly, the higher levels of uric acid in diabetic rats is diminished significantly by the administration of *E. carlinae*, which could be related to a delay in the onset of the complications of diabetes, as has been previously suggested [20]. Abnormalities in the metabolism of lipids are one of the most frequent complications in diabetes. Among the most common lipid abnormalities is the elevation of cholesterol and triglycerides. Hypertriglyceridemia has been identified as a major risk factor for cardiovascular complications [21]. Diabetic group had elevated triglycerides level compared to control group (Table 3). This increase was normalized by treatment with *E. carlinae*. With regard to total cholesterol level, the treatment with *E. carlinae* was able to reduce this level (Table 3). Based on our results we conclude that the ethanolic extract of *E. carlinae* has no hypoglycemic effect; however, by its hypolipidemic effect it could be used as an adjuvant in the treatment of diabetes. Further studies need to be done to characterize the active components of the ethanolic extract of *E. carlinae* and its mechanism of action.

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**Table 3: Effect of *E. carlinae* on biochemical parameters after oral administration during 40 days.**

| Groups          | Glucose (mg/DL) | Creatinine (mg/DL) | Uric acid (mg/DL) | Total cholesterol (mg/DL) | Triglycerides (mg/DL) |
|-----------------|-----------------|--------------------|-------------------|----------------------------|-----------------------|
| Control         | 85 ± 6          | 0.59 ± 0.02        | 1.40 ± 0.04       | 59 ± 3                     | 153 ± 18              |
| Control + *E. carlinae* | 79 ± 3          | 0.56 ± 0.01        | 1.17 ± 0.26       | 54 ± 3                     | 128 ± 7               |
| Diabetic        | 359 ± 8*        | 0.48 ± 0.06        | 2.43 ± 0.50*      | 78 ± 6*                    | 252 ± 19*             |
| Diabetic + *E. carlinae* | 376 ± 21       | 0.32 ± 0.02*       | 1.10 ± 0.33*      | 46 ± 6*                    | 115 ± 7*              |

Values are mean ± SE of at least n = 6. *P ≤ 0.05 versus control group; †P ≤ 0.05 versus diabetic group.
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Review Article

Lipoprotein Lipase as a Candidate Target for Cancer Prevention/Therapy

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Epidemiological studies have shown that serum triglyceride (TG) levels are linked with risk of development of cancer, including colorectal and pancreatic cancers, and their precancerous lesions. Thus, it is assumed that serum TG plays an important role in carcinogenesis, and the key enzyme lipoprotein lipase (LPL), which catalyzes the hydrolysis of plasma TG, may therefore be involved. Dysregulation of LPL has been reported to contribute to many human diseases, such as atherosclerosis, chylomicronaemia, obesity, and type 2 diabetes. Recently, it has been reported that LPL gene deficiency, such as due to chromosome 8p22 loss, LPL gene polymorphism, and epigenetic changes in its promoter region gene, increases cancer risk, especially in the prostate. In animal experiments, high serum TG levels seem to promote sporadic/carcinogen-induced genesis of colorectal and pancreatic cancers. Interestingly, tumor suppressive effects of LPL inducers, such as PPAR ligands, NO-1886, and indomethacin, have been demonstrated in animal models. Moreover, recent evidence that LPL plays important roles in inflammation and obesity implies that it is an appropriate general target for chemopreventive and chemotherapeutic agents.

1. Introduction

A high-calorie diet and low physical activity, part of the so-called “Westernization” of lifestyle, are associated with elevated incidences of the breast, colon, liver, pancreas, and prostate cancers. Moreover, they are also linked with the risk of obesity, type 2 diabetes, and dyslipidemia. The World Cancer Research Fund and American Institute for Cancer Research have evaluated causal relationships between body fat and cancer and provided strong evidence for roles in such as colorectum and pancreas cancers [1]. In Japan, overweight and obesity (body mass index ≥25) are reported to be associated with cancers of specific organs, such as the colorectum (male), postmenopausal breast (female), and the liver in individuals positive for hepatitis C virus infection [2–4].

Greater body fatness is a major risk factor for the metabolic syndrome, which presents as a combination of symptoms, such as dyslipidemia (elevated triglyceride (TG) levels or low high-density lipoprotein (HDL) cholesterol), elevated blood pressure, and elevated fasting glucose levels. Hypertriglyceridaemia is associated with the risk of colon cancer in Japanese men (HR = 1.71) and being overweight with the risk of breast cancer (HR = 1.75) [5]. In addition, most epidemiological studies, including our own, have consistently showed that serum TG levels are associated with the risk of colorectal adenoma, a precursor lesion of colorectal cancer [6–11]. Thus, it is assumed that serum TG could play an important role in carcinogenesis and that the key enzyme lipoprotein lipase (LPL), which catalyzes the hydrolysis of plasma TG, may also be involved. In this paper, we focus on the roles of LPL in cancer development and further discussed possible approaches to cancer prevention/therapy.

2. Function, Structure, and Gene Regulation of LPL

2.1. Functions and Structure of LPL. LPL plays an important role in lipid metabolism as an enzyme responsible for hydrolysis of the TG component in circulating chylomicrons and very-low-density lipoprotein (VLDL) via binding with apolipoprotein C2 [12, 13]. Thus, lowering or deficiency of LPL expression is associated with hyperlipidemia [14, 15]. The LPL enzyme itself is composed of two structurally
distinct regions. The amino-terminal domain is responsible for catalysis with a catalytic center formed by three amino acids (Ser^132, Asp^156, and His^241). The carboxy-terminal domain of LPL is required for its binding to the lipoprotein substrate [3, 16–18].

2.2. LPL Gene Expression and Its Regulation. The human LPL gene is located on chromosome 8p22 and composed of 10 exons [19]. LPL is ubiquitously expressed in the whole body, but especially in the adipose tissue and the skeletal muscle [20, 21] and is regulated by hormonal and inflammatory stimuli, such as insulin [22, 23], glucocorticoid [24, 25], adrenaline [26], tumor necrosis factor (TNF)-α [27, 28], transforming growth factor (TGF)-β [29], and interleukin (IL)-1β [27].

The expression of LPL is controlled transcriptionally and posttranscriptionally. Basal promoter activity has been shown to be regulated by Oct-1 and the NF-Y binding motifs [30, 31], and the 5′-CCTCCCCC-3′ motif, which interacts with Sp1 and Sp3 [32]. Induction of LPL gene transcription is mediated by the peroxisome proliferator response element (PPRE) and the responsible element which binds to sterol regulatory element-binding protein (SREBP) [33, 34]. The effect of insulin on LPL expression is an example of posttranscriptional control, the hormone being suggested to increase LPL mRNA levels via mRNA stabilization [23, 35].

3. Relationship between LPL and Cancer: Human Studies

3.1. Loss of LPL and Resultant Common Disease. LPL has been reported to play key roles in many human diseases, such as atherosclerosis, obesity, type 2 diabetes, chylomicronemia, Alzheimer’s disease, and cachexia [15]. Especially, such as atherosclerosis, obesity, type 2 diabetes, chylomicronemia (familial hyperchylomicronemia) [36]. Homozygous deficiency of LPL in humans is rare, but heterozygous deficiency is observed in around 3% of people with various ethnic backgrounds [37, 38]. Although these individuals have elevated serum levels of TG and decreased HDL cholesterol [39], it is not clear whether they are at increased risk of atherosclerosis, ischemic heart disease, type 2 diabetes, and cancer. There is a report that the LPL S447X mutation is associated with a higher risk of pancreatic calcification and steatorrhea in hyperlipidemic pancreatitis [40]. Since LPL provides fatty acids to the tissues and fatty acids evoke insulin resistance, LPL gene deficiency could affect glucose metabolism. However, whether heterozygous LPL deficiency reduces plasma glucose levels or not is still controversial. One paper described reduction of plasma glucose levels, but two others observed no effect as compared with LPL intact humans [41–43]. On the other hand, it has been reported that patients with poorly controlled diabetes frequently have dyslipidemia due to defects in LPL enzyme activity [44].

3.2. Effects of Chromosome 8p22 Loss and LPL Gene Polymorphisms on Cancer Risk. Alteration in genomic DNA, such as point mutations and deletions/amplifications or epigenetic changes such as CpG island hypermethylation and histone modification, can induce abnormal gene expression, which in the case of tumor suppressor genes or oncogenes could eventually lead to carcinogenesis. The human LPL gene has been mapped to chromosome 8p22 and previous studies on loss of heterozygosity (LOH) in colorectal tumors suggested that a putative tumor suppressor gene may lie within the short arm of chromosome 8, that is, 8p22-p21.3. Loss of 8p23.1-22 is also reported to be an important stage in initiation or promotion of hepatocellular carcinoma development and may also be the most frequent chromosomal alteration in prostate cancer [45]. It has been found that deletion of LPL is observed in 68% (52/76) of localized prostate cancers by FISH analysis [46]. It has further been reported that chromosomal region 8p23.1–8p21.1 may harbor one or more important prostate-cancer susceptible loci based on linkage analyses in 159 hereditary prostate cancer families [47, 48]. To date, several new candidate cancer-susceptible genes have been cloned to 8p22, such as deleted in breast cancer 2 (DBC2), leucine zipper tumor suppressor 1 (LZTS1), deleted in liver cancer 1 (DLC1), and mitochondrial tumor suppressor 1 (MTUS1) [49–52]. Thus, cancer-susceptible genes mapped close to the LPL gene could be affected by LPL gene deletion, and exert combined effects in promoting carcinogenesis.

Moreover, an LPL Ser447Stop polymorphism has been shown to be associated with prostate cancer risk [53] and the LPL gene is commonly methylated in prostate tumors [54]. LPL promoter CpG island methylation has been revealed in 45% of LPL-deleted tumors and in 22% of LPL-retaining tumors [54]. Biallelic inactivation of LPL by chromosomal deletion and promoter methylation may thus contribute to prostate tumorigenesis, but information is lacking regarding pancreatic cancer.

4. Relationship between LPL and Cancer: Animal Studies

4.1. Dyslipidemia Observed in Cancer-High-Susceptibility Animal Models. Elevated serum TG has been shown to promote carcinoen-induced colon carcinogenesis, and rats with hypertriglyceridermia such as the Zucker obese and Nagase albuninemic strains and F344 rats fed a high-fat diet are all known to be more sensitive to carcinogen treatments than rats with normal serum lipid levels [55–57]. In the case of mice, the Apc^1309 (C57BL/6)Apc^Min/+ (Min+) animal models of human familial adenomatous polyposis (FAP) feature development of large numbers of intestinal polyps and hypertriglyceridermia [59, 60]. Although no significant differences between Apc^1309 mice and wild-type mice were observed at 6 weeks of age, the average serum TG value in the former at 12 weeks was obviously increased almost 10-fold (∼600 mg/dl) over that at 6 weeks. Similar increase of TG levels (∼400 mg/dl) was observed in Min mice at 15 weeks compared to 8 weeks of age (Table 1). Along with TG elevation, mRNA levels of LPL in the liver and small intestine of Apc^1309 and Min mice were suppressed. Of note, other lipogenic genes, such as FAS and stearoyl-CoA
Table 1: Summary of animal models with dyslipidemia and cancer high susceptibility.

| Animal Strain | Age (week-old) | Serum TG (mg/dL) | Treatment | Tumor | Reference |
|---------------|----------------|------------------|-----------|-------|-----------|
| *Apc<sup>1309</sup>* (C57BL/6<sup>Δ</sup>Apc<sup>1309</sup>) | 12              | ∼600             | —         | Intestinal adenoma | [59] |
| Min (C57BL/6-Apc<sup>Min/+</sup>) | 15              | ∼400             | —         | Intestinal adenoma | [59, 60] |
| KK-A<sup>y</sup> | 19              | 481              | AOM       | Colon cancer | [61] |
| ICR       | 20              | 159              | AOM + DSS | Colon cancer | [62] |
| Syrian golden hamster | —               | 6                | BOP       | Pancreatic cancer | [63] |

Table 2: Summary of tumor suppressive effects of LPL inducers in animal models.

| Agent | Dose  | Animal model | Value to the untreated control group | Reference |
|-------|-------|--------------|--------------------------------------|-----------|
| Pioglitazone | 200 ppm | *Apc<sup>1309</sup>* | 67% | [59] |
|        | 1600 ppm | Min | 9% | [60] |
|        | 800 ppm | BOP-treated hamster | 40% | [63] |
| NO-1886 | 800 ppm | Min | 42% | [65] |
| Indomethacin | 10 ppm | Min | 25% | [66] |

desaturase-1, β-oxidation genes like acyl-CoA oxidase and carnitine palmitoyl transferase 1, and gluconeogenesis genes, exemplified by phosphoenolpyruvate carboxykinase, demonstrated no variation from wild-type mouse expression.

Obese KK-A<sup>y</sup> mice were found to be highly susceptible to azoxymethane-(AOM-)induced colorectal aberrant crypt foci (ACF) and colorectal carcinoma development compared to lean C57BL/6J mice [61]. Surprisingly, colorectal carcinomas developed within a very short-term period, 19 weeks, after AOM injection. The number of total ACF in KK-A<sup>y</sup> mice was around 70/mouse and almost 8 times higher than that in lean C57BL/6J mice. The incidences of adenomas and adenocarcinoma were 84% and 88%, respectively, in KK-A<sup>y</sup> mice, far higher than the 8% and 4% in C57BL/6J values. KK-A<sup>y</sup> mice exhibit abdominal obesity, hypertriglyceridemia, and hyperinsulinemia at the time of ACF and tumor development. At 13 weeks of age, the average serum levels of TG, total cholesterol, and free fatty acids of KK-A<sup>y</sup> mice undergoing AOM treatment were 484.1 mg/dL, 101.6 mg/dL, and 1,796 mEq/L, respectively (Table 1). It is interesting that hepatic LPL mRNA levels were also suppressed in KK-A<sup>y</sup> mice compared with C57BL/6J mice. Moreover, serum proinflammatory adipokines, such as IL-6, leptin, and plasminogen activator inhibitor-1 (Pai-1), were elevated. Importantly, expression of pro-inflammatory adipokine mRNAs such as for IL-6, leptin, monocyte chemotactic protein (MCP)-1, Pai-1 and TNF-α was significantly increased in the visceral fat tissue; in contrast, that for adiponectin was decreased.

Tanaka et al. have developed a novel colitis-related colorectal carcinogenesis model, using AOM plus dextran sodium sulfate (DSS), a colitis-inducing agent [64]. In this model (AOM + 2% DSS in ICR mice), numerous colorectal adenocarcinomas occur within a short-term period and the serum TG levels demonstrate increase to about 134, 175 and 159 mg/dL at 5, 10, and 20 weeks, respectively [62] (Table 1).

