Adiponectin is an insulin-sensitizing adipokine with anti-diabetic, anti-atherogenic, anti-inflammatory and cardioprotective properties. This adipokine is secreted from adipocytes into the circulation as three oligomeric isoforms, including trimeric, hexameric and the HMW (high-molecular-mass) oligomeric complex consisting of at least 18 protomers. Each oligomeric isoform of adiponectin exerts distinct biological properties in its various target tissues. The HMW oligomer is the major active form mediating the insulin-sensitizing effects of adiponectin, whereas the central actions of this adipokine are attributed primarily to the hexameric and trimeric oligomers. In patients with Type 2 diabetes and coronary heart disease, circulating levels of HMW adiponectin are selectively decreased due to an impaired secretion of this oligomer from adipocytes. The biosynthesis of the adiponectin oligomers is a complex process involving extensive post-translational modifications. Hydroxylation and glycosylation of several conserved lysine residues in the collagenous domain of adiponectin are necessary for the intracellular assembly and stabilization of its high-order oligomeric structures. Secretion of the adiponectin oligomers is tightly controlled by a pair of molecular chaperones in the ER (endoplasmic reticulum), including ERp44 (ER protein of 44 kDa) and Ero1-Lα (ER oxidoreductase 1-Lα). ERp44 inhibits the secretion of adiponectin oligomers through a thiol-mediated retention. In contrast, Ero1-Lα releases HMW adiponectin trapped by ERp44. The PPARγ (peroxisome-proliferator-activated receptor γ) agonists thiazolidinediones selectively enhance the secretion of HMW adiponectin through up-regulation of Ero1-Lα. In the present review, we discuss the recent advances in our understanding of the structural and biological properties of the adiponectin oligomeric isoforms and highlight the role of post-translational modifications in regulating the biosynthesis of HMW adiponectin.

Key words: adipokine, adiponectin, insulin sensitivity, metabolic syndrome, obesity.

INTRODUCTION

Although once thought to be an inert energy-storage depot, adipose tissue is now recognized as a major endocrine organ in the body, secreting a plethora of bioactive molecules termed adipokines or adipocytokines [1]. Notable among adipokines is adiponectin, an abundant serum adipokine secreted predominantly from adipocytes. Unlike most other adipokines, serum levels of adiponectin are decreased in obesity and its associated medical complications [2]. Adiponectin, also termed Acrp30 (adipocyte complement-related protein of 30 kDa), ADIPOQ, apM1 (adipose tissue plasminogen activator-mimicking 1), G0P1 (glycolipid), and G0P2 (glycolipid) in humans and mice, respectively, is largely secreted from fat cells [3–6]. In the last several years, the adipokine has attracted much attention because of its multiple beneficial effects on a cluster of obesity-related metabolic and cardiovascular dysfunctions.

In addition to its role as an insulin sensitizer, adiponectin can protect against almost all of the major obesity-related pathologies, including hypertension [7], atherosclerosis [8], NASH (non-alcoholic fatty liver disease) and NASH (non-alcoholic steatohepatitis) [9], heart failure [10], airway inflammation [11] and several types of cancer [12,13]. Adiponectin exerts its pleiotropic beneficial effects through its direct actions on multiple target tissues (Figure 1). Both adiponectin receptors (Adipor1 and Adipor2) are ubiquitously expressed, although their relative abundance varies in different target tissues [14]. Most biological effects of adiponectin are mediated by the activation of AMPK (AMP-activated protein kinase), a prime therapeutic target for obesity-related metabolic and cardiovascular diseases [15–21]. Adiponectin enhances the binding of APPL1 to both AdipoR1 and AdipoR2, and these interactions are essential for the subsequent phosphorylation of AMPK at Thr172.

The physiological functions and clinical relevance of adiponectin in obesity-related medical complications have been extensively reviewed elsewhere [2,14,23,24]. In the present review, we discuss the recent advances on the regulation of adiponectin oligomeric complex formation at the post-translational level. In addition, we highlight recent clinical, genetic and experimental evidence supporting the role of HMW (high-molecular-mass) oligomeric adiponectin as a major bioactive form mediating the insulin-sensitizing activity of this adipokine.

Abbreviations used: Adip, adiponectin receptor; AMPK, AMP-activated protein kinase; APPL1, adaptor protein containing phosphotyrosine binding, pleckstrin homology domains and leucine zipper 1; bFGF, basic fibroblast growth factor; ER, endoplasmic reticulum; Ero1-Lα, ER oxidoreductase 1-Lα; ERp44, ER protein of 44 kDa; G0SA, Golgi-associated γ-adaptin ear homology domain Arf (ADP-ribosylation factor)-interacting protein 1; HB-EGF, heparin-binding epidermal growth factor-like growth factor; HMW, high-molecular-mass; IL, interleukin; LPS, lipopolysaccharide; PDI, protein disulfide-isomerase; PPARγ, peroxisome-proliferator-activated receptor γ; PTM, post-translational modification; PDGF, platelet-derived growth factor; siRNA, small interfering RNA; SIRT1, sirtuin 1; T2DM, Type 2 diabetes mellitus; TNF, tumour necrosis factor.

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HYDROXYLATION AND GLYCOSYLATION OF ADIPONECTIN

Adiponectin belongs structurally to the soluble defence collagen superfamily sharing significant homology with collagen X, VIII and complement factor C1q [3]. The adiponectin protein is composed of a signal peptide, a variable N-terminal domain, followed by a collagenous domain comprising 22 Gly-Xaa-Yaa repeats and a C-terminal C1q-like globular domain (Figure 2). The primary amino acid sequences of adiponectin are highly conserved, sharing over 80% identity among all the species cloned so far [25]. 

