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Extracellular conversion of adiponectin hexamers into trimers

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Synopsis

Adiponectin is an adipocyte-secreted hormone that exists as trimers, hexamers and larger species collectively referred to as HMW (high-molecular-weight) adiponectin. Whether hexamers or HMW adiponectin serve as precursors for trimers outside the circulation is currently unknown. Here, we demonstrate that adiponectin trimers can be generated from larger oligomers secreted from primary rat adipose cells or differentiated 3T3-L1 adipocytes. Purified hexameric, but not HMW, adiponectin converted into trimers in conditioned media separated from 3T3-L1 adipocytes or, more efficiently, when enclosed in the dialysis membrane in the presence of adipocytes. Several lines of evidence indicate that the conversion is mediated by an extracellular redox system. First, N-terminal epitope-tagged hexamers converted into trimers without proteolytic removal of the tag. Secondly, appearance of trimers was associated with conversion of disulfide-bonded dimers into monomers. Thirdly, thiol-reactive agents inhibited conversion into trimers. Consistent with a redox-based mechanism, purified hexamers reductively converted into trimers in defined glutathione redox buffer with reduction potential typically found in the extracellular environment while the HMW adiponectin remained stable. In addition, conversion of hexamers into trimers was enhanced by NADPH, but not by $NADP^+$. Collectively, these data strongly suggest the presence of an extracellular redox system capable of converting adiponectin oligomers.

Key words: adipocyte, adiponectin, oligomerization, oxidation–reduction, redox, thiol

INTRODUCTION

Adiponectin, an abundant serum protein secreted mainly from adipocytes with anti-inflammatory and anti-atherosclerotic properties [1–3], enhances insulin sensitivity [3–6] and confers protection against cardiac and vascular dysfunctions [7–11]. Mature monomeric adiponectin of 26 kDa [12] multimerizes into trimers {LMW [low-molecular-mass (weight)] adiponectin [13]}, hexamers {MMW [medium-molecular mass (weight) [13,14] and LMW [15,16]}, and several larger species collectively referred to as HMW [high-molecular-mass (weight)] adiponectin [6,13,17]. The largest and most abundant HMW adiponectin is an octadecamer (18mer) [18].

Accumulating evidence indicates that levels of circulating HMW adiponectin correlate more closely with insulin action than the total adiponectin [19–23], suggesting that HMW adiponectin is more important for enhancing insulin action than the other oligomers. Infusion of HMW adiponectin, but not hexamers lowered blood glucose in adiponectin knockout mice [19]. Trimeric adiponectin was shown to inhibit hepatocyte glucose production and lower blood glucose [16]. Furthermore, trimeric adiponectin activates AMPK (AMP-activated protein kinase) in skeletal muscle [24] and cardiac myocytes [25], whereas HMW adiponectin activates AMPK in the liver [13,26,27]. Hexamers, on the other hand, do not appear to be as metabolically active. Interestingly, hexamers constitute the major adiponectin species in male mice [5,15], and are as abundant as the other isoforms in male humans [13,15], raising the question of their biological role.

Both HMW and trimeric adiponectin having similar metabolic actions suggest that the HMW isoform can serve as a precursor to trimers. However, purified and iodinated/fluorophore-tagged or semi-purified but untagged adiponectin oligomers did not undergo net conversion into another oligomeric form in serum following injection into animals [15,16,28]. As a result, we hypothesized that adiponectin oligomer conversion can take place near the cell surface. To test this hypothesis, we sought

Abbreviations used: AMPK, AMP-activated protein kinase; HMW, high-molecular mass (weight); HSD, honestly significant difference; Hsp70, heat-shock protein 70; LMW, low-molecular mass (weight); MWCO, molecular-mass cut-off; NEM, *N*-ethylmaleimide.