Injection of N-nitrosobis(2-oxopropyl)amine (BOP) into Syrian golden hamsters is known to induce pancreatic ductal adenocarcinomas, with a histology very similar to typical human pancreatic ductal adenocarcinomas. Moreover, associated genetic mutations, that is, K-ras point mutations and p16 aberrant methylation/homozygous deletions, are found in common in both hamster and human lesions. Interestingly, Syrian golden hamsters exhibit a hypertriglyceridemic state, almost 300 mg/dL at 6 weeks of age, even when not fed a high-fat diet [63] (Table 1). Also, in the case of this animal model, a low activity of LPL could be one of the causes of hypertriglyceridemia, activity of this enzyme in the liver being only 20% and 30%, respectively, of the values in C57BL mice and F344 rats.

5. Tumor Suppressive Effects of LPL Inducers

Pioglitazone, \{([±]-5-[4-[2-(5-ethyl-2-pyridyl)ethoxy]benzyl]thiazolidine-2,4-dione monohydrochloride), is a potent peroxisome proliferator-activated receptor (PPAR)<sub>γ</sub> ligand with a weak binding affinity for PPAR<sub>α</sub>. In the promoter region of the LPL gene, there exists a PPARE, and pioglitazone treatment successfully induced LPL expression in the liver and intestinal epithelial cells in *Apc*-deficient mice. The total numbers of polyps in the groups treated with 100 and 200 ppm pioglitazone in the *Apc<sup>1309</sup>* were reduced to 67% of the value in the untreated control group [59] (Table 2). With another *Apc*-deficient model, Min mice given 100–1600 ppm pioglitazone for 14 weeks showed decrease of intestinal polyps to 63–9% of the control number [60] (Table 2 and Figure 1).
Pioglitazone possesses other functions rather than just simply inducing LPL, such as causing cell growth arrest and apoptosis. Thus, data regarding LPL selective inducers are necessary for determining the relationship between hypertriglyceridemia and intestinal carcinogenesis. NO-1886, 4-[(4-bromo-2-cyanophenyl)carbamoyl] benzylphosphonate, chemically synthesized at Otsuka Pharmaceutical Factory [67] is one useful tool for clarifying this issue. Using a reporter gene assay, NO-1886 demonstrated no PPAR agonistic activity, unlike bezafibrate and pioglitazone [68].

Administration of 400 and 800 ppm NO-1886 also significantly decreased the total number of intestinal polyps by 48% and 42% of the untreated control value, respectively, in Min mice, along with causing marked increase in LPL mRNA levels in the liver and the small intestine. Moreover, treatment with NO-1886 also significantly decreased the numbers of colon polyps [65] (Table 2, Figure 1).

In the case of BOP-treated hamsters, pioglitazone has been demonstrated to improve hyperlipidemia and suppress ductal adenocarcinoma development. The incidences of ductal adenocarcinoma in the BOP plus 800 ppm pioglitazone and BOP alone groups were 38% and 80%, and the multiplicities were 0.55 and 1.37, respectively [63] (Table 2). Expression levels of hepatic LPL mRNA were elevated by treatment with 800 ppm pioglitazone. Moreover, quantitative real-time RT-PCR assays demonstrated almost 1.7-fold higher mRNA levels of LPL than that of pioglitazone-non-treated hamsters.

Indomethacin is a conventional nonsteroidal anti-inflammatory drug which has long been clinically employed to improve inflammation. It has demonstrated potent chemopreventive activity against intestinal tumor development in animal models, and a clinical trial in FAP patients also showed reduction in intestinal polyp development [69, 70]. We earlier reported that indomethacin suppresses intestinal polyp formation in Min mice together with ameliorating the hyperlipidemic state by regulating LPL, other lipid metabolic factors and inflammatory pathways [66]. Reduction of serum TG levels was 90% in Min mice with 10 ppm indomethacin treatment and higher than that with 400 ppm pioglitazone (83%) observed in our other previous study [59, 60]. The PPAR agonistic activity of indomethacin is reported to be 50 times weaker than that of troglitazone, a well-established PPAR agonist [71]. These results indicate that functions other than agonistic activity of indomethacin are responsible for its strong lipid-lowering effects (Figure 1).

6. Involvement of LPL in Inflammation, Obesity, and Others

6.1. LPL and Inflammation and Apoptosis. In addition to the lipid modifying function of LPL, two different mechanisms might be involved in LPL influence on carcinogenesis. The first involves anti-inflammatory action of LPL. It has been reported that LPL suppresses TNF-α- and interferon (IFN)-γ-evoked inflammation-related gene expression in endothelial cells through inactivation of transcription factor nuclear factor kappa B (NF-κB) [72]. Conversely, TNF-α, IFN-γ, IL-1β, IL-6, and leukemia inhibitory factor (LIF) decrease LPL activity.

It is well known that cyclooxygenase-2 (COX-2) is markedly elevated in human colon cancers, in AOM-treated rats, and in intestinal polyps of Apc-deficient mice. COX-2 is in fact thought to play important roles in both cancer cell proliferation and angiogenesis. Experiments conducted to clarify the mechanisms of NO-1886 effects on colon carcinogenesis revealed that the expression levels of mRNA for COX-2, in DLD-1 human colon cancer cells, were reduced under conditions of TGFα stimulation. On the other hand, there was no obvious change in the mRNA levels for COX-1 and inducible nitric oxide synthase (iNOS). The results obtained by RT-PCR analysis were also confirmed by
β-gal reporter gene assay in DLD-1 cells [65]. Consistent with the in vitro data, administration of 400 and 800 ppm NO-1886 reduced COX-2 mRNA levels in normal parts of small intestine of Min mice at 20 weeks of age [65]. In addition, NO-1886 ameliorates and induces regression of experimental steatohepatitis through increasing LPL activity and suppression of proinflammatory agents, such as TNF-α, IL-6, and COX-2 [73]. Recently, mice lacking angioptietin-like protein family 4 (Angptl4), which is the inhibitor of LPL, showed a severe and lethal phenotype characterized by fibrinopurulent peritonitis, ascites, intestinal fibrosis, and cachexia in response to a saturated fat diet [74].

The second mechanism is modification of the apoptosis pathway by LPL activation. Phosphatase type 2Cβ activation by unsaturated fatty acids has been demonstrated to induce apoptosis [75]. Unlike ester bodies of fatty acids, free fatty acids have cytotoxic effects in vitro and the products produced by hydrolysis of plasma TG may be implicated in such an apoptotic effect.

6.2. LPL and Obesity. Given the importance of LPL for lipid metabolism, its activity would be expected to be intimately involved in obesity effects and development of the metabolic syndrome. A large number of studies in rodents and humans have revealed that obesity results in increased LPL activity in adipose tissue [15, 35, 76–78]. Interestingly, LPL is regulated in opposite directions in adipose tissue and muscle. Feeding increases adipose LPL activity with a corresponding decrease in muscle LPL activity [35, 79]. Exercise stimulates LPL activity in the muscle and leads to increase fatty acid oxidation [80]. In an animal study, NO-1886 suppressed high-fat diet-induced fat accumulation in rats due to the increase of muscle LPL activity [81].

7. Conclusion

Targeting LPL activity or expression levels for development of reagents against cancer seems particularly challenging, because LPL is expressed ubiquitously and plays essential roles in maintaining homeostasis in the body. Data from LPL homozygous knockout mice, which die within one day of birth, underline its importance. However, appropriate suppression of serum TG levels could be achieved by using drugs, even if the number of selective inducers of LPL is limited. Thus, it might be important to develop selective LPL inducers or search for agents focusing on the aspect of “drug repositioning” to obtain the tools for investigating correlation between LPL and cancer. It should be borne in mind that LPL is inhibited by intrinsic factors, such as angptl3, angptl4, and C3 (Figure 1). These could clearly be candidate target molecules for development of LPL inducers. Considering that LPL activity has impact on obesity and metabolic syndrome, its targeting may also affect the regulation of adipocytokines, which may also be involved in carcinogenesis. Further investigations are warranted to clarify the importance of LPL and to accumulate evidence as to the worthiness as a target for cancer chemopreventive and chemotherapeutic agents.

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Research Article

Endocytosis and Sphingolipid Scavenging in Leishmania mexicana Amastigotes

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Leishmania species are the causative agents of the leishmaniases, a spectrum of neglected tropical diseases. Amastigote stage parasites exist within macrophages and scavenge host factors for survival, for example, Leishmania species utilise host sphingolipid for synthesis of complex sphingolipid. In this study L. mexicana endocytosis was shown to be significantly upregulated in amastigotes, indicating that sphingolipid scavenging may be enhanced. However, inhibition of host sphingolipid biosynthesis had no significant effect on amastigote proliferation within a macrophage cell line. In addition, infection itself did not directly influence host biosynthesis. Notably, in contrast to L. major, L. mexicana amastigotes are indicated to possess a complete biosynthetic pathway suggesting that scavenged sphingolipids may be nonessential for proliferation. This suggested that Old and New World species differ in their interactions with the macrophage host. This will need to be considered when targeting the Leishmania sphingolipid biosynthetic pathway with novel therapeutics.

1. Introduction

Leishmania species are insect vector-borne kinetoplastid protozoan pathogens causing a wide spectrum of neglected tropical diseases (the leishmaniases—cutaneous, mucocutaneous, and visceral) in humans across the globe. Moreover, the spread and severity of disease are exacerbated by its status as an important coinfection in AIDS patients [1]. Leishmania species exhibit a digenetic lifecycle: (a) existing as flagellated promastigotes within the sand fly vector; (b) following an insect bite and uptake by professional phagocytic cells, especially macrophages, differentiating into the aflagellate amastigote form. Within the host macrophage the parasite proliferates within acidic, fusogenic, endosome-derived phagolysosomes [2]. This compartment intersects with the phagosomal and autophagosomal pathways meaning that the parasites have access to the rich mix of nutrients resulting from degradation of phagocytosed macromolecules within the lytic environment of the phagolysosome. However, residency within such a compartment could also lead to contact with components of the host major histocompatibility complex (MHC), subsequent macrophage activation, and parasite killing. To overcome this certain species of Leishmania sequester and degrade MHC molecules [3, 4]; presumably this occurs via endocytosis although the mechanism has not been characterised. Endocytosis is also the process by which nutrients can be taken into the parasite, for example, L. amazonensis amastigotes were reported to endocytose transferrin from the phagolysosome [5]. However other species, including L. mexicana, do not show this behaviour [6]. Low-density lipoprotein [7] and haemoglobin [8] have also been shown to be endocytosed by Leishmania promastigote forms.

Sphingolipids are amphipathic lipids consisting of a sphingosine backbone with a long-chain fatty acid and a polar alcohol as attachments. Ceramide is an unmodified sphingolipid that functions as a secondary messenger in ubiquitous, eukaryotic signalling mechanisms. Modified (complex) sphingolipids are major components of the outer leaflet of eukaryotic plasma membranes believed to be
involved, with sterols, in the formation of microdomains or lipid rafts. Rafts are proposed to function in a diverse array of processes from the polarised trafficking of lipid-modified proteins, to the assembly of signal transduction complexes [9]. The first, rate-limiting, enzyme in sphingolipid biosynthesis is serine palmitoyltransferase (SPT). SPT catalyses the condensation of L-serine and palmitoyl CoA to form 3-ketodihydrosphinganine. Subsequently, N-acetylation of sphingoid base in the endoplasmic reticulum (ER) leads to the formation of ceramide, which is converted to complex sphingolipids (e.g., sphingomyelin SM or inositol phosphoceramide—IPC) via sphingolipid synthases. The animal sphingolipid synthase, SM synthase (SMS), transfers phosphorylcholine from phosphatidylcholine (PC) to ceramide to give SM; in contrast yeast, plants, and the kinetoplastid protozoan parasite Leishmania utilise IPC synthase to catalyse the formation of IPC via the transfer of phosphorylinositol from phosphatidylinositol (PI) to (phyto)ceramide [10]. This non-mammalian enzyme has long been established as a drug target for antifungals [11], and more recently, the sphingolipid synthase from Trypanosoma brucei (a kinetoplastid relative of Leishmania) has been demonstrated to be essential [12, 13].

Intriguingly, biosynthesis of sphingoid base and ceramide, precursors of complex sphingolipids, is absent and nonessential for the proliferation of L. major amastigotes within phagolysosomes in a model animal system [14, 15]. However, intramacrophage amastigotes synthesize the complex sphingolipid IPC de novo utilising precursors from alternative, host sources and maintain this lipid species at equivalent cellular levels to promastigotes [15]. It has been shown that L. donovani stimulate host macrophages to upregulate the production of ceramide, a substrate for IPC synthase [16] and a downstream product of SPT. Furthermore, recent work has identified the L. major SMase (LmjISCL) and shown it to be essential for pathogenicity in an animal model. This indicated that the generation of ceramide from host SM, via LmjISCL, is key to proliferation within the phagolysosome [17, 18]. Taken together, these studies indicated that L. major (and perhaps L. donovani, another Old World species) scavenge host sphingolipids to generate ceramide for amastigote proliferation, pathogenicity, and IPC biosynthesis.

Like amastigote Leishmania, the bacterial pathogen Coxiella burnetti (the aetiologic agent of Q fever) replicates within an acidified endocytic compartment and scavenge the sphingolipid SM from the plasma membrane via endocytosis [19]. In this work we sort to establish whether Leishmania employs a similar strategy to acquire and utilise essential host sphingolipids.

2. Materials and Methods

2.1. Cell Culture. Leishmania mexicana (MNYC/BZ/62/M379) promastigotes were maintained at 26°C in Schneider’s media (Sigma Aldrich) pH7, supplemented with 15% foetal bovine sera (FBS, Biosera Ltd). Promastigotes were differentiated to amastigote forms in Schneider’s media with 20% FBS at pH 5.5 and 32°C according to the published protocol [20]. The continuous murine macrophage cell line RAW264.7 was maintained in DMEM (Gibco-BRL) with 10% FBS, at 37°C and 5% CO₂. The cytotoxicity of myricin (Sigma Aldrich) was established using the AlamarBlue (Invitrogen) assay according to manufacturer’s protocol and as previously [21, 22]. The efficacy of myricin was confirmed using a yeast diffusion assay [23].

2.2. Metabolic Labelling. L. mexicana axenic promastigotes and amastigotes (10^7 mL⁻¹) were incubated in serum-free Schneider’s media for 30 minutes before labeling in the same with 5 μM of BSA conjugated BODIPY FL C₅-ceramide (Invitrogen) at 26°C and 32°C, respectively, for 2 hours. Promastigote parasites were similarly labeled for 16 hours in DMEM (ICN) supplemented with 10% FCS (Gibco BRL) and 20 mCi mL⁻¹ [myo-³H]-inositol (102 Ci mmol⁻¹ Amersham) [14]. For serine-containing lipid analysis axenic amastigotes (5 × 10^7 mL⁻¹) were incubated for 45 minutes in MEM Eagle (Sigma Aldrich) then labeled for 8 hours in the same medium containing 20 mCi mL⁻¹ [³H]-L-serine (20 Ci mmol⁻¹, ICN) [14]. Lipids were extracted and analysed as previously described [23].