Adiponectin is modified extensively at the post-translational level during its secretion from adipocytes [25]. Analysis of endogenous adiponectin by two-dimensional gel electrophoresis showed that this adipokine is heavily glycosylated to form a cluster of heterogeneous ‘spots’ with different pI values and molecular masses. Our early studies on mouse adiponectin secreted from adipocytes and bovine adiponectin purified from

Figure 1  Summary of multiple biological actions of adiponectin in its major target tissues

I/R, ischaemia/reperfusion; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.

Figure 2  Structural domains of adiponectin and its PTMs

The sequence alignment above shows that all the amino acid residues with known PTMs are highly conserved among different species of adiponectin. The cysteine residue in the variable region mediates the disulfide bond formation during the formation of hexameric and HMW oligomeric adiponectin.
plasma have identified four conserved proline residues in the collagenous domain that undergo hydroxylation, and five conserved lysine residues (one in the hypervariable region and four in the collagenous domain) that are hydroxylated and subsequently glycosylated by a glucosyl α(1-2)galactosyl group, as determined by NMR analysis (Figure 1) [25,26]. A recent MS-based study by Whitehead and colleagues confirmed the presence of these PTMs (post-translational modifications) in human adiponectin and identified three additional hydroxylations on Pro\textsuperscript{31}, Pro\textsuperscript{36} and Pro\textsuperscript{65} [27].

The precise enzymes responsible for PTMs remain elusive at this stage. A family of lysyl hydroxylases (EC 1.14.11.4), consisting of lysyl hydroxylase-1, -2a, -2b and -3, has been shown to catalyse the conversion of lysine into hydroxylysine [28]. Pharmacological inhibition of these enzymes by minoxidil results in a reduced glycosylation of adiponectin secreted from rat adipocytes [19]. Among these four members, lysyl hydroxylase-3 is the most likely candidate involved in PTMs of adiponectin. In addition to its lysyl hydroxylase activity, lysyl hydroxylase-3 also possesses a low level of glucosyltransferase and galactosyltransferase activities, suggesting that this enzyme alone is sufficient for catalysing lysine hydroxylation and its further attachment with an glucosyl α(1,2)galactosyl group [29]. Lysine hydroxylation and glycosylation play an obligatory role in mediating the effects of adiponectin to inhibit hepatic gluconeogenesis [25], through enhancing the oligomerization of this adipokine (see the detailed discussion below). In addition, these PTMs have been implicated to be involved in the binding of adiponectin to its putative receptor T-cadherin [30], a glycosyl-phosphatidylinositol-linked cell-surface molecule.

In addition to hydroxylation and/or glycosylation on those lysine and proline residues, adiponectin is modified by a α,2,8-linked disialic acid moiety (Neu5Acor2→8Neu5Acor2,3Gal) [31], although the site(s) and functional significance of this modification have not been determined.

**OLIGOMERIZATION OF ADIPONECIN**

The unique feature of adiponectin structure is its ability to assemble into several characteristic oligomeric isoforms, including trimeric, hexameric and the HMW oligomeric complexes consisting of 18 protomers or more. In the circulation, adiponectin is present predominantly as these three oligomeric complexes [17,32–34]. The monomeric form of adiponectin has never been detected under native conditions. Mounting evidence from clinical and genetic as well as animal-based studies suggest that oligomerization represents a key mechanism that regulates the multiple biological activities of adiponectin.

**Biochemical and structural properties of different adiponectin oligomers**

The trimeric adiponectin, also called the LMW (low-molecular-mass) isoform, is the basic building block of adiponectin. The trimer is formed via hydrophobic interactions within its globular heads of adiponectin and stabilized by the non-covalent interactions of the collagenous domains in a triple-helix stalk. Freeze–etch electron microscopy showed the trimer to exhibit a ball-and-stick-like structure containing a large globular sphere, an extended collagen stalk and a smaller sphere on the opposite end of the stalk [17]. The crystal structure of the globular domain of adiponectin, which forms exclusively as the trimeric complexes, has been resolved at a resolution of 2.1 Å (1 Å = 0.1 nm) [35]. Notably, this structure reveals an unexpected homology with the TNF (tumour necrosis factor) family of cytokines. Despite the lack of homology at the primary amino acid sequence level, the structural features between TNFα and globular adiponectin are highly conserved. Both TNFα and globular adiponectin have a ten-β-strand jellyroll folding topology and form bell-shaped homotrimeric oligomers.

The hexameric adiponectin, also termed the MMW (medium-molecular-mass) oligomeric complex, is formed through the disulphide bond-mediated self-association of two homotrimers [17,32,36]. The cysteine residue located in the N-terminal variable region (Cys\textsuperscript{66} in human and Cys\textsuperscript{69} in mouse adiponectin respectively) is essential for the disulphide bond formation. The adiponectin mutant with this cysteine residue being replaced by either alanine or serine loses the capacity to assemble further into hexameric and the HMW oligomers [17,36,37]. The architecture of hexameric adiponectin visualized by high-resolution electron microscopy shows two trimers lying adjacent to each other in parallel head-to-head fashion and is reminiscent of the letter Y.

The structural properties of HMW adiponectin remain poorly characterized at this stage, owing to the heterogeneous nature of this isoform. A recent sedimentation equilibrium-based study suggests that HMW adiponectin purified from both bovine serum and culture of NIH 3T3-L1 adipocytes is octadecameric [38]. Analysis of bovine HMW adiponectin by dynamic light scattering and transmission electron microscopy shows an asymmetric bouquet-like architecture resembling that of complement C1q, Six globular objects can be seen atop a thin stalk, which presumably corresponds to the six trimers on a collagen triple helix that are required to form the octadecameric complex. The stalks bunch together in a manner that is consistent with the requirement for N-terminal disulphide bonding. The side views of HMW adiponectin suggest a conical structure of the oligomer with the C-terminal portion forming the base. Interestingly, these globular domains are arranged in a tight ring. This circular arrangement might enable multivalent interactions of globular domains with a single receptor. It is important to note that the composition of human HMW adiponectin oligomers differ from those observed in rodents and cows. Analysis of adiponectin oligomers by non-reducing and non-heating gel electrophoresis shows that the human HMW adiponectin isoforms are composed of multiple species, ranging from 18–30-mers or even larger molecular mass species [27,53], whereas murine and bovine HMW adiponectin contains only the octadecamers [38].