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evidence of conversion in both 3T3-L1 and primary rat adipocyte culture media. It has been proposed that a reductase near the cell surface could convert HMW adiponectin into trimers [19], but the potential mechanisms for inter-conversion of adiponectin oligomers are not known. Cells actively maintain extracellular redox state within a specific range [29]. Altering extracellular redox state affects a variety of cellular processes including proliferation, apoptosis and NF-κB (nuclear factor κB) signalling [29]. Plasma redox state is significantly more oxidative in a number of disease states including Type 2 diabetes [30]. Extracellular reduction of adiponectin leading to formation of trimers from larger oligomers is potentially important because it may lead to differential signalling resulting from changes in oligomer composition. In this paper, we present evidence showing extracellular conversion of adiponectin hexamers into trimers outside adipocytes via a thiol-sensitive and redox-based mechanism.

MATERIALS AND METHODS

Cell culture and primary rat adipocyte preparation

The 3T3-L1 preadipocyte cell line (A.T.C.C.) was maintained and differentiated as previously described [6,31]. In the present study, 8–10-day-differentiated 3T3-L1 adipocytes were used. Primary adipocytes were isolated from male Sprague–Dawley rats and cultured as previously described [32,33]. All experimental protocols involving animals were approved by the appropriate Institutional Animal Care and Use Committees in accordance with the Public Health Service Animal Welfare Policy. BSA in adipocyte-conditioned media was partially removed by mixing media with washed Cibacron blue 3GA–agarose beads (Sigma) suspended in PBS at 1:1 volume ratio for 3 h at 4 °C on a rotating shaker. No adiponectin was lost during the procedure (results not shown).

Cycloheximide treatment of 3T3-L1 adipocytes

First, 8–10-day-differentiated 3T3-L1 adipocytes were washed twice in Opti-MEM I (Invitrogen) and incubated in ascorbate-supplemented (50 mg/l) Opti-MEM I media containing 20 μg/ml cycloheximide. At specific time points, the conditioned media were collected and cells were washed twice in cold PBS and lysed in 20 mM Tris, pH 7.5, 1 % Triton X-100 and Complete protease inhibitor cocktail (Roche). The conditioned media were centrifuged at 12000 ***g*** for 5 min to remove cell debris. Lysates and conditioned media were frozen at -70 °C until analysis.

Expression and purification of 5′ FLAG epitope-tagged adiponectin

Mouse adiponectin cDNA with a FLAG epitope sequence located downstream of the signal peptide sequence near the N-terminus was a gift of Dr Christopher Hug (Children's Hospital, Boston, MA, U.S.A.) [34]. FLAG-epitope-tagged adiponectin was expressed in HEK-293T (human embryonic kidney cells expressing the large T-antigen of SV40) cells as described in [6]. Adiponectin secreted into the conditioned media was collected and purified using resin-bound M2 monoclonal antibody (Sigma) and eluted with FLAG peptide according to the manufacturer's instructions. Adiponectin oligomers were enriched using gel filtration chromatography [6].

Purification of adiponectin 18mers from bovine serum and conversion into hexamers

Adiponectin was purified to homogeneity from foetal bovine (Atlanta Biologicals) or calf (Invitrogen) serum as described previously [12] and stored in sterile PBS. Oligomerization states of purified adiponectin were confirmed using gel filtration chromatography [6]. Approximately 95 % of purified adiponectin consists of 18mers with the balance being hexamers. Hexamers were derived from purified 18mers by adjusting the pH to 4 using glycine and re-neutralized to pH 7 using Hepes or PBS at pH 7.4 as previously described [35,36].

Incubation of recombinant FLAG epitope-tagged adiponectin in cell culture

First, 8-day-differentiated 3T3-L1 adipocytes in six-well plates were rinsed twice in PBS^{++} (PBS with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$) and replenished with 1.5 ml of fresh serum-free Opti-MEM I (Invitrogen) containing 2 μg/ml FLAG-epitope-tagged adiponectin oligomers per well. Isolated rat primary adipocytes in the KRH (Krebs Ringer's/Hepes) medium [32] containing 4 % BSA were incubated with 2 μg/ml FLAG-tagged adiponectin oligomers. Amounts of adiponectin oligomers remaining in the incubation media over time were determined by immunoblot analysis following separation of the incubation media from the cells and cell debris by centrifugation.