2.3. Endocytosis Assay. L. mexicana cells were washed three times using warm serum-free media and counted using a Neubauer haemocytometer. 10^7 cells were then incubated with 50 μg mL⁻¹ 3 k Texas Red Dextran (Invitrogen) in 500 μL of serum-free media for 2 hours at 32°C. Controls were incubated on ice. Cells were subsequently washed 5 times with ice cold PBS and fixed with 3.7% formaldehyde. After washing, cells were resuspended in 500 μL of PBS and the fluorescence quantified using a plate reader (FLx800TM, BioTek; 590/20 Ex 645/40 Em). Observation of cells by fluorescence microscopy indicated that the dextran had been taken up by the parasites.

2.4. Macrophage Infection. 1 × 10^5 RAW264.7 murine macrophages in DMEM were allowed to adhere to coverslips within each well of a 24-well tissue culture plate (Nunc) and then incubated for 24 hours in appropriate media (DMEM with 10% FBS or with 1% Nutridoma, Roche) with or without myricin. L. mexicana amastigotes were then applied at a ratio of 10:1 in media and the infection allowed to proceed for 48 hours at 32°C and 5% CO₂, with daily changes of media with myricin where appropriate.

2.5. Expression Analyses. Denatured parasite lysates were separated and immobiloblotted as described [24] and the filters probed with mouse anti-LmjLCB2 or rabbit anti-LmjNMT polyclonal primary antibodies [14] at 1:1000, followed by horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich). Complexes were detected using the ECL system (Amersham Pharmacia). For the mRNA analyses, total RNA from equivalent numbers of 48 hours infected, or noninfected, RAW264.7 cells were extracted using the RNeasy kit (Qiagen) according to the manufacturer’s protocol. Following DNase treatment (RQ1,
Promega) cDNA was synthesized using the ImProm-II Reverse Transcription System (Promega) according to manufacturer’s protocol. Quantitative PCR was performed in a Rotor-Gene RG3000 (Corbett Research) using SYBR Green Jump-Start Taq Ready Mix (Sigma Aldrich) according to the manufacturer’s instructions. The murine MmLcb2 (encoding subunit 2 of SPT) was amplified using the primer pair 5′-AGGTGGATATCATGGAGAGA 3′ and 5′-GATCCAGTTCTCTCGTCC 3′. The reference gene, MmCasc3, was amplified using primers as previously [25]. The qPCR was carried out in triplicate on 3 replicates with annealing temperature 52°C for MmLcb2 and 55°C for MmCasc3.

3. Results and Discussion

3.1. Endocytosis in Promastigote and Amastigote L. mexicana. Like Leishmania species, Trypanosoma brucei (which causes the neurological NTD African sleeping sickness) is a kinetoplastid parasite. However, whereas the pathogenic amastigote forms of Leishmania species shelter within macrophages, the mammalian form of T. brucei proliferates extracellularly within the bloodstream of the host. Here it must avoid the full force of the immune system, and the switching of the predominant surface molecule, Variable Surface Glycoprotein (VSG), is key to this. Endocytosis of VSG is an important part of this antigenic variation and immune evasion. Notably, the rate of endocytosis in bloodstream form (BSF) T. brucei is 10 times higher than in the insect stage procyclic form (PCF), and the entire VSG coat is replaced every 7 minutes [26]. This allows the internalisation and degradation of bound antibodies and complement factors which helps protect the free-living parasite from the humoral immune response. This upregulation of endocytosis is facilitated by an increase in expression of factors involved in the uptake of VSG and its subsequent trafficking and processing [27, 28]. Before this study it remained unknown whether Leishmania species increased their rate of endocytosis on differentiation to the mammalian amastigote form. This may be predicted to facilitate the observed uptake of MHC and immune avoidance [6] or allow the acquisition of host sphingolipid for use in IPC synthesis [15, 18].

To address this the New World species L. mexicana was employed. Unlike L. major, this species can be grown in both promastigote and amastigote stages in axenic cell culture [20] allowing direct comparative analyses to be undertaken [29]. To quantify endocytosis fluorescently labelled dextran was utilised. Previously, dextran has been demonstrated to be taken up, via the endocytic pathway, by L. donovani promastigotes [30]. Here, axenic cell equivalents of promastigotes (both noninfective, proliferating procyclics, and infective, nonproliferating metacyclics) and amastigotes were labelled under identical conditions (32 °C in serum-free media). Dextran is measurably taken up by procyclic promastigotes; however the quantity endocytosed by amastigotes is more than 4 times greater (Figure 1). Interestingly the nondiving metacyclic forms demonstrate a similar up-regulation of this activity.

These data indicate that endocytosis is up regulated in amastigote forms, and this could be speculated to facilitate efficient immune evasion or substrate scavenging as discussed. The observation of similar levels of endocytic activity in metacyclic promastigotes was surprising given their apparent quiescence [20]. However, one may speculate that this indicated that the machinery for this process is acquired at this stage ready for the establishment of amastigote infection in a mammalian host.

3.2. The Role of Host Sphingolipid Biosynthesis in L. mexicana Invasion and Proliferation. To investigate IPC synthase activity in L. mexicana, axenic promastigotes and amastigotes were metabolically labeled with fluorescent BODIPY FL-ceramide (Figure 2). Both cell types produced a labelled product that comigrated with IPC, although the level in amastigotes was relatively low reflecting the slower growth of this form. These data indicated that L. mexicana has an active IPC synthase in both lifecycle stages, as does L. major [15].

As discussed above, host sphingolipid must be acquired as a source of ceramide substrate for the IPC synthase of amastigote L. major [15]. In support of this several studies have identified host cell glycosphingolipids in intramacrophage amastigotes of Leishmania species [31–33]. In addition, it has been reported that fumonisin B1 (an inhibitor of dihydroceramide synthase and ceramide
synthesis) inhibits the replication of intramacrophage \textit{L. donovanii} [34]. Utilising myriocin (a potent, specific SPT inhibitor [35]) the role of host synthesized sphingolipids was investigated in \textit{L. mexicana} infected RAW264.7 cells, a continuous murine macrophage cell line. The viability of axenic promastigotes and amastigotes (data not shown) was unaffected by the presence of 50 $\mu$M myriocin as previously reported [36]. Similarly, the RAW264.7 host cells were viable at 50 $\mu$M myriocin; levels above this lead to detachment of cells from the tissue culture well (data not shown).

In this study 50 $\mu$M of myriocin was applied to RAW264.7 cells for 24 hours prior to challenge with \textit{L. mexicana} axenic amastigotes at a ratio of 10:1. With daily application of the drug the infection was allowed to continue for 48 hours prior to fixation, staining, and counting as described in Section 2. This experiment was conducted either in the presence of full (10%) serum or in serum-reduced (1% Nutridoma) media. Under both sets of conditions the proportion of macrophages infected with amastigotes is unaffected by the presence of myriocin (Figure 3), as is the average number of parasites per host cell (Figure 4). These data indicate that host sphingolipid biosynthesis and exogenous sphingolipids are not central to the invasion and proliferation of \textit{L. mexicana} in RAW264.7 macrophages.

Similarly, myriocin has been reported to inhibit \textit{L. major} amastigote replication in cultured murine macrophages [15]. In addition, unlike in \textit{L. donovanii} [34], the specific ceramide synthase inhibitor fumonisin B1 had no demonstrable affect on the proliferation of intra-macrophage \textit{L. major} amastigotes [15]. Together, these results indicated that host sphingolipid synthesis is not central to the proliferation of \textit{L. major} or \textit{L. mexicana} amastigotes within macrophages. However, it remains possible that free ceramide is generated by the host via complex sphingolipid catabolism—a process up regulated in \textit{L. donovanii} infected macrophages [16]. In this study the macro-

\textbf{Figure 2:} HPTLC analysis of axenic procyclic promastigotes (aP) and amastigotes (aA) metabolically labelled with BODIPY FL C$_5$-ceramide. IPC: inositol phosphorylceramide; Cer: ceramide.

\textbf{Figure 3:} Invasion, % infected macrophages, determined 48 hours after infection in the presence or absence of the SPT inhibitor myriocin and with (FBS-DMEM) or without (FBS-reduced DMEM) exogenous serum in the media. Results of three independent experiments.
infection with \textit{L. mexicana} (Table 1) under the conditions described. This indicated that host sphingolipid biosynthesis is not up regulated on infection.

These results were perhaps surprising given the previously observed down-regulation of the \textit{Lm}Lcb2 protein in \textit{L. major} amastigote forms [14, 38], coupled with the maintenance of IPC biosynthesis [15] and the requirement for catabolism of scavenged SM [17, 18]. In light of these data it was chosen to examine the expression profile of the \textit{L. mexicana} SPT. Utilising the cross reactive anti-\textit{Lmj}Lcb2 antibody [14, 39] the expression of \textit{Lmx}Lcb2 was probed using Western blotting (Figure 5). The levels of the constitutively expressed \textit{N}-myristoyltransferase (NMT) were used as a loading control [24].

This indicated that \textit{Lmx}Lcb2, and so \textit{Lmx}SPT, is constitutively expressed throughout the lifecycle of \textit{L. mexicana}. These data have been recently confirmed by proteomic analyses of isolated, intra-macrophage \textit{L. mexicana} amastigotes [40]. Furthermore, metabolic labelling of axenic amastigotes demonstrated the incorporation of tritiated serine into the primary complex sphingolipid, IPC (Figure 6).

Taken together, these data indicated that this New World species has a complete and active \textit{de novo} sphingolipid biosynthetic pathway in the amastigote stage, and this contrasts with Old World \textit{L. major} [14, 38]. In addition, the possibility that myriocin may inhibit \textit{Lmx}SPT in intra-macrophage \textit{L. mexicana} amastigotes needs to be considered in relation to the data shown in Figure 4. If this compound is able to access and inhibit the parasite enzyme in these assays, then the results obtained would imply that both host and parasite SPT activity are nonessential for \textit{L. mexicana} proliferation, a similar scenario to that seen in \textit{L. major} [15].

3.4. Summary. These data are the first to demonstrate that the endocytic rate of the pathogenic, amastigote stage of \textit{Leishmania} species is raised when compared with insect stage procyclic promastigotes. This is reminiscent of the situation in \textit{T. brucei}, where endocytosis is up regulated on differentiation to mammalian BSF parasites in order to facilitate immune evasion [36]. This dramatic adaptation is facilitated by an increase in expression of factors involved in the uptake of molecules and their subsequent trafficking and degradation [27, 28]. In \textit{Leishmania} species proteolytic activity is highly up regulated during differentiation from promastigotes to amastigotes, and this coincides with the appearance of multivesicular lysosomes (megasomes) [41]. This correlates with the uptake of host MHC molecules [41] and, as shown here, an increase in endocytosis. However, unlike in \textit{T. brucei}, none of the known molecular machinery of endocytosis so far studied in \textit{Leishmania} species has demonstrated any increased expression on differentiation [24, 42]. However, a RAB-like GTPase in \textit{L. major} has been shown to have increased expression at the level of mRNA in amastigote forms, although the role of this factor in the cell remains unknown [43].

Both promastigotes and amastigotes of \textit{L. major} [15] and \textit{L. mexicana} are able to synthesize the primary complex sphingolipid, IPC. Old World \textit{L. major} is able to do this in the absence of \textit{de novo} SPT activity, therefore is the observed increase in endocytic activity related to an ability to scavenge host sphingolipid for use as substrate [15]? The
data presented here suggest that *L. mexicana* amastigotes can invade and proliferate normally when host sphingolipid biosynthesis is inhibited and/or when the exogenous source of lipid (serum) is removed. This suggested that the parasite is not wholly dependent on ongoing host sphingolipid synthesis, although residual host complex sphingolipid may remain and be sufficient for the parasite. Analyses of host *MmLcb2* and parasite *LmLcb2* expression indicated that infection does not influence host sphingolipid biosynthesis and that promastigote and amastigotes stages of *L. mexicana* equivalently express SPT, the first enzyme in sphingolipid biosynthesis [40]. This is in contrast to *L. major* where *LmjLcb2* is down-regulated and nonessential for pathogenesis and complex sphingolipid biosynthesis [14, 38]. This implies that there are profound differences between the Old and New World species in terms of the relationship between the *Leishmania* parasite and its mammalian host cell. Further knowledge of these differences will be required when considering the targeting of lipid biosynthesis for the development of novel therapies.

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Review Article

Behaviour of Human Erythrocyte Aggregation in Presence of Autologous Lipoproteins

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The aim of this work was to evaluate in vitro the effect of autologous plasma lipoprotein subfractions on erythrocyte tendency to aggregate. Aliquots of human blood samples were enriched or not (control) with their own HDL-C, LDL-C, or VLDL-C fractions obtained from the same batch by density gradient ultracentrifugation. Plasma osmolality and erythrocyte aggregation index (EAI) were determined. Blood aliquots enriched with LDL-C and HDL-C showed significant higher EAI than untreated aliquots, whereas enrichment with VLDL-C does not induce significant EAI changes. For the same range of lipoprotein concentrations expressed as percentage of osmolality variation, the EAI variation was positive and higher in presence of HDL-C than upon enrichment with LDL-C \( (P < 0.01) \). Particle size, up to LDL diameter values, seems to reinforce erythrocyte tendency to aggregate at the same plasma osmolality (particle number) range of values.

1. Introduction

There is scientific agreement that a high serum level of low-density lipoproteins cholesterol (LDL-C) is a risk factor for atherosclerosis and cardiovascular diseases \([1–3]\). A linear relationship between LDL-C levels and the occurrence of coronary artery disease is well documented in two meta-analysis \([4, 5]\). Conversely, it has been shown that HDL-C when at normal or high serum levels acts as a vascular protector and consequently without contribution such as a risk factor for atherosclerosis \([6]\). However, if its antioxidant capacity is diminished in patients with systolic heart failure, it will predict a higher risk of incident long term for adverse cardiac events \([7]\).

Several clinical studies evidenced associations between complex lipid macromolecules; for example, high LDL-C concentrations and blood rheological behaviour, like blood hyperviscosity, that are both referred to as cardiovascular risk factors \([8–10]\). Blood viscosity is dependent on macro-(hematocrit and plasma viscosity) and micro-(erythrocyte deformability and aggregation) hemorheological parameters. Disturbances in blood rheological behaviour, such as high values of the blood and plasma viscosity and increased erythrocyte aggregation tendency, have been described in patients with ischemic heart diseases \([11]\). Red blood cells (RBCs) participate in acute coronary occlusion, mainly under conditions of lower shear rate, for example, within the microcirculation in the peri-infarct domain of myocardium \([12]\).