**Methods to measure different oligomeric complexes of adiponectin**

In the last several years, a handful of methods have been developed to analyse the composition and distribution of the three adiponectin oligomeric isoforms. Scherer and colleagues developed a method of velocity sedimentation followed by Western blot analysis to separate and quantify oligomeric complex distribution of adiponectin in human and animal serum samples [36,39]. However, this method cannot discriminate between the hexameric and trimeric complexes of adiponectin. The non-heating and non-reducing SDS/PAGE analysis originally described by Kadowaki and colleagues is a relatively simpler and less time-consuming method for the separation and semi-quantification of different adiponectin oligomers, and is now routinely used in many basic research laboratories [33]. Gel filtration followed by accurate quantification of adiponectin using sandwich ELISA is perhaps the most reliable method for measurement of different adiponectin oligomeric complexes [17,34,40]. Although this method is tedious and demands specific instrumentation, gel filtration enables the separation of different oligomeric complexes of adiponectin with sufficient quantities for further functional investigations [17,37,40].
A versatile ELISA method recently developed by Kadowaki and colleagues allows the sequential measurement of different adiponectin oligomers in a single assay [41]. These authors found that protease A can selectively digest both trimeric and hexameric forms of adiponectin, whereas protease K can only digest the trimeric complex. Therefore, by sequential pre-treatment of the same samples with these two proteases, the concentrations for all the three oligomers can be calculated indirectly. The assay method enables the total and various oligomeric forms of adiponectin to be measured in a small volume of blood samples, and is particularly useful for clinical studies which involve a large number of human samples. More recently, the same authors improved further the sensitivity of this ELISA method to enable the measurement of very low concentrations of the three adiponectin oligomers in human cerebrospinal fluid [42]. Another immunoassay recently developed by Nakano et al. [43] uses a monoclonal antibody that is specific to HMW adiponectin and does not detect the trimeric and hexameric isoforms. The assay enables the easy and rapid (2–3 h) measurement of HMW adiponectin without the need for specific instrumentation and technical expertise. This simplified assay is useful for large-scale epidemiological studies to investigate the clinical relevance of HMW adiponectin.

The oligomeric complex distribution of adiponectin in health and diseases

With the availability of the aforementioned assay methods, a large number of epidemiological studies have been conducted in diverse ethnic groups to investigate the association of adiponectin oligomer distribution with various cardiometabolic parameters in health and various disease conditions. Scherer and colleagues reported that the increases in the ratio of HMW to total adiponectin, but not the total adiponectin level itself, correlated well with improved insulin sensitivity during treatment with the insulin-sensitizing drug thiazolidinediones in both diabetic mice and patients with T2DM (Type 2 diabetes mellitus) [39]. The lower serum levels of total adiponectin observed in patients with T2DM and cardiovascular disease compared with healthy controls are attributed almost entirely to the selective reduction in the HMW oligomer, with no obvious changes in the concentrations of the other two isoforms [40,44]. On the other hand, weight reduction by either calorie restriction or gastric bypass surgery results in a selective elevation of the HMW adiponectin, but not the trimeric and hexameric complexes [45–47]. In both db/db diabetic mice and ob/ob obese mice, the ratio of HMW to total adiponectin is markedly decreased compared with their lean controls, although the serum levels of total adiponectin are comparable among these mice [39,48].

A recent study by Garvey and colleagues has comprehensively examined the relationship between total serum adiponectin and its different oligomers with the key features of the metabolic syndrome in Caucasians [49]. These authors showed a close association of serum total adiponectin with increased insulin sensitivity, reduced abdominal fat, high basal lipid oxidation, and high concentrations of less atherogenic LDL (low-density lipoprotein) and more cardioprotective HDL (high-density lipoprotein). The HMW adiponectin, but not the other two oligomeric isoforms, is primarily responsible for these relationships. Furthermore, reduced concentrations of HMW adiponectin independently recapitulate the lipoprotein subclass profile associated with insulin resistance even after adjusting for glucose disposal rate and body mass index, suggesting that the deficiency of HMW adiponectin is a key factor in explaining the metabolic syndrome [49]. Consistent with this, serum levels of HMW adiponectin correlate better with the metabolic abnormalities associated with childhood obesity [50] and better reflect insulin resistance in subjects with hepatitis C genotype-3 infection than total [51]. In addition, Sweeney and colleagues reported that the HMW, but not trimeric or hexameric forms of adiponectin, correlates with markers of the metabolic syndrome and liver injury in Thai subjects [52]. Several prospective studies suggest that serum HMW adiponectin is a better marker than total adiponectin in the prediction of insulin resistance and the metabolic syndrome [53], endothelial dysfunction [54] and T2DM [55,56]. Furthermore, a recent 7-year follow-up study by Inoue et al. [57] showed that only serum HMW adiponectin, but not the other oligomeric isoforms, serves as an independent predictor of future cardiovascular events in patients with coronary heart disease.