Under in vitro stasis conditions, RBCs in normal human blood form loose aggregates with a characteristic morphology, similar to a stack of coins. Such aggregation is frequently named as rouleaux formation \([13]\). After prolonged stases, individual rouleaux can cluster, thereby forming three-dimensional structures, \([14, 15]\). Under circulation, the attractive forces involved are relatively weak, and aggregates can be dispersed during flow by the shear rate \([16]\). RBCs aggregation increasing at low shear rate affects blood viscosity and microvascular flow dynamics being markedly enhanced in several clinical states \([17–21]\).
Factors influencing RBCs aggregation can be divided into (i) extrinsic factors such as levels of plasma proteins (e.g., fibrinogen, lipoproteins, macroglobulins, or immunoglobulins), hematocrit, and shear rate, and (ii) intrinsic factors, for example, RBCs shape, deformability and membrane surface properties [22–32]. RBC membrane surface properties and structure, such as surface charge and the ability of macromolecules to penetrate the membrane glycocalyx, greatly affect aggregation for cells suspended in a defined medium [33, 34]. Different studies have shown that hyperlipoproteinemia is associated with erythrocyte hyper-aggregation [35–37]. The inverse correlation of erythrocyte aggregation with HDL2-C subfraction was reported in hypercholesterolemia middle-aged male population without apparent symptoms of cardiovascular disease [38]. It was evidenced in vitro that LDL-C enhances the RBCs aggregation induced by fibrinogen according to two aggregation models [39]. Considering the particle-like nature of the lipoproteins we raise the hypothesis that increased amounts of lipoprotein particles may change plasma osmolality with repercussions in erythrocyte aggregation.

The aim of our work was to study in vitro the erythrocyte aggregation tendency in blood samples collected from healthy male adults and enriched with their own plasma lipoproteins subfractions.

2. Material and Methods

2.1. Blood Samples. On consecutive days, venous blood samples were obtained with previous consent from healthy fasting volunteers adult males (n = 10) after 15 min in the recumbent position and collected (for two plastic tubes) with anticoagulant (10 I.U. of heparin/mL or 0.1% EDTA).

2.2. Lipoprotein Fractions. Lipoproteins fractions were prepared by a discontinuous NaCl/KBr density gradient ultracentrifugation using an SW 50.1 rotor (Beckman) [40]. Lipoprotein fractions were characterised by electrophoresis (Electra HR Helena Laboratories) buffer tris-barbital-sodium buffer pH 8.8) in cellulose acetate by comparison with serum controls (Lipotrol, Helena Laboratories).

2.3. Erythrocyte Aggregation Index. Erythrocyte aggregation was determined using the MA1 aggregometer from Myrenne GMBH (Roetgen, Germany). The MA1 aggregometer consists of a rotating cone plate chamber which disperses the sample by high shear rate of 600 s\(^{-1}\) and a photometer that determines the extent of aggregation. The intensity of light (emitted by a light emitting diode) is measured after transmission through the blood sample. The aggregation was determined in stasis for 10 seconds after dispersion of the blood sample [41].

2.4. Plasma Osmolality. Plasma osmolality was determined with the Osmomat 030 Cryoscopic Osmometer from Gonotec (Berlin, Germany).

2.5. Experimental Design. Blood samples from each donor were divided on aliquots, and after centrifugation and small volumes of plasma (0, 5 μL, 10 μL, 20 μL, and 40 μL) were discharged and replaced by equal values of their own previously enriched lipoprotein subfractions prepared a day before. With this procedure, no hematocrit variations were obtained. Blood aliquots were gently mixed by inversion, and erythrocyte aggregation was assessed. At the end of each assay, the aliquots were centrifuged at 12000 rpm for 1 minute in the Biofuge 15 centrifuge from Heraeus, and plasma osmolality was determined. HDL-C, LDL-C, and VLDL-C concentrations were expressed as percentage of osmolality variation values.

2.6. Statistical Analysis. The statistical evaluation performed utilized the “one-way” ANOVA with homogeneity test, cluster analysis, and average method.

3. Results

The major plasma lipoprotein subfractions were obtained by the discontinuous density gradient centrifugation between the density range of 1.006 and 1.300 g/mL. Each lipoprotein fraction was well banded with VLDL-C at the top, LDL-C in the upper middle and the HDL-C in the lower middle portion of the tube. After that, each fraction was pooled and submitted to electrophoresis where their obtained migration was confirmed by comparison with the serum control.

The volume of each lipoprotein sub-fraction added to the autologous blood samples aliquots caused variation of osmolality concentrations in relation to its absence. Using the cluster analysis and the beverage method, four classes of concentration range expressed as osmolality variation were grouped for each LDL-C; the VLDL-C and the HDL-C enriched blood aliquots, namely, Class I 0.005–0.025; Class II 0.030–0.035; Class III 0.045–0.055; Class IV 0.077–0.095 (Figure 1).

The erythrocyte aggregation variation for each enriched LDL, HDL, and VLDL blood aliquots in relation to the initial value is grouped by the different osmolality class variation values (Figure 2). The variation of erythrocyte aggregation in relation to the initial values depends on the plasma osmolality values, as well as of the type of lipoprotein subfraction. The enriched VLDL-C blood samples aliquots do not induce statistical significant variation on erythrocyte aggregation values (Figure 3). At variance in relation to the initial erythrocyte aggregation values, the enriched LDL-C blood samples presented significant statistical enhanced values at all range of percentage of osmolality variation (Figure 4). The same behaviour was verified in the enriched HDL-C blood aliquots, with exception for the higher values of percentages of osmolality variation, where a very significant (P < 0.0001) decrease was obtained for the erythrocyte aggregation (Figure 5). For the same range of osmolality variation, the two types of lipoproteins LDL-C and HDL-C induced different variation of erythrocyte aggregation (P < 0.01). When the values of osmolality variation were plotted against the respective values of aggregation variation
In the present in vitro study, we investigated the induction of human erythrocyte tendency to aggregation by autologous lipoproteins sub-fractions. With the amount of lipoprotein sub-fractions added, changes in plasma osmolality were observed as a consequence of the increase number of particles. We used cluster method on the values of osmolality to define four classes (Figure 1).

In blood aliquots enriched with VLDL-C, no changes in erythrocyte aggregation index were verified (Figure 3). For the same amount of particles corresponding to the same class of osmolality variation, both HDL-C and LDL-C enrichment induce enhancement of erythrocyte aggregation (Figures 4 and 5). VLDL has higher diameter than the other two lipoprotein classes [42] and may either rest in the plasma bulk not interfering with erythrocyte aggregation tendency. LDL-C particles bind in a nonabsolute specific way with erythrocyte membrane, while 60% of membrane area can be occupied by HDL-C as has been described [43]. The occupancy of some areas of erythrocyte membrane by HDL-C or LDL-C, causing some interference in the promotion of EAI tendency, may be an explanation for our results. Significant ($P < 0.01$) higher values of erythrocyte aggregation were obtained in HDL-C-enriched aliquots more than for LDL-C-enriched ones at the same class of osmolality variation (under the same number of particles). The exception was obtained in each enriched HDL-C aliquot, a significant ($R^2 = 0.383$) inverse linear regression was obtained (Figure 6).

4. Discussion

In the present in vitro study, we investigated the induction of human erythrocyte tendency to aggregation by autologous lipoproteins sub-fractions. With the amount of lipoprotein sub-fractions added, changes in plasma osmolality were observed as a consequence of the increase number of particles. We used cluster method on the values of osmolality to define four classes (Figure 1).

In blood aliquots enriched with VLDL-C, no changes in erythrocyte aggregation index were verified (Figure 3). For the same amount of particles corresponding to the same class of osmolality variation, both HDL-C and LDL-C enrichment induce enhancement of erythrocyte aggregation (Figures 4 and 5). VLDL has higher diameter than the other two lipoprotein classes [42] and may either rest in the plasma bulk not interfering with erythrocyte aggregation tendency. LDL-C particles bind in a nonabsolute specific way with erythrocyte membrane, while 60% of membrane area can be occupied by HDL-C as has been described [43]. The occupancy of some areas of erythrocyte membrane by HDL-C or LDL-C, causing some interference in the promotion of EAI tendency, may be an explanation for our results. Significant ($P < 0.01$) higher values of erythrocyte aggregation were obtained in HDL-C-enriched aliquots more than for LDL-C-enriched ones at the same class of osmolality variation (under the same number of particles). The exception was
for the HDL-C-enriched aliquots with the highest percentage of osmolality variation which significantly ($P < 0.0001$) decreased EAI to lower values than the aliquot control obtained. We raise the hypothesis that the higher number of unbinding HDL-C particles may increase the ionic strength to a threshold and consequently pull away the erythrocytes decreasing their tendency to aggregate.

Our results suggest that in healthy human blood aliquots enriched with autologous HDL-C or LDL-C, (where fibrinogen is present at normal range), when submitted to shear rate to disaggregate RBCs, and stopped after that, EAI tendency increases. The association between EAI values and HDL-C obtained in our experimental design is in accordance with others studies [39], which is a decrease of fibrinogen-induced RBCs aggregation in presence of the HDL2 subclass as not observed in a different experimental approach.

Recently [44], it was verified that the LDL particles number must be considered as an indicator for atherosclerosis risk factor, which has been previously remembered by others in the prevention of cardiovascular disease [45].

Two models, the cross-bridging and the depletion layer, were described to explain the reversible erythrocyte aggregation process at physiological conditions [46], but there are controversies and difficulties to adopt one or reject an other. The depletion model developed for polymer solutions of dextran demonstrated that there is an optimal molecular weight value to reach the greater erythrocyte aggregation tendency [47]. The hydrodynamic radius (Rh) determined for VLDL [48] is in the range between 15 nm and 40 nm which is much above of others belonging to macromolecules promoters of erythrocyte aggregation increase [49]. Particle size is an influent factor that may explain the absence of VLDL effect in the erythrocyte aggregation obtained. Regarding our results, they may also fit the cross-bridging model if, by an unknown mechanism, we assume that the particles numbers and size favour fibrinogen binding in a similar way as previously reported for immunoglobulin effects on fibrinogen-mediated erythrocyte aggregation [50, 51]. More studies are needed to explain in what model fit the effects of lipoproteins in erythrocyte aggregation. Our results may contribute to better understand the direct associations between high erythrocyte aggregation tendency and other cardiovascular risk factors such as hyperlipoproteinemia.

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Research Article

Overexpression of PGC-1α Increases Fatty Acid Oxidative Capacity of Human Skeletal Muscle Cells

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We investigated the effects of PGC-1α (peroxisome proliferator-activated receptor γ coactivator-1α) overexpression on the oxidative capacity of human skeletal muscle cells ex vivo. PGC-1α overexpression increased the oxidation rate of palmitic acid and mRNA expression of genes regulating lipid metabolism, mitochondrial biogenesis, and function in human myotubes. Basal and insulin-stimulated deoxyglucose uptake were decreased, possibly due to upregulation of PDK4 mRNA. Expression of fast fiber-type gene marker (MHCIIa) was decreased. Compared to skeletal muscle in vivo, PGC-1α overexpression increased expression of several genes, which were downregulated during the process of cell isolation and culturing. In conclusion, PGC-1α overexpression increased oxidative capacity of cultured myotubes by improving lipid metabolism, increasing expression of genes involved in regulation of mitochondrial function and biogenesis, and decreasing expression of MHCIIa. These results suggest that therapies aimed at increasing PGC-1α expression may have utility in treatment of obesity and obesity-related diseases.

1. Introduction

The genesis of obesity is multifactorial. However, there is evidence that reduced energy expenditure and in particular reduced capacity to utilise fat for metabolic fuel are important factors, particularly in the weight reduced state [1]. PGC-1α (peroxisome proliferator-activated receptor γ coactivator-1α) is a transcriptional coactivator initially isolated from brown adipose tissue [2], but now known to be abundant in many metabolically active tissues, such as skeletal muscle, liver, heart, and brain, where PGC-1α plays a major role in transduction of nutritional and physiological stimuli to transcriptional metabolic and contractile responses [2, 3].

Among many transcription factors coactivated by PGC-1α are nuclear respiratory factors (NRF1/2) [4], myocyte enhancer factor-2 (MEF2) [4], and several members of nuclear hormone receptors, including peroxisome proliferator-activated receptor (PPAR) subtypes—a family of lipid activated nuclear hormone receptors that play a key role in mediating adaptive regulation of muscle fatty acid oxidation [5]. The most common function of PGC-1α across tissues is regulation of mitochondrial physiology, but in addition, this family of coactivators controls separate, tissue-specific biological programs. In liver, expression of PGC-1α is strongly induced by fasting and stimulates hepatic gluconeogenesis and ketogenesis [6, 7]; in heart, it is a powerful stimulant of mitochondrial gene expression and biogenesis [8], while PGC-1α deficiency in brain has been shown to lead to behavioral abnormalities associated with axonal degeneration [9].

In skeletal muscle, PGC-1α is powerfully induced in conditions of increased physical activity [10–15], when ATP demand is high and induction of mitochondrial oxidative function becomes essential in order to adapt and maintain whole body energy balance. Enhancement of mitochondrial function and biogenesis occur through PGC-1α coactivation of nuclear respiratory factor (NRF) [16], and regulation of genes involved in oxidative phosphorylation is mediated through interactions with estrogen-related receptor α (ERRα) [17]. In addition, adaptation to increased contractile
activity involves conversion from type II (fast twitch) to type I (slow twitch) fibers [18], a process also shown to be driven by PGC-1α in transgenic animals [16], while musclespecific knock out of PGC-1α in animals has been shown to lead to conversion from type I to type II muscle fibers, exercise intolerance, decrease in mitochondrial proteins and myopathy [19]. Also in primary skeletal muscle cells from rats, overexpression of PGC-1α has been shown to confer a switch toward a more slow myofiber phenotype [20]. Further, overexpression of PGC-1α improved lipid utilization, insulin signaling and glucose transport in vivo animal studies [21, 22], while whole body overexpression of PGC-1α appears to have opposite effects on hepatic and muscle insulin sensitivity [23]. In cell cultures of rodent myotubes (C2C12 and L6), overexpression of PGC-1α has been shown to increase the level of the insulin-regulated glucose transporter 4 (GLUT4) mRNA and glucose uptake [24].

Peroxisome proliferator-activated receptor δ (PPARδ) is coactivated by PGC-1α in vivo and in vitro, and has been linked to increased fatty acid oxidation [25]. In addition, PPARδ, along with PGC-1α, has been identified as one of key players mediating effects of exercise at the cellular level [16, 26, 27] and suggested as a new target for the treatment of metabolic syndrome [28].

Cultured myotubes are a useful tool in investigating metabolic processes at cellular level, but limited in their usefulness in that they are characterized by glycolytic properties [29, 30]. PGC-1α has been referred to as “master regulator” of the coordination of mitochondrial biogenesis, mainly because increase in PGC-1α has been shown to activate transcription factors that switch muscle cells towards oxidative metabolism [31–33]. However, significant reduction of PGC-1α levels in muscle cell cultures compared to in vivo muscle extracts has been reported in chicken [34], and the role of PGC-1α in metabolic processes in skeletal muscle in vivo has previously been highlighted using adenosiviral overexpression in cell cultures [24], but data describing metabolic effects of PGC-1α overexpression in primary human skeletal muscle cells are limited [35].

The aim of the present work was to study whether overexpression of PGC-1α in cultured human skeletal muscle cells from healthy individuals would increase the oxidative capacity of the cells, promote fiber type conversion, and increase expression of genes involved in mitochondrial biogenesis and function. Moreover, we wanted to compare cultured myotubes to skeletal muscle in vivo. Finally, a possible interplay between PGC-1α overexpression and pharmacological activation of PPARδ on fatty acid oxidation and expression of key genes in lipid metabolism was studied.