In line with these data from epidemiological studies, there is also genetic evidence supporting the role of HMW adiponectin as a major insulin-sensitizing isoform in humans. Kadowaki and colleagues have reported two rare genetic mutations (G84R and G90S) within the collagenous domain closely associated with insulin resistance and T2DM [33]. Interestingly, subjects with either of these two mutations have extremely low levels of HMW adiponectin. Moreover, recombinant adiponectin with either of these two mutations expressed in NIH 3T3 fibroblasts exhibited an impaired ability to form the HMW oligomers. Taken together, these epidemiological and genetic data suggest that the beneficial metabolic effects of adiponectin in humans might be mediated primarily by its HMW isoform, and the deficiency of this oligomer is an important aetiological factor that links obesity with its medical complications.

Distinct biological functions of different adiponectin oligomers

The functional studies of adiponectin have been complicated by the heterogeneity of its oligomeric complexes. Recombinant adiponectin produced from various sources often have different oligomeric complex distribution. Increasing evidence suggests that different adiponectin oligomers act on different target tissues and exert distinct biological functions.

Consistent with clinical observations, recent evidence from both in vitro and animal-based studies also supports the role of the HMW oligomer as the major active form in mediating the multiple metabolic actions of adiponectin in the liver. First, Scherer and colleagues showed that recombinant adiponectin produced from mammalian cells, which can form the HMW oligomers, potently decreased hyperglycaemia in diabetic mice through inhibition of hepatic glucose production [58]. However, bacterially generated full-length adiponectin, which lacks the capacity to form the HMW adiponectin, was almost inactive. These authors also compared the acute effects of each adiponectin oligomer prepared by gel filtration in adiponectin-deficient mice, and showed that intravenous injection of the HMW adiponectin, but not the hexameric adiponectin, led to a dose-dependent decrease in serum glucose levels [39]. Secondly, our ex vivo study on primary rat hepatocytes demonstrated that the formation of the HMW oligomers is obligatory to mediate the insulin-sensitizing effects of adiponectin in suppression of hepatic gluconeogenesis [25]. More recently, we have comprehensively evaluated both acute and chronic effects of recombinant adiponectin without or with different percentage composition of the HMW oligomers in several types of rodent models [19]. Our results showed that acute injection of recombinant adiponectin enriched with the HMW oligomers resulted in marked activation of AMPK in the liver, whereas chronic infusion with this protein led to prolonged alleviation of hyperglycaemia and insulin resistance in db/db diabetic mice. However, these beneficial metabolic effects became less obvious in mice treated with recombinant
adiponectin with reduced composition of the HMW oligomers, and were abolished in mice treated with recombinant adiponectin deficient in the HMW oligomers [19]. These animal-based findings are consistent with the clinical observations showing that the ratio of HMW/total adiponectin correlated closely with hepatic insulin sensitivity as measured by the hyperinsulinaemic–euglycaemic clamp [39]. Thirdly, the role of the HMW oligomer as a predominant active form of adiponectin mediating its hepatic actions is also supported by two recent independent reports demonstrating that the insulin-sensitizing effects of the PPARγ (peroxisome-proliferator-activated receptor γ) agonists thiazolidinediones were diminished in oβb ob obese mice with the targeted mutation of the adiponectin gene [59,60]. Notably, treatment with thiazolidinediones has been shown to cause selective elevation of the HMW oligomeric adiponectin [39].

Together, these data suggest that the insulin-sensitizing effects of thiazolidinediones in the hepatic tissue are mediated, at least in part, by the HMW adiponectin. In addition to the hepatic insulin-sensitizing activity, the HMW adiponectin has also been suggested to be the most potent isoform for alleviation of fatty liver disease in high-fat-diet-induced obese mice [26], and inhibition of apolipoprotein B and E release from human hepatocytes [61].

The bioactive isoform of adiponectin responsible for its actions in skeletal muscle is still a matter of debate. Early studies by both the Lodish and Kadowaki groups showed that a bacterially generated globular domain of adiponectin, which forms exclusively as the trimeric complexes, potently increased fatty acid β-oxidation and enhanced insulin sensitivity in skeletal muscle [62,63]. On the other hand, full-length adiponectin derived from Escherichia coli, which contains a high percentage of hexameric and a small portion of trimeric adiponectin is much less effective than the globular domain of adiponectin in this tissue. Lodish and colleagues also demonstrated that different oligomers of adiponectin activated different signalling pathways in C2C12 myotubes and in isolated rat extensor digitorum longus [17,32,62,63]. The HMW and hexamer isoforms of adiponectin-activated NF-κB (nuclear factor κ B), but the trimeric isoform did not have this activity. In contrast, the trimeric adiponectin, but not the HWM and hexameric oligomers, induced phosphorylation of the AMPKα subunit at Thr172 and activation of this kinase in both C2C12 myotubes and rat extensor digitorum longus. In addition, the trimeric globular head of adiponectin also shows a much higher binding affinity than bacterially generated full-length adiponectin to Adipor1, the predominant form of adiponectin receptor expressed in skeletal muscle [64]. On the basis of these findings, the prevailing view in the last several years was that the trimer is the most potent isoform mediating the beneficial metabolic effects of adiponectin in skeletal muscle. However, it is important to note that these functional studies on skeletal muscle have used the bacterially generated globular domain of adiponectin as a source of the trimeric adiponectin. Bacterially generated adiponectin does not possess PTMs and therefore differs from endogenous adiponectin. Although the findings are certainly of pharmacological interest, the physiological significance is still uncertain. On the other hand, a more recent study from Kadowaki and colleagues has purified three oligomeric species of adiponectin from human serum, and compared the bioactivities of these endogenous adiponectin oligomers in C2C12 myotubes [65]. In contrast with the aforementioned observations, this study showed that the HMW oligomeric adiponectin had the highest binding activity to the membrane fractions of C2C12 myotubes and the most potent effect in activating AMPK in this cell line. Since adiponectin oligomeric complexes used in this study resemble closely those under physiological conditions, it raises the possibility that the HMW adiponectin is also the most potent form mediating the metabolic actions of adiponectin in skeletal muscle, and warrants further investigation.