2. Materials and Methods

2.1. Materials. DMEM-Glutamax, FCS, Ultroser G, penicillin-streptomycin-amphotericin B, and trypsin-EDTA were obtained from Life Technology (Paisley, UK). Skeletal Muscle Growth Medium Bullet Kit was obtained from Clonetics (BioWittaker, Verviers, Belgium).

2.2. Human Skeletal Muscle Biopsies and Cell Cultures. Muscle biopsy samples of the musculus obliquus internus abdominis or vastus lateralis were taken from seven healthy volunteers, and a cell bank of satellite cells was established. The biopsies were obtained with informed consent and approval by the National Committee for Research Ethics (Oslo, Norway). Muscle cell cultures free of fibroblasts were established by the method of Henry et al. [36]. Briefly, muscle tissue was dissected in Ham’s F-10 medium at 4°C and dissociated by three successive treatments with 0.05% trypsin/EDTA, and satellite cells were resuspended in skeletal muscle cell growth medium 2% FCS, 50 U/mL penicillin, 50 μg streptomycin, 1.25 μg/mL amphotericin B, and no added insulin. The cells from each donor were grown separately on culture wells or flasks coated with extracellular matrix gel [37]. After 1-2 weeks, at ~80% confluence, growth medium was replaced by DMEM with 2% FCS, 50 U/mL penicillin, 50 μg/mL streptomycin, 1.25 μg/mL amphotericin B, and 25 μM insulin to induce differentiation of myoblasts into multinucleated myotubes. The cells were cultured in a humidified 5% CO₂ atmosphere at 37°C, and medium was changed every 2-3 days. All myotube cultures were used for analysis on day 7 or 8 after the onset of differentiation.

2.3. Transient Retroviral Vector Production. The three retroviral constructs used in this study were pBABE (empty vector), pBABE-hPGC-1α, and pBABE-zsGreen (positive control for infection efficacy). 293T/17 cells were seeded 8 h prior to transfection at a density of 4 × 10⁶ cells per 100 mm culture dishes in 15 mL of DMEM with 2 mM L-glutamine and 10% foetal calf serum (FCS). Transfection was performed using Profection Mammalian Transfection System (Promega Biotech, Sweden). DNA mixture was prepared by diluting following amounts of plasmids: 16 μg genome, 16 μg pVPack and 8 μg pME-VSV-G to 438 μL distilled water per 100 mm culture dish. Fifteen min prior to transfection, 65.5 μL CaCl₂ and 500 μL HEPES were added to DNA mixture, and 1 mL of DNA/CaCl₂/HEPES was then added to each culture dish. The cells were allowed to incubate at a humidified 5% CO₂ atmosphere at 37°C for 24 h, when medium was replaced with 10 mL fresh prewarmed DMEM with 10 mM sodium
butyrate (per 100 mm culture dish) and incubated at 5% CO₂ atmosphere and 37°C for an additional 6 h. The medium was then removed, and fresh medium without sodium butyrate was added, and the cells incubated for another 16 h. The medium was collected and the supernatant filtered through 0.45 μm pore filter and used directly to infect cultured myoblasts. The described protocol was applied identically for production of all three retroviral constructs: pBABE, pBABE-hPGC-1α, and pBABE-zsGreen.

2.4. Retroviral Infection of Cultured Human Myoblasts. Infection of cultured myoblasts with 100% viral supernatant in the presence of 2 μg/mL polybrene for 24 h gave highest infection efficiency in pilot experiments, and these conditions were chosen for further infections. Cultured myoblasts were seeded at a density of 8000–14000 (depending on growth rate) cells/cm² and infected 1-2 days after seeding with 2 μL (per well in a 6-well plate) 100% retroviral supernatant containing either pBABE (empty vector), PGC-1α or pBABE-zsGreen (positive control) in the presence of 2 μg/mL polybrene, and incubated for 24 h. Thereafter, the virus-containing medium was replaced by Clonetics SkGM-BulletKit without insulin. The cells were allowed to reach ~80% confluence, when medium was replaced by DMEM with 2% FCS, 50 U/mL penicillin, 50 μg/mL streptomycin, 1.25 μg/mL amphotericin B and 25 pM insulin to induce the differentiation of myoblasts into multinucleated myotubes. All experiments on myotube cultures were performed on day 7 or 8 after the onset of differentiation. Pretreatments with GW501516 or control (DMSO) were performed 48 h prior to experiments.

2.5. Palmitic Acid Uptake and Oxidation. Myotubes grown on six-well plates were rinsed with 3 mL of PBS and exposed to [3H]palmitic acid (50 μM, 0.5 μCi/mL in PBS with 0.5% fatty acid free albumin (BSA) 1 mL/well), in a humidified 5% CO₂ atmosphere at 37°C. After 30 or 60 min of incubation, myotubes were placed on ice, and the incubation medium was transferred to new vials and assayed for labeled acid-soluble metabolites (ASMs) as previously described [38]. Briefly, incubation medium was precipitated by addition of 150 μL of 20% BSA and 80 μL of 1 M perchloric acid and centrifuged twice (20000 g, 5 min, 4°C). The supernatant was counted for radioactivity by liquid scintillation. No-cell controls were included. Cells were washed twice with PBS and lysed in 0.3 mL M-PER (Mammalian Protein Extraction Reagent, Thermo Scientific). Cell-associated radioactivity was measured by liquid scintillation. PA uptake was calculated as the sum of ASM (corrected for no cell control) in the medium and the cell-associated radioactivity in the cells. Protein content was determined using the BCA assay (Pierce).

2.6. Deoxyglucose Transport and Glycogen Synthesis. Myotubes were incubated for 60 min in serum-free DMEM with 5.5 mM glucose, with or without 10 μM cytochalasin B at 37°C. Deoxyglucose uptake was measured for 15 min in the presence of 10 μM unlabeled deoxyglucose and (1 μCi/mL) 2-deoxy-D-[3H]glucose in glucose uptake buffer (140 mM NaCl, 20 mM HEPES, 5 mM KCl, 2.5 mM MgSO₄, 1 mM CaCl₂, pH 7.4). After incubation, the cells were washed three times with ice-cold PBS and lysed with 0.3 mL M-PER, and the radioactivity was counted by liquid scintillation. Noncarrier mediated glucose transport was determined in the presence of cytochalasin B (10 μM) and subtracted from all values. For measurement of glycogen synthesis, myotubes were incubated for 2 h (37°C, 5% CO₂) in serum-free DMEM (±100 nM insulin), before adding DMEM with 2 μCi/mL D-[U-14C]-glucose in the presence or absence of 100 nM insulin for 60 min. After 60 min, the cells were washed three times with ice-cold PBS and lysed with 1 M NaOH. Synthesised glycogen was measured as described [39]. Glycogen synthesis increased linearly within 4 h after insulin stimulation and is presented as nmol·mg cell protein⁻¹·h⁻¹. The protein content of each sample was determined according to Bradford using BSA as the reference protein.

2.7. RNA Isolation and Analysis of Gene Expression by Quantitative Real-Time PCR. Human skeletal muscle biopsies and myotubes grown in 6-well plates were lysed in 1 mL Trizole reagent (Invitrogen, Oslo, Norway) according to the supplier’s total RNA isolation protocol. The isolated RNA was dissolved in RNase-free water, and concentration was determined by spectrophotometric measurement at 260 nm. All samples were also electrophoresed in 1% agarose gel to assess integrity of ribosomal bands.

Total RNA from above was treated with DNase (DNA-free, Ambion) to remove contaminating genomic DNA before cDNA synthesis. Shortly, 0.1 volume of 10 times DNase buffer, 4−6 units of DNase and water up to 30 μL was added to each sample. Samples were incubated at 37°C for 40−45 min. DNase inactivation reagent was added to each sample and removed by centrifugation after 2 min incubation at room temperature.

First strand cDNA synthesis was performed with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Oslo, Norway) according to supplier protocol. 1 μg of DNase treated total RNA was used in each synthesis. Priming of synthesis was accomplished with Oligo(dT)₁₂−₁₈. For each synthesis reaction a negative control with no enzyme was set up. Obtained cDNA was used for relative quantification on Applied Biosystems 7700. Quantitative real-time PCR was carried out using either TaqMan Universal PCR Master Mix or SYBR Green PCR Master Mix (both from Applied Biosystems) in 25 μL reactions run in triplicates. Template for the PCR was first strand cDNA from above (amount equivalent of 5−25 ng total RNA). Following forward and reverse primers and in case of TaqMan Universal PCR Master Mix, labelled probes, specific for the different genes, were used at concentrations of 200 nM and 400 nM, respectively: CD36: F: GGGAAAGTCACTGCGACATGA,
For all PCR reactions, two types of negative controls were used. As control for possible amplification of remaining genomic DNA, no enzyme controls from cDNA synthesis were set up for all primer-probe sets. The other negative control was with no template included. All signals were set up for all primer-probe sets. As control for possible amplification of remaining genomic DNA, no enzyme controls from cDNA synthesis were used. As control for possible amplification of remaining genomic DNA, no enzyme controls from cDNA synthesis were used.

**Gene Expression Microarray Analysis**

Retroviral overexpression of PGC-1α in cultured skeletal muscle cells. To determine whether experimental alterations in PGC-1α expression would affect oxidative capacity of cultured human skeletal muscle cells, myoblasts were infected with either empty vector or PGC-1α (data not shown). PGC-1α mRNA expression in cells infected with either empty vector or PGC-1α increased 150–200-fold compared to cells infected with empty control vector (Figure 2(a)). In skeletal muscle biopsies, PGC-1α mRNA expression was about 35-fold higher in differentiated cultured skeletal muscle cells (Figure 2(b)), thus PGC-1α infection restored the reduction in PGC-1α mRNA expression observed during cell culturing.

For all PCR reactions, two types of negative controls were used. As control for possible amplification of remaining genomic DNA, no enzyme controls from cDNA synthesis were set up for all primer-probe sets. The other negative control was with no template included. All signals were set up for all primer-probe sets. The other negative control was with no template included. All signals were set up for all primer-probe sets.

2.8. Immunoblots. Aliquots of 20 μg cell protein from total cell lysates prepared in Laemmli buffer were electrophoretically separated on NuPage 4–12% (w/v) Bis-Tris gel (Invitrogen), followed by immunoblotting with specific antibodies (Anti OxPhos Complex IV, mouse monoclonal 20E8, Cat. A-21248 from Molecular Probes, Anti Cytochrome C, mouse monoclonal 7H8. 2C12, Cat. 556433 from BD Pharmingen).

**Immunoassays**

Immunoreactive bands were visualized with ECL Western blotting detection reagents (Amersham International).

2.9. Statistical Analyses. All statistical analyses were performed using GraphPad Prism 4.0 for Windows (GraphPad Software Inc., San Diego, CA). Two-tailed paired t-tests were performed to determine the effects of PGC-1α overexpression. All values in figures are presented as mean ± SEM. Statistical significance was set at P < 0.05. For gene analysis, a fold change ≥2 or ≤0.5 was considered an increase or a decrease in expression level, respectively.

**3. Results**

3.1. Retroviral Overexpression of PGC-1α in Cultured Human Skeletal Muscle Cells. To determine whether experimental alterations in PGC-1α expression would affect oxidative capacity of cultured human skeletal muscle cells, myoblasts were infected with either empty vector or PGC-1α (data not shown). PGC-1α mRNA expression in cells infected with either empty vector or PGC-1α increased 150–200-fold compared to cells infected with empty control vector (Figure 2(a)). In skeletal muscle biopsies, PGC-1α mRNA expression was about 35-fold higher in differentiated cultured skeletal muscle cells (Figure 2(b)), thus PGC-1α infection restored the reduction in PGC-1α mRNA expression observed during cell culturing.

3.2. PGC-1α Overexpression Increased Palmitic Acid Oxidation. Oxidation of palmitic acid (PA) was measured 7–8 days after the onset of differentiation. Rate of PA oxidation, measured as acid soluble metabolites (ASM), was significantly increased by 71% (P < 0.05) in cells overexpressing PGC-1α compared to control cells infected with empty vector (Figure 3(a)). PA oxidation rate in uninfected cells (4.3 ± 0.7 nmol/mg/h) did not differ from the cells infected with empty control vector (4.0 ± 0.7 nmol/mg/h), showing that the observed increase in fatty acid oxidation rate (6.8 ± 1.4 nmol/mg/h) was due to overexpression of PGC-1α. Overexpression of PGC-1α did not affect the uptake of PA compared to cells infected with empty control vector (Figure 3(b)).

3.3. PGC-1α Overexpression Increased mRNA Expression of Genes Important for Lipid Metabolism, Mitochondrial Biogenesis, and Function. To identify potential mechanisms responsible for the observed effects of PGC-1α overexpression on PA oxidation, mRNA expressions of selected genes encoding key enzymes regulating fatty acid transport, storage and oxidation pathways and mitochondrial function were examined (Figure 4).

Among genes involved in lipid metabolism, PGC-1α-mediated increase in mRNA expression was most distinct for MCAD (medium chain acyl-coenzyme A dehydrogenase) (7.4-fold), but also PPARα was increased 3.2-fold, and
Figure 1: Retrovirus-mediated PGC-1α overexpression in human skeletal muscle cells. Cultured myoblasts were infected 24–48 h post seeding with 100% medium containing positive control virus pBABE-zsGreen in the presence of 2 μg/mL polybrene. Cells were incubated in virus-containing medium for 24 h, as described in Section 2. Images were taken 48 h post infection. (a) Fluorescence microscope image. (b) Light microscope image.

Figure 2: mRNA level of PGC-1α in human skeletal muscle cells infected with retrovirus coding for PGC-1α compared to control cells infected with empty virus (a), and in muscle biopsies compared to cell cultures (b). mRNA was isolated from muscle biopsies and cultured myotubes, and mRNA expression was assessed by RT-PCR, as described in Section 2. Values are presented as means ± SEM of 3 experiments, each representing one donor, with 3 replicates each, normalized to levels of housekeeping gene 36B4.

CPT1b (carnitine palmitoyltransferase 1b) and HSL (hormone sensitive lipase) both 2.7-fold (Figure 4(a)). In comparison, mean mRNA expression levels of MCAD, PPARα and CPT1b in skeletal muscle biopsies were 24-, 22.5- and 191-fold higher than in cultured cells, respectively (Table 1). Levels of PPARγ, PPARδ, and fatty acid transport protein CD36 were not affected by PGC-1α overexpression in human myotubes (Figure 4(a)).

Overexpression of PGC-1α resulted in a marked increase in mRNA levels of several important components of the respiratory chain: cytochrome C (4.5-fold), COX IV (cytochrome C oxidase IV) (3.6-fold), SOD-2 (superoxide dismutase-2) (3.6-fold), and SOD-3 (2.9-fold) (Figure 4(b)). Protein levels of cytochrome C and COXIV were also increased in cells overexpressing PGC-1α compared to both cells infected with empty control vector and uninfected cells (Figure 4(c)). Two important genes involved in regulation of mitochondrial biogenesis, ERRα (estrogen related receptor α) and mtTFA (mitochondrial transcription factor A), were also noticeably increased in PGC-1α cells, by 3.6-fold and 2.2-fold, respectively (Figure 4(b)). Expressions of NRF1 (nuclear respiratory factor 1) and uncoupling proteins UCP-2 and UCP-3 did not appear to be affected by PGC-1α overexpression (Figure 5(b)). In biopsies, mean mRNA
Figure 3: Effects of PGC-1α overexpression on palmitic acid oxidation (a) and uptake (b) in human myotubes. Myotubes infected with either empty vector or PGC-1α were incubated with [3H]palmitic acid and assayed for labeled acid soluble metabolites (ASM), as described in Section 2. (a) Values are presented as means ± SEM (n = 4 experiments, representing 3 different donors; with 3 replicates each). (b) Values are presented as means ± SEM (n = 3 experiments, representing 2 different donors; with 3 replicates each). ∗Significantly different from empty control vector at P < 0.05.

Table 1: Ratio of mean mRNA expression levels in skeletal muscle biopsies compared to cell cultures. mRNA was isolated from muscle biopsies and cultured myotubes, and expression was assessed by RT-PCR, as described in Materials and Methods. Values are presented as mean ± SEM of 4 experiments, each representing one donor, with 3 replicates each, and normalized to levels of the housekeeping gene 36B4.

| Gene      | Ratio biopsy/cells (+/- SEM) |
|-----------|------------------------------|
| COXIV     | 5.0 (0.34)                   |
| CPT1b     | 191.1 (52.7)                 |
| Cytochrome C | 9.1 (0.38)                  |
| ERRα      | 7.0 (0.5)                    |
| MCAD      | 24.0 (1.7)                   |
| PDK4      | 1203 (300)                   |
| PPARα     | 22.5 (7.3)                   |
| PPARδ     | 1.6 (0.23)                   |
| PPARγ     | 6.5 (2.7)                    |

3.4. PPARδ Stimulation Potentiates PGC-1α-Induced Palmitic Acid Oxidation. We further examined whether effects of PGC-1α overexpression on fatty acid metabolism in human myotubes could be potentiated by simultaneously stimulating PPARδ, a known target of PGC-1α coactivation [25]. We used a highly potent and selective PPARδ agonist, GW501516 [40], which we have previously shown to increase the rate of oleic acid oxidation in cultured human skeletal muscle cell [41]. In myotubes overexpressing PGC-1α, 48 h of GW501516 (10 nM) treatment seemed to potentiate PA oxidation (from 7.6 ± 1.7 nmol/mg/h to 18.2 ± 5.9 nmol/mg/h) compared to myotubes infected with empty vector (from 4.6 ± 0.1 nmol/mg/h to 9.5 ± 2.2 nmol/mg/h) (Figure 5(a)). At the mRNA level, there was an additive effect of PPARδ activation by GW501516 on PGC-1α-induced CPT1b expression (Figure 5(b)). Expression of genes involved in mitochondrial function (cytochrome C, COXIV, ERRα, UCP-2, and UCP-3) was not affected by GW501516 treatment (data not shown).

3.5. Overexpression of PGC-1α Decreased Glucose Transport. To further assess metabolic effects of PGC-1α overexpression, glucose transport and glycogen synthesis were measured. In cells overexpressing PGC-1α, deoxyglucose uptake was decreased compared to cells infected with empty vector (Figure 6(a)). The rate of deoxyglucose transport was unchanged in cells infected with empty control vector compared to uninfected cells (31.7 ± 8.9 nmol/mg/min and 33.3 ± 9.3 nmol/mg/min, resp.), while it decreased to 15.6 ± 8.9 nmol/mg/min in myotubes overexpressing PGC-1α. Insulin increased glucose uptake by about 25% both in control cells and in cells overexpressing PGC-1α (Figure 6(a)). Glycogen synthesis was also induced by insulin, but neither basal nor insulin-induced glycogen synthesis were affected by PGC-1α overexpression (Figure 6(b)). The level
of GLUT1 mRNA was unaffected by PGC-1α expression, while GLUT4 mRNA level increased 6-fold (Figure 6(c)). PDK4 (pyruvate dehydrogenase kinase 4) was also markedly increased in PGC-1α infected cells (6-fold) (Figure 6(c)).

3.6. PGC-1α Overexpression Reduced Expression of Fiber Type IIa Gene Marker. To assess the role of PGC-1α on fiber-type gene markers in myotubes, we further investigated expression of genes specifically enriched in either type I (slow) fibers (MHCI) or type IIa (fast) fibers (MHCIIa). Expression of MHCIIa mRNA was significantly decreased in cells overexpressing PGC-1α (P < 0.005) (Figure 7), while the increase in MHCI mRNA level was not significant. Thus, the MHCI/MHCIIa mRNA ratio was nearly doubled in cells overexpressing PGC-1α compared to control cells infected with empty vector (from 3.4 to 5.9, resp.).

4. Discussion
The aim of the present study was to determine whether overexpression of the transcriptional coactivator PGC-1α would increase oxidative capacity of human skeletal muscle cells and to compare these cells to skeletal muscle in vivo. PGC-1α overexpression increased palmitic acid oxidation.
and expression of key genes involved in regulation of lipid metabolism: CPT1b, MCAD, PPARα and HSL, as well as genes involved in mitochondrial function and biogenesis. Compared to skeletal muscle in vivo, PGC-1α overexpression increased expression of several genes which were downregulated during the process of cell isolation and culturing. Further, our results showed that uptake of glucose in skeletal muscle cells infected with PGC-1α was decreased, while mRNA expression of PDK4 was increased, as well as mRNA level of GLUT4. Moreover, when PGC-1α was overexpressed, the ratio of the mRNA level of MHCIIa (a gene marker of type I, slow oxidative fiber type) to that of MHCIIb (a gene marker of glycolytic, fast-twitch skeletal muscle fibers) was increased.

Human primary myotubes retain some of the metabolic characteristics of mature skeletal muscles, but are generally limited, in vitro, by their low oxidative capacity [29, 30]. This is possibly due to downregulation of PGC-1α and lack of proliferation of mitochondria in the absence of necessary cell environmental signals. In the present study, the rate of palmitic acid oxidation was significantly increased when PGC-1α was overexpressed; an effect which cannot be attributed to increased uptake of palmitic acid from the medium, as this was unchanged. The increase in the oxidation rate of palmitic acid was accompanied by enhanced expression of key genes regulating lipid oxidation: CPT1b, MCAD, and PPARα. ATGL has been shown to be upregulated in human skeletal muscle after exercise [42], along with increased utilization of intramyocellular triacylglycerols, while protein levels of HSL remain unchanged [42, 43]. In the present work, ATGL and HSL mRNA levels were increased by 1.6 and 2.4 fold, respectively, when PGC-1α was overexpressed. Interestingly, an in vivo study has shown that in human skeletal muscles, ATGL is expressed exclusively in type I (oxidative) fibers [44], so upregulation of this gene could indicate a shift toward more fiber type I-like myotubes when PGC-1α is overexpressed.

PGC-1α has been shown to increase mitochondrial biogenesis by several cellular mechanisms [10, 26, 45–47] and to improve lipid oxidation at rest and during submaximal exercise. We found several components of the respiratory chain to be increased at the mRNA level, such as cytochrome C and COXIV, and increase in protein levels of these two components were also compared by immunoblotting (Figure 4(c)). mtTFA, a transcription factor downstream of PGC-1α, critical for mtDNA replication [48], was also increased, suggesting that biogenesis of mitochondria might be increased when PGC-1α is overexpressed in cultured human myotubes. Furthermore, both superoxide dismutases (SODs) investigated in the present study, the mitochondrial SOD-2 and the extracellular SOD-3, were upregulated at mRNA level when PGC-1α was overexpressed. SODs catalyze the reduction of superoxide anions into hydrogen peroxide and oxygen, contributing as such to antioxidant defense of nearly all cells exposed to oxygen, and recent findings have shown that SOD-2 overexpression in mice preserves myoblast mitochondrial mass and function with aging [49].

Recently, PGC-1α and PPARδ have been identified as key players in exercise-signaling cascade leading to mitochondrial biogenesis [16, 27]. In the present work, we also wanted to investigate whether some of the effects
Figure 6: Deoxyglucose transport (a), glycogen synthesis (b) and mRNA expression (c) of GLUT1, GLUT4 and PDK4 in human myotubes infected with either empty vector or PGC-1α. (a): Cultured myotubes infected with either empty vector or PGC-1α were incubated with 1 mL/well of serum-free DMEM ± cytochalasin B 10 μM for 60 min, followed by a 15 min exposure to 2-deoxy-D-[3H]glucose (1 μCi/mL) with or without cytochalasin B, and with or without insulin, as described in Section 2. Values are normalized to the levels of uptake in control cells infected with empty vector and presented as means ± SEM of 4 experiments, representing 3 different donors, with 3 replicates each. *Statistically significant compared to basal uptake in control cells infected with empty vector (P < 0.05) (b): Myotubes infected with either empty vector or PGC-1α were incubated for 2 h in serum-free DMEM ± insulin, and then exposed to D-[14C(U)]glucose ± insulin. After 60 min, the cells were washed three times with ice-cold PBS and lysed with 1 M NaOH. Synthesised glycogen was measured as described in Section 2. Values are presented as means ± SEM of 2 experiments, each representing one donor, with 3 replicates each. (c): mRNA expression of GLUT1, GLUT4 and PDK4. mRNA was isolated from cultured myotubes infected with either empty control vector or PGC-1α eight days after the onset of differentiation. Expression was assessed by RT-PCR, and values are presented as means ± SEM of 3 experiments, each representing one donor, with 3 replicates each, normalized to the levels of housekeeping gene 36B4. A fold change ≥2 or ≤0.5 was considered an increase or decrease in expression level, respectively.
of PGC-1α overexpression on lipid metabolism and mitochondrial function could be potentiated by simultaneously activating endogenous PPARδ with a selective agonist GW501516. PPARδ stimulation appeared to enhance PGC-1α-mediated increase in palmitic acid oxidation and CPT1b expression. The effect of PPARδ activation on expression of CPT1b is in agreement with results from in vitro studies using mouse myotubes [50]. In our study, we could not see any effect of PPARδ activation on the expression of genes in mitochondrial function, which is in contrast to what has been reported in the muscle-specific VP16-PPARδ transgenic mice but not in the transgenic mice overexpressing the native PPARδ protein [27], and in agreement with results found in C2C12 cells (skeletal muscle muscle cells) [50], PPARδ activation did not promote mitochondrial gene expression, even though it increased fatty acid oxidation, since none of the genes in electron transport chain were increased by PPARδ stimulation.

Both PGC-1α and GLUT4 are often deficient in cultured skeletal muscle cells [24, 34], and in primary human myotubes, basal glucose uptake is generally mediated by other glucose transporters, such as GLUT1 and GLUT3 [51, 52]. In the present work, basal glucose uptake was significantly decreased in human myotubes overexpressing PGC-1α, while GLUT1 mRNA expression was unchanged.

A previous study with adenoviral overexpression of PGC-1α in C2C12 and L6 cells showed total restoration of GLUT4 mRNA levels to those observed in vivo, with a 3-fold increase in insulin-stimulated glucose transport [24]. In our study, overexpression of PGC-1α caused increased GLUT4 mRNA but did not increase insulin-stimulated glucose uptake. Inconsistencies between mRNA levels of GLUTs and functional data have been reported in human cultured skeletal muscle cells previously [53–55]. However, it is possible that the observed decrease in glucose uptake could be due to increased mRNA level of PDK4, an inhibitor of pyruvate dehydrogenase complex, which switches oxidation towards lipids.

Muscle fibre type and oxidative capacity have been linked to obesity and insulin resistance with a higher percentage of type 1 being positively related to insulin action and inversely with obesity [56, 57]. In addition it has been shown in studies with transgenic animals that PGC-1α appears to be an important factor in regulating muscle fibre type determination [16]. In the present study, myotubes infected with retro virus coding for PGC-1α had in increased MHCI/MCHIIa ratio based on gene markers for type I and type IIa fibers respectively. To our knowledge, shift in fiber-type composition has not previously been shown in cultured human skeletal muscle cells. The fact that there is a tendency towards increased MHCI/MCHIIa ratio indicates the possibility of a more complete switching between fiber types at conditions when PGC-1α is overexpressed, also in cultured skeletal muscle cells.

In summary, in the present study, overexpression of PGC-1α in cultured human myotubes increased fatty acid oxidative capacity of the cells and increased expression of genes involved in regulation of mitochondrial function. Compared to skeletal muscle in vivo, PGC-1α overexpression increased expression of several genes, which were downregulated during the process of cell isolation and culturing. We have also shown that mRNA expression of a fast fibre-type gene marker (MCHIIa) was decreased, suggesting that PGC-1α may play a role in fiber-type regulation in human myotubes.

Conflict of Interests
None of the authors have a conflict of interests to declare.

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Research Article

The Increased Activity of Liver Lysosomal Lipase in Nonalcoholic Fatty Liver Disease Contributes to the Development of Hepatic Insulin Resistance

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We tested the hypothesis that TAG accumulation in the liver induced by short-term high-fat diet (HFD) in rats leads to the dysregulation of endogenous TAG degradation by lysosomal lipase (LIPA) via lysosomal pathway and is causally linked with the onset of hepatic insulin resistance. We found that LIPA could be translocated between qualitatively different depots (light and dense lysosomes). In contrast to dense lysosomal fraction, LIPA associated with light lysosomes exhibits high activity on both intracellular TAG and exogenous substrate and prandial- or diet-dependent regulation. On standard diet, LIPA activity was upregulated in fasted and downregulated in fed animals. In the HFD group, we demonstrated an increased TAG content, elevated LIPA activity, enhanced production of diacylglycerol, and the abolishment of prandial-dependent LIPA regulation in light lysosomal fraction. The impairment of insulin signalling and increased activation of PKCε was found in liver of HFD-fed animals. Lipolysis of intracellular TAG, mediated by LIPA, is increased in steatosis probably due to the enhanced formation of phagolysosomes. Consequent overproduction of diacylglycerol may represent the causal link between HFD-induced hepatic TAG accumulation and hepatic insulin resistance via PKCε activation.

1. Introduction

NAFLD (nonalcoholic fatty liver disease) is often associated with insulin resistance (IR) and type 2 diabetes [1]. High-fat diet-induced liver triacylglycerol (TAG) accumulation results in the hepatic IR even after three days of administration and without significant impairment of insulin-mediated peripheral glucose disposal [2]. However, the mechanism by which hepatic fat accumulation might lead to the hepatic insulin resistance is far from being clearly understood [3]. The TAG metabolism in the liver is subject to a highly sensitive regulation in order to fulfil the actual needs of the organism. It has been shown that the liver is a site of continuous lipolysis of endogenous TAG and partial reesterification of released free fatty acids (FFA) back to the intracellular lipid storage pool [4]. The rate of intracellular lipolysis is 2-3 times greater than required to maintain the observed rate of TAG secretion [5].