In contrast with the peripheral effects of adiponectin on insulin sensitivity, the central actions of this adipokine appear to be mediated predominantly by its hexameric and trimeric isoforms, but not the HMW oligomers. In both humans and rodents, only the trimeric and hexameric complexes of adiponectin are present in cerebrospinal fluid [21,42,66]. The HMW oligomeric adiponectin is virtually undetectable in cerebrospinal fluid, perhaps due to the extremely large size of this complex (> 500 kDa) which makes it difficult to translocate across the blood–brain barrier. Intracerebroventricular injection of the hexameric adiponectin is sufficient to activate AMPK through Adipor I in the hypothalamus, resulting in increased food intake and decreased energy expenditure in mice. Interestingly, in oβb ob obese mice, the levels of both hexameric and trimeric adiponectin in cerebrospinal fluid are much higher than those in their lean control [21]. The elevation of hexameric and trimeric adiponectin in cerebrospinal fluid can further aggravate obesity by increasing food intake and by suppressing energy expenditure. Consistent with this finding, a recent study by Scherer and colleagues showed that the adiponectin transgenic mice are morbidly obese, with much higher levels of adipose tissue when compared with their wild-type controls, despite the significant improvement in insulin sensitivity in this transgenic animal model [67].

All three types of the adiponectin oligomers have been implicated in the multiple biological actions of this adipokine in the vasculature. In the endothelium, both the trimeric globular domain of adiponectin and full-length adiponectin enriched with the hexameric and/or HMW oligomers have been shown to be effective in enhancing nitric oxide production, and in suppressing hyperglycaemia-induced oxidative stress and inflammation [20,68–70]. On the other hand, the protective effect of adiponectin against apoptosis of endothelial cells is mediated exclusively by its HMW oligomer, but not by the hexameric and trimeric complexes [40]. In monocytes and macrophages, trimeric and HMW adiponectin have been reported to exert opposite biological effects [71]. Trimeric adiponectin inhibits LPS (lipopolysaccharide)-mediated IL (interleukin)-6 release and stimulates the secretion of the anti-inflammatory cytokine IL-10. In contrast, the HMW oligomeric adiponectin enhances the monocyte release of IL-6 [71]. In smooth muscle cells, adiponectin suppresses cell proliferation stimulated by several atherogenic growth factors [PDGF (platelet-derived growth factor)-BB, bFGF (basic fibroblast growth factor) and HB-EGF (hepin-binding epidermal growth factor-like growth factor)] in an oligomerization-dependent manner [37]. PDGF-BB binds to the HMW and hexameric complexes, but not to the trimeric adiponectin. bFGF preferentially interacts with the HMW isoform, whereas HB-EGF binds to all three forms with comparable affinities. The three adiponectin oligomers act as decoys for these atherogenic growth factors to decrease their bioavailability and to preclude their bindings to the respective membrane receptors, leading to the attenuation of smooth muscle proliferation and new intima formation [37,72]. In addition, different adiponectin oligomers exhibit preferential binding affinities to LPS, various chemokines and albumin [65,73,74].

Differential effects of different adiponectin oligomers on suppression of tumour cell growth have also been reported in several in vitro studies [12,75,76]. The inhibition of adiponectin on prostate cancer cell growth is mediated exclusively by its HMW oligomers [75]. On the other hand, recombinant adiponectin enriched with different oligomeric complexes exhibits comparable activities in suppression of cell growth and in induction
of cell apoptosis in both oestrogen-dependent and -independent breast cancer cell lines [12,76].

**Contribution of lysine hydroxylation and glycosylation to the formation of HMW adiponectin**

In light of the major role of HMW adiponectin in mediating the insulin-sensitizing activities of this adipokine, great effort has been made in the last several years to elucidate the molecular basis underlying the formation of this high-order oligomeric structure. The disulfide bond mediated by a cysteine residue within the variable region of adiponectin is obligatory, but not sufficient to form the HMW oligomeric complex [17,36]. Several lines of evidence from our laboratory and others have demonstrated a critical role of lysine hydroxylation and glycosylation within the collagenous domain in the formation and stabilization of HMW oligomeric adiponectin. First, bacterially generated recombinant adiponectin, which lacks PTMs by lysine hydroxylation and glycosylation, can assemble into trimeric and hexameric forms, but not into HMW oligomers [17,19,27,58]. Secondly, HMW adiponectin has a much higher content of carbohydrates than the trimeric and hexameric complexes of adiponectin [19]. A recent study on human plasma adiponectin showed that the higher level of glycosylation in the HMW oligomeric adiponectin is primarily attributed to the increased level of a glucosylgalactosyl moiety, rather than sialic acid [77]. Thirdly, ablation of lysine hydroxylation and glycosylation by either pharmacological inhibition or mutagenesis led to impaired intracellular assembly and secretion of the HMW adiponectin *in vitro* and *in vivo* [19,27]. In adiponectin-knockout mice infused with adenovirus expressing a mutant adiponectin in which the four lysine residues (Lys80, Lys104, Lys150 and Lys250) were replaced with arginine residues, HMW adiponectin was virtually undetectable in the circulation, despite a similar level of its mRNA expression compared with the mice injected with adenovirus expressing wild-type adiponectin [19]. Fourthly, destruction of glucosylgalactosyl residues by treatment with 10 mM metaperiodate resulted in the destabilization of HMW adiponectin and an increased susceptibility of adiponectin to protease K digestion [77]. On the other hand, depletion of sialic acids had no obvious effects on the HMW oligomeric adiponectin. Furthermore, the important role of lysine hydroxylation and glycosylation in the formation of HMW adiponectin was also confirmed by the clinical observation, showing that glycosylation of plasma adiponectin from patients with T2DM was significantly lower when compared with age- and sex-matched healthy controls [19]. Notably, this change was closely associated with a significant decrease in the proportion of HMW to total adiponectin in these diabetic patients, suggesting that these two events might be causally linked with this disease. On the other hand, glucose-induced elevation in the production of HMW oligomeric adiponectin from human adipose explants correlated with the increases in adiponectin glycosylation [27].

The precise role(s) of lysine hydroxylation and glycosylation in the biosynthesis of HMW adiponectin remains poorly understood at this stage. Sequential mutation of one, two, three and all four lysine residues within the collagenous domain resulted in a progressive decrease in the ratio of HMW to total adiponectin, suggesting that the glucosylgalactosyl moiety attached on these hydroxylsine residues might function in a co-operative manner to facilitate the formation of the HMW oligomeric adiponectin [19]. On the other hand, mutation of the hydroxylated and glycosylated lysine in the variable region had no obvious effect on the formation of HMW adiponectin [27]. Since electron microscopic analysis of HMW adiponectin indicates that each oligomer contains a single stalk of collagenous domains [38], the glucosylgalactosyl moiety attached on the four lysine residues might facilitate and/or stabilize the interactions between the collagenous regions of dimers within the HMW oligomeric isoform, but not the dimer and monomer within the trimeric isoform. In addition, lysine hydroxylation and glycosylation might enhance the secretion of HMW adiponectin from adipocytes [19].

**Sexual dimorphism in the HMW oligomeric complex of adiponectin**

The sexual dimorphism of adiponectin has been reported in both humans and rodents, with males having significantly lower plasma levels of total adiponectin than females [78–80]. The gender difference in adiponectin is attributed primarily to the inhibitory effects of the male hormone testosterone on adiponectin production. Abrogation of androgen action by castration increases serum levels of adiponectin to a level comparable with that in female mice, whereas testosterone treatment had opposite effects [79,80]. Notably, neither castration nor testosterone treatment interferes with the mRNA abundance of the adiponectin gene in adipocytes, suggesting that the regulation occurs at a post-transcriptional level.

Recent studies have demonstrated that the lower plasma levels of total adiponectin in males are mainly due to the selective reduction of HMW adiponectin, whereas the plasma concentrations of trimeric and hexameric adiponectin are similar between the two sexes [34,39]. Testosterone selectively decreases the circulating levels of HMW adiponectin by preferentially inhibiting the secretion of this oligomeric complex from adipocytes. In mice, castration results in a selective elevation of HMW adiponectin in the circulation, but has no obvious effects on serum concentrations of either hexameric or trimeric adiponectin [34]. The ratio of HMW to total adiponectin in adipose tissues is similar between male and female mice, but is substantially higher than that in the circulation, suggesting that the HMW oligomeric complex of adiponectin is selectively retained inside the cells [34,39]. The pulse–chase experiment with [35S]methionine revealed that the secretion of HMW adiponectin from adipocytes is much slower than that of the other two oligomeric complexes, and testosterone treatment decreases further the secretion of HMW adiponectin [34]. These data suggest that the three oligomeric complexes of adiponectin are released from adipocytes via distinct secretory pathways, which renders it possible for testosterone to selectively impede the secretion of HMW adiponectin from the cells. In line with this hypothesis, an early study showed that the secretion of adiponectin from adipocytes involves at least two distinct secretory pathways: one for the constitutive secretion and another one for the regulated secretion of adiponectin [81]. Immunofluorescence microscopy data revealed that adiponectin is localized in at least two distinct compartments, which are different from the secretory pathways responsible for secretion of leptin, type IV collagen and transferrin.

The selective suppression of testosterone on production of HMW oligomeric adiponectin is also supported by a number of clinical observations. In hypogonadal patients with primary testicular failure, testosterone replacement therapy for a period of 3 months led to a marked reduction in the percentage composition of HMW oligomeric adiponectin in the circulation, although this treatment caused only a modest decrease in serum levels of total adiponectin [34]. A recent study on 859 Danish school children showed a significant reduction in serum total adiponectin levels during puberty in males, but not in females [82]. The ratio of HMW to total adiponectin was also decreased when comparing pre-pubertal and post-pubertal males. Furthermore, the ratio of HMW to total adiponectin correlated negatively with testosterone,
implying that the decrease in HMW adiponectin during puberty in males is caused by testosterone.

Testosterone has been proposed as a causative factor for insulin resistance, coronary heart disease and hypertension [83,84]. In light of the potent insulin-sensitizing and vasculoprotective activities of HMW adiponectin, selective inhibition of this oligomeric complex by testosterone might explain, at least in part, why men have higher risk of insulin resistance and atherosclerosis than women.

**Regulation of the HMW adiponectin oligomer by insulin**

Recent data from both animal-based and clinical investigations provides convincing evidence to support the role of insulin as a negative regulator of adiponectin production. An inverse correlation between serum levels of adiponectin and insulin has been observed in both human subjects and animal models [2]. In Type 1 diabetic patients with insulin deficiency, serum levels of total adiponectin are much higher than their healthy controls [85]. Adipose-specific deletion of the insulin receptor in mice resulted in a ~60% increase in circulating levels of total adiponectin when compared with their wild-type controls [86]. Furthermore, serum levels of total adiponectin are markedly increased in patients with a genetically defective insulin receptor [87], or with acquired loss of insulin receptor function and extreme insulin resistance due to insulin-receptor-blocking antibodies (type B insulin resistance) [88]. On the other hand, elevation of plasma insulin concentrations in healthy individuals during a euglycaemic–hyperinsulinaemic clamp caused a significant decrease of adiponectin plasma levels under euglycaemic conditions [89]. Transcriptional down-regulation of adiponectin gene expression in response to insulin has also been reported in the murine 3T3-L1 adipocyte cell line [90,91].