Nevertheless, in spite of intensive research in this field, there are many uncertainties concerning the enzyme(s) responsible for intracellular TAG degradation. One possible candidate is lysosomal lipase (LIPA) [6]. It belongs to a group of more than 50 acid hydrolases that are characterised by low pH optimum (4.5–5). Because these enzymes require a pH range that is incompatible with the neutral cytoplasmic milieu, they are sequestered in specific cytoplasmic particles
2. Materials and Methods

2.1. Animals and Experimental Protocol. Male rats were kept in temperature-controlled room at 12:12 h light-dark cycle. Animals had free access to drinking water and diet if not stated otherwise. All experiments were performed in agreement with the Animal Protection Law of the Czech Republic 311/1997 which is in compliance with Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985) and were approved by the ethical committee of the Institute for Clinical and Experimental Medicine. Starting at age 3 months (b.wt. 300 ± 20 g), all animals were fed either HFD (70 cal% as saturated fat, 20 cal% as protein, and 10 cal% as carbohydrate) or standard laboratory chow diet (SD) for 2 weeks. The groups labelled SD fed or HFD fed had free access to the diet until decapitation (10–11 am), and the groups designated as SD fasted or HFD fasted were deprived of food for the last 24 hours. Glucose tolerance was determined as the rate of disappearance of glucose from circulation after a single dose of glucose (3 g/kg b.wt.) administered intragastrically to overnight-fasted animals.

2.2. Preparation of Lysosomal and Phagolysosomal Fractions. The lysosomes and phagolysosomes represent a heterogeneous population of organelles. 20% (wt/vol) homogenate prepared by homogenization of liver tissue in 0.25 M sucrose; 0.001 M EDTA pH = 7.4; heparin 7 IU/m, 1 mM PMSE, leupeptin 10 μg/mL, and aprotonin 10 μg/mL by Teflon pestle homogenizer. The crude impurities were removed by brief centrifugation at 850 g. The fat cake was removed carefully in order to prevent contamination of liquid fraction. An aliquot of the homogenate was kept at 4°C until lipase assay (maximum 2 hour), the rest was centrifuged for 10 000 g 20 min 4°C, and the resulting pellet and supernatant were separated. The supernatant contains preferentially the less dense lysosomes with higher TAG content (light lysosomes), and the pellet is formed by more dense particles (dense lysosomes).

2.3. Assay of Triacylglycerol Lipase Activity on Exogenous Substrate. The optimal conditions for the lipase assay (substrate concentration, reaction temperature, and linear range of the assay) were determined in the pilot experiments. The data are provided in supplements (a–c). 4% liver homogenate or lysosomal subfractions prepared from the fresh tissue under iso-osmotic conditions were used for the assay. The reaction medium (92.5 kBq 3H triolein, 100 μM triolein, 110 μM lecithin, 0.15 M NaCl, and 0.1 M acetate buffer pH 4.5) was emulsified by sonication (Hielsler sonicator UP200S). The assay itself was performed under hypooosmotic conditions (50 mM sucrose) in order to ensure the release of the enzyme sequestered within the lysosomes. The liver homogenate or isolated fractions were incubated for 60 minutes at 30°C. The released fatty acids were extracted according to [18] and counted for radioactivity.

2.4. Assay of Triglyceride Lipase Activity on Endogenous Substrate. This approach takes advantage of the coordinated changes in the intracellular localisation of LIPA and its intracellular substrate. The optimal conditions for the lipase assay were determined in the pilot experiments. The data are provided in supplements (e, f). The liver homogenate and subcellular fractions were prepared as described above under iso-osmotic conditions that prevent the disruption of lysosomes. The lysis of lysosomes was induced only after separation of fractions during the assay. 20% homogenate was mixed 1:1 with 0.2 M acetate buffer pH = 4.5 and incubated for 60 min in 30°C in shaking water bath. The reaction mixture was extracted in chloroform-methanol, and phases were separated by 1 M NaCl.

Aliquots of lower chloroform phase were separated for further determination of FFA and DAG content. An aliquot of chloroform phase was evaporated, and 100 μL...
of Krebs-Ringer phosphate buffer (pH = 7.6) containing
6% FFA-free BSA was added. The tubes were incubated in
shaking incubator at 37°C for 2 hours. FFA concentration
in final KRB/BSA solution was measured using commercially
available kit. In order to check the efficiency of FFA
solubilisation, the emptied tubes were washed with fresh
KRB + 6% BSA, and then 100 μL of chlorophorm was added.
An aliquot was separated by TLC, but no substantial traces of
FFA were detected.

2.5. Determination of DAG Content. This method is based
on the phosphorylation of DAG in the sample to DAG-
3-phosphate using γ35-ATP followed by quantification of
radioactivity in a chlorophorm extract. Lipids from liver
tissue or incubation mixture were extracted in chlorophorm-
methanol and an aliquot of chlorophorm phase was evapo-
rated under the stream of nitrogen. The sample was then sol-
ubilised by sonication in detergent buffer (7.5% n-octyl-β-D-
glucopyranoside, 5 mM cardiolipin, and 1 mM DETAPAC).
Reaction buffer (50 mM imidazole/HCl, pH = 6.6, 50 mM
NaCl, 12.5 mM MgCl2, and 1 mM EGTA), diacylglycerol
kinase, and γ35-ATP were added and incubated 30 min in
25°C. Lipids were extracted into chlorophorm-methanol,
were separated with 1% HClO4, and the exact volume of
lower chlorophorm phase was determined. An aliquot
was evaporated, resolved in 5% chlorophorm-methanol, and
separated by TLC. Individual populations of lipids were
visualised by iodine vapours, the bands corresponding to
DAG were scraped off, and the radioactivity was determined
by scintillation counting.

2.6. Incubation of Liver Slices In Vitro. The production of β-
hydroxybutyrate from liver slices in vitro was measured in
the absence of exogenous FFA. Liver slices (width approximately
1 mm) were quickly dissected and incubated for 2 hours in
Kreb’s Ringer bicarbonate buffer with 5 mmol/L glucose, 2%
bovine serum albumin, gaseous phase 95% O2, and 5% CO2.
All incubations were carried out at 37°C in sealed vials in a
shaking water bath. The aliquots of the incubation medium
were stored frozen until the further analysis.

2.7. Electrophoretic Separation and Immunodetection.
The homogenate, light lysosomal fraction, and dense lysosomal
fraction prepared as described above were used for the
assessment of LIPA protein content. A separate group of rats
were used to assess the impact of hepatic fat accumulation
on the insulin signalling pathway. The animals were either
deprived of food for 24 hours (fasted) or had free access to
food, and insulin (6 U/kg i.p.) was administered 30 min prior
decapitation (fed + insulin). Liver samples (200 mg) were
harvested in situ and stored in liquid nitrogen until further
utilization. The homogenate was prepared by Ultra-Turax
homogenizer (IKA Werte, Staufen, Germany) in homoge-
nization buffer (150 mM NaCl, 2 mM EDTA, 50 mM TRIS,
20 mM glycerophosphate, 1 mM Na3VO4, 2 mM sodium
pyrophosphate, 1 mM PMSF, leupeptin 10 μg/mL, and apro-
tinin 10 μg/mL). The homogenate was used for determina-
tion of mTOR and Akt phosphorylation. The proteins were
separated by electrophoretic separation under denaturing
conditions and electroblotted onto PVDF membranes. The
level of phosphorylation of Akt and mTOR kinases was
assessed by immunodetection using specific phospho-Akt
(Ser473) antibody and phospho-mTOR (Ser2448) antibody,
respectively. The total expression of Akt and mTOR protein
was determined on the same membrane after stripping and
reblotting using specific antibodies. All these antibodies were
purchased from Cell Signalling Technology, (Boston, MA).
The immunodetection of LIPA protein was performed using
mouse monoclonal (9G7F12) antibody to lysosomal acid
lipase (Abcam, Cambridge, UK). The loading control was
performed using rabbit polyclonal antibody to beta actin
(Abcam, Cambridge, UK). The bands were visualized using
ECL and quantified using FUJI LAS-3000 imager (FUJI
FILM, Japan) and Quantity One software (Biorad, Hercules,
CA).

2.8. PKC Membrane Translocation. The liver homogenate
was prepared as described above. The total membrane
and cytosolic fractions were prepared by centrifugation of
the homogenate at 100 000 g. Solubilisation of membrane
fraction was carried out in 1% Triton X-100, 0.1% SDS,
and 0.5% deoxycholate. After electrophoretic separation and
blotting, the PKCe was detected using anti-PKCε antibody
(Sigma, St. Louis, USA). PKC translocation was expressed
as the ratio of arbitrary units of membrane bands over the
cytosol bands.

2.9. Biochemical Analysis. TAG content in liver homogenate
or phagolysosomal fraction was determined after the extrac-
tion according to Folch et al. [19]. The glycogen content
was determined in fat-free dry mass after hydrolysis in 30%
KOH and expressed as a glucose equivalent (μmoles per g dry
weight).

FFA, insulin, TAG and glucose serum content, and
β-hydroxybutyrate production were determined using com-
mercially available kits (FFA: FFA half microtest, Roche Diag-
nostics GmbH Mannheim, Germany; triglycerides and glu-
cose; Pliva-Lachema, Brno CR; insulin: Mercodia, Uppsala,
Sweden; β-hydroxybutyrate: RanBut, RANDOX Crumlin,
UK).

2.10. Statistical Analysis. Data are presented as mean ± SEM.
Statistical analysis was performed using Kruskal-Wallis test
with multiple comparisons (n = 5–7). Differences were
considered statistically significant at the level of P < 0.05.

3. Results

3.1. The Effect of HFD on Physical and Metabolic Parameters.
The two-week period of HFD resulted in higher body weight
and increased fat accumulation determined as epididymal fat
pad: body weight ratio (Table 1). The impairment of glucose
metabolism was indicated by increased fasting glycemia,
increased fasting insulinemia, and impaired glucose toler-
ance measured by oral glucose tolerance test and expressed
as AUC1–180 min. The alterations in glucose metabolism were
**Table 1:** The effect of HFD on physical and metabolic parameters.

| Parameter                                | Standard diet | High-fat diet |
|-------------------------------------------|---------------|---------------|
| **Fasted**                                | **Fed**       | **Fasted**    | **Fed**       |
| Body weight (g)                           | 331 ± 6.9     | 379 ± 10.7x   |
| Epididymal fat pad/b.w. (g/100 g)         | 0.9 ± 0.07    | 1 ± 0.06      |
| Glycemia (mmol/L)                         | 5.1 ± 0.1     | 7.9 ± 0.5†    |
| Insulinemia (pmol/L)                      | 56 ± 15       | 135 ± 21x     |
| AUC<sub>0-180</sub> (mmol glucose/L)      | 1168 ± 26.6   | 1325 ± 32.9x  |
| Serum Tg (mmol/L)                         | 0.7 ± 0.1     | 1.4 ± 0.08†   |
| Serum FFA (mmol/L)                        | 0.7 ± 0.05    | 0.4 ± 0.02†   |
| ALT (μkat/L)                              | 1.2 ± 0.1     | 1.2 ± 0.1     |
| AST (μkat/L)                              | 4.3 ± 0.6     | 3.9 ± 0.3     |
| β-hydroxybutyrate (μmol/L)                | 1.67 ± 0.05   | 0.05 ± 0.01†  |

Data are given as means ± SEM, n = 7. *P < 0.05 HFD versus SD group; †P < 0.05 fasted versus fed animals; *P < 0.05 SD- versus HFD-fasted animals; ‡P < 0.05 SD- versus HFD-fed animals.

**Figure 1:** The effect of HFD on the LIPA activity measured as FFA release from artificial substrate (3H-triolein). (a) Homogenate; (b) dense lysosomes; (c) light lysosomes. The lipase activity was measured as the release of fatty acids at pH = 4.5 from 3H-triolein. Open bars = fasted animals; hatched bars = fed animals. ++P < 0.01 fed versus fasted; ‡P < 0.01 HFD fed versus SD fed.
not accompanied by dyslipidemia. Serum β-hydroxybutyrate concentration was significantly elevated in both HFD-fed as well as HFD-fasted group compared to corresponding SD groups what indicates increased utilisation of fatty acids for ketogenesis in the liver. Short-term HFD administration did not alter ALT and AST serum concentrations.

As expected, compared to the SD group, the HFD-administered animals accumulated increased amount of TAG (fasted: 14.6 ± 1.4 versus 3.2 ± 0.2; \( P < 0.001 \); fed: 16.2 ± 2.5 versus 2.9 ± 0.2 \( \mu \text{mol/g} \); \( P < 0.001 \)) and DAG (fasted: 138 ± 17 versus 83 ± 12; \( P < 0.01 \); fed: 145 ± 19 versus 53 ± 9 \( \text{nmol/g} \); \( P < 0.001 \)) in the liver. The insulin-stimulated increase of glycogen content in liver was lower in HFD compared to SD animals (fasted: 27 ± 7 versus 41 ± 9 n.s.; fed: 123 ± 10 versus 261 ± 15 \( \mu \text{mol/g} \); \( P < 0.001 \)).

3.2. The Effect of HFD on Lysosomal Lipase Activity. In order to determine maximal LIPA activity in liver homogenate and in particular lysosomal subpopulations, we employed emulsified \(^3\text{H}\)-labeled triolein as a substrate. In this experimental setting, the substrate is present in excess, and the only limiting factor is the amount of enzyme. The total LIPA activity measured in the whole homogenate was not affected either by prandial status (fasted or fed state) or diet intervention (SD or HFD) (Figure 1(a)). Similar results were observed in the fraction of dense lysosomes that represent primary lysosomes (Figure 1(b)). On \(^3\text{H}\)-triolein as a substrate, we found most of total LIPA activity in this fraction. The LIPA activity determined in light lysosomes represents only minor portion of total activity, but unlike homogenate or dense lysosomes, it responds to different metabolic states (Figure 1(c)). In the SD group, it is elevated in fasting and depressed in fed state. HFD feeding abolished the prandial regulation of LIPA activity especially due to its upregulation in fed state.

A separate set of experiment was designed in order to evaluate the contribution of LIPA associated with dense and
light lysosomes to the degradation of intracellular TAG. In this experimental design, the intracellular TAGs contained in particular fraction are the only source of substrate, and the intensity of lipolysis depends not only on the amount of enzyme but also on the amount of substrate available in the sample (Figures 2(a), 2(b), and 2(c)). Compared with the same experiments carried on $^3$H-triolein, we found two differences. First, HFD administration led to a significant increase of total LIPA activity measured in homogenate. Second, after separation of lysosomal subpopulations, LIPA activity associated with light lysosomes was higher than those associated with dense lysosomes. This observation could be explained by the previous “in vivo” translocation of both the substrate (TAG droplets) and the enzyme (LIPA) into light lysosomal fraction (phagolysosomes). In accordance with this presumption, we found higher TAG content in phagolysosomal fraction in HFD compared with SD group (fasted: $2.3 \pm 0.2$ versus $4.1 \pm 0.7$; fed: $1.02 \pm 0.3$ versus $5.6 \pm 0.48 \mu$mol·mg prot$^{-1}$). Similarly with the results obtained on $^3$H-triolein, the effect of fasting was manifested only in SD group and only in light lysosomal fraction. HFD feeding resulted into the elevation of LIPA activity in light lysosomes and into the abolishment of prandial regulation. Taken together, our results indicate that in the liver most of the enzyme is present in inactive form in dense (primary) lysosomes, and the physiologically active portion of the enzyme could be determined in light lysosomal fraction.