Insulin-mediated reduction in serum levels of total adiponectin observed in mice and human subjects is mainly due to a preferential decrease of the HMW oligomeric adiponectin, rather than the other two isoforms. In mice, acute injection of insulin resulted in a selective reduction of the HMW oligomeric adiponectin in the circulation, without obvious effects on the other two oligomeric isoforms [36]. In a recent euglycaemic–hyperinsulinaemic clamp study, hyperinsulinaemia was found to reduce selectively the circulating concentrations of HMW adiponectin in human subjects [44]. Conversely, in type B insulin-resistant patients with acquired loss of insulin receptor function, the paradoxical elevation in serum levels of total adiponectin was accounted for largely by the increases in the HMW oligomers [88]. Hyperinsulinaemia had little effect on the clearance rates of HMW adiponectin in the circulation [44]. Together, these data suggest that insulin preferentially decreases the circulating levels of HMW adiponectin by inhibiting its secretion from adipocytes.

The inhibition of HMW adiponectin by insulin might trigger the formation of a vicious cycle during the initial stage of hyperinsulinaemia, whereby high insulin levels lead to a down-regulation of HMW adiponectin, which in turn decreases insulin sensitivity, subsequently promoting further higher levels of circulating insulin to maintain glucose homeostasis.

**Stimulatory effects of the PPARγ agonists on secretion of HMW adiponectin**

The PPARγ agonists thiazolidinediones, such as rosiglitazone and pioglitazone, have been shown to increase serum levels of adiponectin in both humans and rodents [92–94]. In ob/ob obese mice lacking adiponectin, the ability of thiazolidinediones to improve glucose tolerance and insulin sensitivity is markedly attenuated, suggesting that the therapeutic effects of these drugs were mediated, at least in part, by induction of adiponectin production [59,60]. Early studies by Shimomura and colleagues have provided evidence showing that the PPARγ agonists increase adiponectin production by promoting transcription of the corresponding mRNA through a PPARγ-responsive element in the promoter of its gene [94,95]. Several other studies, however, have shown that thiazolidinedione-induced elevation of adiponectin primarily occurs at the post-translational level, without obvious effects on mRNA expression [92,96,97]. There is growing evidence suggesting that a major mode of action of thiazolidinediones is to preferentially increase the circulating levels of HMW adiponectin [14]. In diabetic patients and animal models, treatments with thiazolidinediones lead to a selective elevation in the circulating levels of the HMW oligomeric adiponectin, but not the other isoforms [39]. Furthermore, changes in HMW oligomeric adiponectin, but not total adiponectin, correlate strongly with a thiazolidinedione-mediated increase in insulin sensitivity [39]. Addition of thiazolidinediones into the human adipose tissue explants dose-dependently increases the secretion of the HMW isoform into the extracellular medium, with no significant effect on adiponectin mRNA expression and secretion of the other isoforms [96]. Similar results were also observed in mouse adipocytes, 3T3-L1 adipocytes, NIH 3T3-F422A adipocytes and SGBS human adipocytes, but not in HEK-293 (human embryonic kidney) cells [39], suggesting that additional adipocyte-specific or -enriched PPARγ agonist-responsive factors are required for this process. More recent studies demonstrated that the PPARγ agonists stimulate the secretion of HMW adiponectin by inducing the adipocyte expression of the ER (endoplasmic reticulum) oxidoreductase 1-Lα (Ero1-Lα) [48,98] (see below for a detailed discussion).

**CELLULAR MACHINARIES INVOLVED IN THE SECRETION OF ADIPONECTIN OLIGOMERS**

Despite the fact that different adiponectin oligomers possess distinct biological functions, these oligomeric forms, once released from adipocytes, are not interchangeable, suggesting that adiponectin oligomeric complex distribution in the circulation is primarily controlled at the level of secretion from adipocytes [36]. Recent studies have demonstrated that the secretion of adiponectin oligomers from adipocytes is tightly regulated by a pair of molecular chaperones in the ER, including ERp44 (ER protein of 44 kDa) and Ero1-Lα, both of which are induced during adipogenesis [48,98]. Using a pulse–chase experiment with [35S]cysteine/[35S]methionine, Scherer and colleagues showed that a significant portion of de novo-synthesized adiponectin is not secreted, but is retained in adipocytes, even after a prolonged chase period [48]. Treatment of cells with reducing reagents such as 2-mercaptoethanol stimulates the release of intracellular adiponectin by 7–8-fold, suggesting that adiponectin is trapped inside the cells through a thiol-mediated retention. This thiol-mediated intracellular retention of adiponectin is caused by a covalent interaction between adiponectin and the ER-resident protein ERp44, a phenomenon reminiscent of that observed in the immunoglobulin M complexes [99,100]. ERp44 forms a mixed disulfide bond with adiponectin through the cysteine residue within its variable region (Cysγ in humans and Cysγ in mice) [48]. Adiponectin secretion is inhibited by overexpression of ERp44, and is enhanced by siRNA (small interfering RNA)-mediated down-regulation of this ER-resident protein. Because maturation of adiponectin oligomers needs extensive PTM, this thiol-mediated retention by ERp44 within the ER may prolong the resident time for this adipokine in the secretory pathway and thus
provide a better chance for proper folding into the higher-order complexes.