3.3. The Effect of HFD on Lysosomal Lipase Protein Distribution. In order to distinguish whether the higher LIPA activity found in the light lysosomal fraction in HFD group is consequent to the increased amount of enzyme in this fraction or only to the increased availability of the substrate, we determined the amount of LIPA protein in liver homogenate and in particular fractions. We found that the LIPA protein content in homogenate (Figure 3(a)) and dense lysosomal fraction (Figure 3(b)) is similar in both fasted and fed animals and that it is not affected by short-term HFD administration. In contrast to these findings, the abundance of LIPA protein in light lysosomal fraction is lower than in homogenate or dense lysosomes, but it varies according to several factors (Figure 3(c)). In SD group, it strongly depends on prandial status. In SD-fasted rats, LIPA protein abundance in this fraction is significantly higher compared with their fed counterparts. Short-term HFD diet has no effect on the content of LIPA protein in fasted animals, but it significantly increases its amount in the fed ones. Consequently, the prandial-dependent regulation is completely abolished in HFD group.

3.4. Diacylglycerol Production in Incubated Liver Homogenate. DAG is one of the major products of LIPA action on TAG molecule as this enzyme has lower affinity to DAG or monoacylglycerol compared with its affinity to TAG [13]. In our experimental conditions (incubation of liver homogenate or isolated fraction in pH $= 4.5$), DAG could not be further utilised for TAG biosynthesis, and the difference in DAG concentrations at the end and at the beginning of incubation represents the net DAG production from TAG degradation. Nevertheless, we cannot exclude some
degradation of DAG by lysosomal carboxylesterases. In homogenate, DAG production in SD group was significantly lower compared with those in HFD, and it was prandial dependent, that is, elevated in fasting and downregulated in fed state. In HFD group, a significant DAG production was detected in both fasted and fed animals (Figure 4(a)). The stimulatory effect of HFD on DAG production was found in both dense (Figure 4(b)) and light (Figure 4(c)) lysosomes. In HFD group, approximately 60% of DAG formation occurred in light lysosomal fraction, and in contrast to the SD group, it was independent of prandial status.

3.5. The Effect of HFD on Ketogenesis In Vitro. LIPA is expressed not only in hepatocytes but also in many other cell types including Kupffer cells present in the liver. In order to address the issue whether the above-mentioned changes in LIPA activity could be ascribed to hepatocytes, we measured ketone bodies production from liver slices in vitro (Figure 5). Ketogenesis is the metabolic pathway occurring exclusively in hepatocytes and tightly reflects the intracellular TAG metabolism. We found an elevated ketogenesis due to the HFD administration what under these experimental set up implicates the accentuation of TAG hydrolysis. When liver slices were incubated in the absence of exogenous FFA, HFD-fasted group exhibited significantly higher β-hydroxybutyrate production compared with SD fasted. A similar trend was found also in fed animals.

3.6. The Effect of HFD on Key Components of Insulin Signalling Pathway. To determine the effect of HFD on hepatic insulin sensitivity, the activation of key components of insulin signalling pathway was measured by immunodetection of their phosphorylation status. As shown in Figure 6(a), the insulin-stimulated phosphorylation of Akt kinase was significantly impaired in HFD-compared to SD group. Similar
First, alterations in LIPA activity associated with diabetes-induced IR. We have previously shown that LIPA is involved in the degradation of intracellular TAG in the liver [20]. The essential role of LIPA for hydrolysis of TAG is supported by findings of Du et al. [21] who reported that LIPA knock-out mice (Lipa−/−) exhibited progressive hepatosplenomegaly and massive TAG accumulation in the liver.

Our data indicate that the principal factor regulating the LIPA activity is not the total amount of the enzyme itself but rather its intracellular localisation. We did not find any significant differences in LIPA mRNA expression in response to either fasting or diet intervention (not shown), and in accordance with this, we did not find any difference in total LIPA protein content determined in the whole homogenate. According to Seglen and Solheim [22], active phagolysosomes have a lower density than the small, inactive lysosomes, allowing their separation by differential centrifugation. Based on this observation, we separated the total lysosomes into two subpopulations according to their density. We expected that the active lysosomes containing the TAG substrate would remain in the less dense fraction (light lysosomes), while the inactive lysosomes would sediment (dense lysosomes). In our experimental setting, the effect of fasting or HFD was manifested predominantly in light lysosomal fraction what supports the physiological relevance of this methodology. In SD group, we observed a significant prandial-dependent regulation, LIPA activity being upregulated in fasted and downregulated in fed animals. HFD feeding was associated with a significant elevation of LIPA protein content and LIPA activity in light lysosomal fraction particularly in fed animals and consequently with the abolishment of prandial-dependent regulation of LIPA activity. Similar trends were observed on both exogenous and endogenous substrates. The changes in LIPA activity were reflected by the corresponding changes in LIPA protein content in light lysosomal fraction. The interesting conclusions come from the comparison of LIPA activity in the light and dense lysosomal fractions determined on either 3H-triolein or intracellular TAG. The activity measured on 3H-triolein depends only on the amount of the enzyme present in the particular fraction as the substrate is available in excess. In contrast, when intracellular TAGs are the only source of substrate, the activity in particular fractions depends on the coordinated translocation of the enzyme and the substrate. The main difference in LIPA activity determined by these two approaches was found in the distribution of LIPA activity among dense and light lysosomal fractions. In dense lysosomes, we found high LIPA activity on 3H-triolein but only low LIPA activity on intracellular TAG. This difference indicates that dense lysosomal fraction contains an enzyme that is not active in physiological situation but that could be activated after addition of the artificial substrate. The LIPA activity determined in light lysosomes represented the bulk of total LIPA activity on intracellular TAG substrate but only minor portion of total activity determined on 3H-triolein. It is possible to speculate that the LIPA activity on endogenous substrate quantitatively reflects the formation of activated lysosomes, that is particles containing both the substrate and the enzyme. Taken together, these data indicate that LIPA

4. Discussion

In the present study, we provide evidence that in steatosis the increased degradation of TAG mediated by LIPA and associated with the increased production of DAG may be one of the mechanisms determining the rapid onset of hepatic IR. Our hypothesis is based on following findings. First, alterations in LIPA activity associated with different metabolic states are based on prandial-dependent translocation of the enzyme from the inactive pool of dense lysosomes into light lysosomal fraction, and it is upregulated in fasting and downregulated in the fed state. After short-term HFD administration, this prandial-dependent regulation of LIPA activity is abolished. The fed state-associated downregulation of LIPA activity is impaired, and the portion of the active enzyme is permanently increased. These changes were demonstrated on both endogenous TAG and exogenous substrate (emulsified 3H-triolein). Second, in steatosis, the production of TAG degradation intermediates, FFA and DAG, by lysosomal lipase was significantly elevated. Finally, we proved an increased PKCe activation together with the defects in the insulin-signalling cascade in the fatty liver. Taken together, these data indicate that the enhanced activity of LIPA in HFD-fed animals and following overproduction of PKCe activator DAG contribute to the establishment of HFD-induced IR. We have previously shown that LIPA is involved in the degradation of intracellular TAG in the liver [20]. The essential role of LIPA for hydrolysis of TAG is supported by findings of Du et al. [21] who reported that LIPA knock-out mice (Lipa−/−) exhibited progressive hepatosplenomegaly and massive TAG accumulation in the liver.

Our data indicate that the principal factor regulating the LIPA activity is not the total amount of the enzyme itself but rather its intracellular localisation. We did not find any significant differences in LIPA mRNA expression in response to either fasting or diet intervention (not shown), and in accordance with this, we did not find any difference in total LIPA protein content determined in the whole homogenate. According to Seglen and Solheim [22], active phagolysosomes have a lower density than the small, inactive lysosomes, allowing their separation by differential centrifugation. Based on this observation, we separated the total lysosomes into two subpopulations according to their density. We expected that the active lysosomes containing the TAG substrate would remain in the less dense fraction (light lysosomes), while the inactive lysosomes would sediment (dense lysosomes). In our experimental setting, the effect of fasting or HFD was manifested predominantly in light lysosomal fraction what supports the physiological relevance of this methodology. In SD group, we observed a significant prandial-dependent regulation, LIPA activity being upregulated in fasted and downregulated in fed animals. HFD feeding was associated with a significant elevation of LIPA protein content and LIPA activity in light lysosomal fraction particularly in fed animals and consequently with the abolishment of prandial-dependent regulation of LIPA activity. Similar trends were observed on both exogenous and endogenous substrates. The changes in LIPA activity were reflected by the corresponding changes in LIPA protein content in light lysosomal fraction. The interesting conclusions come from the comparison of LIPA activity in the light and dense lysosomal fractions determined on either 3H-triolein or intracellular TAG. The activity measured on 3H-triolein depends only on the amount of the enzyme present in the particular fraction as the substrate is available in excess. In contrast, when intracellular TAGs are the only source of substrate, the activity in particular fractions depends on the coordinated translocation of the enzyme and the substrate. The main difference in LIPA activity determined by these two approaches was found in the distribution of LIPA activity among dense and light lysosomal fractions. In dense lysosomes, we found high LIPA activity on 3H-triolein but only low LIPA activity on intracellular TAG. This difference indicates that dense lysosomal fraction contains an enzyme that is not active in physiological situation but that could be activated after addition of the artificial substrate. The LIPA activity determined in light lysosomes represented the bulk of total LIPA activity on intracellular TAG substrate but only minor portion of total activity determined on 3H-triolein. It is possible to speculate that the LIPA activity on endogenous substrate quantitatively reflects the formation of activated lysosomes, that is particles containing both the substrate and the enzyme. Taken together, these data indicate that LIPA

![Figure 5: The effect of HFD on β-hydroxybutyrate production from liver slices in vitro.](image-url) Liver slices were incubated in oxygenated KRB without exogenous fatty acids. Open bars = fasted animals; hatched bars = fed animals. * P < 0.05 fed versus fasted; ** P < 0.01 HFD versus SD fasted; + P < 0.05; # P < 0.05 HFD fed versus SD fed.
Figure 6: Alterations of insulin signalling cascade associated with hepatic fat accumulation. All results are expressed as a fold increase in the insulin-stimulated state relative to the basal state. Representative Western blots are shown above each graph. (a) Fold increase in Akt (Ser473) phosphorylation, (b) fold increase in mTOR (Ser2448) phosphorylation. The basal level of protein phosphorylation was determined in the homogenate prepared from the liver of 24 hours fasted animals. The effect of insulin was determined in identically processed samples from animals which had free access to food and 40 min prior to decapitation were administered insulin 6 U/kg. The total protein (Akt or mTOR) expression was determined after stripping the membrane and reblotting with anti-Akt or anti-mTOR antibody. Values represent means ± S.E.M. of 7 animals. *P < 0.05.

Associated with light lysosomes represents the physiologically active enzyme.

We suppose that in NAFLD, characterised by high TAG intracellular content, one of the factors determining the phagolysosomal formation may be the substrate availability itself. The increased amount of intracellular lipid droplets in steatosis could promote the phagolysosome formation and stimulate the lysosomal lipolysis. Only recently, Singh et al. [23] described direct involvement of autophagy and lysosomal pathway in the degradation of intracellular lipid droplets in the liver. They found that lipid droplets can enter the autophagic degradation pathway in the same manner as proteins and damaged organelles via formation of autophago(lipo)somes that further fuses with primary lysosomes. As the only known lysosomal enzyme with lipolytic activity is LIPA, we believe that our results are in accordance with findings of Singh et al..

The ketone body formation tightly reflects the liver lipid metabolism. Debeer et al. [24] demonstrated that both ketogenesis and FFA oxidation are a particularly good markers of lysosomal TAG degradation. We observed higher \( \beta \)-hydroxybutyrate concentration in serum and higher ketone body production from isolated liver slices in the absence of exogenous fatty acids in HFD group. We conclude that these data provide indirect evidence that confirms the stimulatory effect of short-term HFD on lysosomal lipolysis.

Figure 7: The effect of hepatic fat accumulation on PKC\(\varepsilon\) activation. Representative Western blot is shown in the upper part of the figure; TM, total membrane fraction, C, cytosol fraction. The PKC\(\varepsilon\) membrane to cytosol ratio is shown in the graph. The relative densities of the bands in the membrane fraction were compared with corresponding ones in cytosol fraction in order to obtain the measurable parameter of activation. Values represent means ± S.E.M. of 7 animals. *\( P < 0.05.\)
Concomitantly occurring stimulation of lipolysis and the accumulation of endogenous TAG after HFD administration seem to be contradictory. However, in hepatocytes, a significant portion of FFA released from intracellular TAG (approximately 70%) is reesterified back [25]. HFD impairs VLDL secretion [26], and most of FFA reenter the intracellular storage pool. We have previously reported that DGAT-1 expression is increased in fatty liver what indicates enhanced esterification of fatty acids and may result in the intensification of lipolytic/reesterification cycle in hepatocytes [20]. The increased lipolysis thus does not result in decreased TAG content but only in higher TAG turnover.

In the liver, PKCε, member of novel PKCs subfamily, is involved in the development of HFD-induced IR [16, 17]. Samuel et al. showed that fat-induced hepatic IR may result from activation of PKCε and its downstream targets. Nevertheless, the nature of the signal that activates PKCε has not been fully explained. Systemic increase in FFA serum levels, as one possible underlying factor, has not been described after HFD administration. Another candidate, 1,2-sn-DAG, is an important intracellular signalling molecule, and it is the known activator of novel PKCs isoform family [27]. The increased DAG content due to the increased flux through TAG synthetic pathway and the following PKCε activation was described in skeletal muscle in HFD-administered animals [28]. However, DAG is also an intermediate in TAG degradation pathway that, in contrast to muscle, is quite active in the liver. Our findings suggest that DAG originating from the increased lipolytic activity of LIPA and accentuated TAG breakdown could act as PKCε activator in fatty liver. This hypothesis is supported by the fact that in fatty liver LIPA is activated specifically in the fed state, and possible PKCε activator is available during the period of insulin action.

In conclusion, we found that short-term HFD-induced TAG accumulation in the liver is associated with the increased degradation of intracellular TAG by lysosomal lipase and with higher production of lipolytic products—DAG and FFA. Our findings suggest that the elevated DAG production by LIPA activated by increased supply of dietary lipids may represent the causal link between dietary fat-induced hepatic TAG accumulation and hepatic IR via the PKCε activation. In the light of these findings, lysosomal lipolysis may represent a new promising therapeutic target.

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