In contrast with the inhibitory effects of ERp44, overexpression of Ero1-Lα selectively enhances the secretion of HMW adiponectin [48,98]. Moreover, co-expression of Ero1-Lα with ERp44 disrupts the covalent interaction between ERp44 and adiponectin and releases the thiol-mediated retention of adiponectin by ERp44. In contrast, siRNA-mediated down-regulation of Ero1-Lα inhibits the secretion of HMW adiponectin, but enhances the release of trimeric adiponectin [48]. Ero1-Lα has been proposed to stimulate the production of HMW through at least two mechanisms. First, as an ER-membrane-associated oxidoreductase, Ero1-Lα might participate in adiponectin maturation by utilizing the oxidizing power of oxygen to generate disulfide bonds in itself, which it then transfers to PDI (protein disulfide-isomerase), and the resulting oxidized PDI is then able to transfer its disulfide bonds to adiponectin to facilitate the formation of its high-order structures. Secondly, as a preferred binding partner of ERp44 [100], Ero1-Lα can displace the ERp44-retained passenger proteins such as HMW adiponectin, and therefore release this oligomeric complex trapped by ERp44. Therefore ERp44 and Ero1-Lα appear to act as a Yin and Yang pair in the ER to tightly control the maturation and secretion of adiponectin oligomers.

The role of ERp44 and Ero1-Lα in regulating the secretion of adiponectin oligomeric complexes is also corroborated by the observation that changes in expression of these two proteins are closely associated with alterations in the oligomeric complex distribution of adiponectin under various pathophysiological conditions [48]. In ob/ob obese mice, the decreased expression of ERp44 and Ero1-Lα in adipose tissue is associated with the reduced ratio of HMW to total adiponectin in the circulation. Conversely, the PPARγ agonists thiazolidinediones, which selectively induce the secretion of HMW adiponectin, induce Ero1-Lα expression in 3T3-L1 adipocytes and adipose tissue of ob/ob obese mice [48,98]. Moreover, regulation of the HMW oligomeric adiponectin by the NAD-dependent deacetylase SIRT1 (sirtuin 1) is also mediated by Ero1-Lα [98]. The SIRT1 activator resveratrol suppresses the secretion of HMW oligomeric adiponectin through deacetylation and inhibition of PPARγ, resulting in the down-regulation of the PPARγ-responsive gene Ero1-Lα. On the other hand, glucose and lactate selectively enhance the secretion of HMW oligomeric adiponectin through inhibition of SIRT1, thus leading to activation of PPARγ and up-regulation of Ero1-Lα.

The release of adiponectin from the ER into the extracellular medium is mediated by a trafficking vehicle containing GGA1 [Golgi-associated γ-adaptin ear homology domain Arf (ADP-ribosylation factor)-interacting protein 1] [101]. Overexpression
This possibility warrants further investigation.

PROTEOLYSIS OF ADIPONECTIN

In addition to the aforementioned PTMs (hydroxylation, glycosylation, and oligomerization), there is evidence to suggest that adiponectin undergoes proteolysis after its secretion [4]. The presence of a small amount of a truncated globular domain of adiponectin has been demonstrated in human serum by immunoprecipitation [62]. A study by Kadowaki and colleagues has identified a leucocyte elastase secreted from the human monocyte cell line THP1 that cleaves adiponectin in vitro [102]. The cleavage of adiponectin by the leucocyte elastase occurs at four distinct sites in the collagenous domain. Nevertheless, it is important to note that the preponderance of serum adiponectin is present as its full-length protein [26]. Whether or not the proteolytic cleavage of adiponectin observed in vitro also occurs in vivo remains to be clarified further. Alternatively, it is also possible that the proteolysis of adiponectin occurs at the target sites of this adipokine by the proteases released from local tissues. This possibility warrants further investigation.

SUMMARY AND FUTURE PROSPECTIVES

Recent data from clinical, genetic and animal-based studies unanimously supports the role of adiponectin as an important insulin-sensitizing adipokine with multiple beneficial effects on obesity-associated medical complications. The diverse biological functions of adiponectin are attributed to its three oligomeric forms, which act on different targets and activate distinct signalling pathways. HMW adiponectin is the major bioactive form responsible for insulin-sensitization at the periphery tissues, whereas the central actions of this adipokine appear to be mediated exclusively by its hexameric and trimeric oligomers. The composition of adiponectin oligomers in the circulation, particularly the ratio of HMW to total adiponectin, varies under different obesity-related pathological conditions, and can be reversed by pharmacological and lifestyle interventions.

The biosynthesis and secretion of adiponectin in adipocytes is a complex process that involves several types of PTM (Figure 3). Secretion of adiponectin oligomers, especially HMW adiponectin, is tightly controlled by a pair of ER-resident proteins (ERp44 and Ero1-Lα). A number of pathophysiological conditions, metabolic hormones and pharmacological agents cause alterations in the distribution of adiponectin oligomeric complexes by modulating the expression of these two rate-limiting chaperones. The finding that the PPARγ agonists selectively increase the circulating concentrations of HMW adiponectin through up-regulation of Ero1-Lα expression raises the possibility of targeting the cellular components involved in the adiponectin secretory pathway as a new therapeutic strategy to combat obesity-related medical complications.

Despite these exciting research advances, it is important to note that regulation of adiponectin oligomer composition is complex and occurs at multiple levels through multiple mechanisms, and our understanding of this process is still at a very early stage. Further studies should focus on identification of additional cellular components involved in the adiponectin secretory pathways and elucidation of how these cellular components are co-ordinated to tightly control the secretion of different adiponectin oligomers under various pathophysiological conditions. The structural and molecular basis underlying the differential effects of different adiponectin oligomers remain largely uncharacterized at this stage. In particular, whether or not different oligomers of adiponectin exert their actions through distinct receptors, or the same receptors with different binding affinity, should be clarified further by future studies.

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