Exclusion of purified bovine serum or FLAG-epitope-tagged adiponectin oligomers from cells using a dialysis membrane

First, 8-day-differentiated 3T3-L1 adipocytes in six-well tissue culture plates were replenished with 3 ml of DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % calf serum (Gemini Bio-Products) per well and incubated for 8 h at 37 °C prior to addition of adiponectin. FLAG-tagged adiponectin enriched in hexamers or bovine serum hexameric or HMW oligomers prepared as described above were placed in a dialysis apparatus that floated on top of the adipocyte culture and separated from the media and the cells through a dialysis membrane with MWCO (molecular-mass cut-off) of 12–14 or 6–8 kDa (Spectrum Laboratories) as indicated. The dialysis apparatus was fabricated from 1.5-ml microcentrifuge tubes with most of the tube body removed from the bottom using a sharp blade, leaving an open ring approximately 3 mm in height and an intact cap. In total, 50 μl of adiponectin at 2 μg/ml in PBS was applied to the open cap and covered with washed dialysis membrane. The dialysis membrane was affixed to the cap using the remaining microcentrifuge body in the shape of an open ring. The apparatus was placed on top of the adipocyte culture media with the dialysis membrane facing the media and incubated at 37 °C for

the indicated amount of time. One individual apparatus was used per time point.

Incubation of recombinant FLAG epitope-tagged adiponectin in cell culture-conditioned media

Differentiated 3T3-L1 adipocytes were washed twice in PBS^{++} and incubated with Opti-MEM I (Invitrogen) for 24 h. The ratio of initial media volume to growth area was 0.14–0.16 ml/cm^2. Cell-conditioned media were collected and passed through 0.2 μm filters prior to storage at $-70\,^{\circ}$C. The pH of the 24 h-conditioned media was 0.1–0.35 unit lower than that of the fresh media but did not fall below 7.1. In experiments addressing the potential role of a thiol-based redox mechanism for adiponectin oligomer conversion, the conditioned media were first treated with 10 mM NEM (*N*-ethylmaleimide) or diamide at 37 °C for 10 min prior to addition of adiponectin. FLAG-tagged adiponectin oligomers were incubated at 37 °C in 2 μg/ml conditioned media. In experiments testing the effects of NADPH, NADP$^+$ and auranofin on oligomer conversion, the concentration of NADPH or NADP$^+$ was 100 μM, whereas the concentration of auranofin was 15 or 20 nM.

Reduction of adiponectin by glutathione

Bovine adiponectin hexamers (0.1 mg/ml) prepared from 18mers as described above were incubated at 37 °C for 6 h in 140 mM NaCl, 7 mM phosphate, 140 mM Hepes at pH 7 in the presence of 14.3 mM GSSG or 13.1 mM GSSG/2.4 mM GSH. Stock concentrations of GSH (400 mM) were prepared in PBS and titrated to pH 7 using NaOH. Reduction potential was calculated using the Nernst equation with a standard state potential of -240 mV at pH 7. Reactions in microcentrifuge tubes were capped with rubber septa and ambient air was flushed out with pressurized argon gas for 10 min. Purified HMW adiponectin was incubated under identical conditions without being first treated with glycine. For HMW adiponectin, additional reactions with 28.6 mM GSH were also performed. Reactions with HMW adiponectin were incubated for 48–72 h to ensure that equilibrium was reached. Oligomerization and redox states of adiponectin were determined using native and non-reducing denaturing gels [12] following addition of 10 mM NEM to alkylate-free thiols. Gels were stained using Krypton IR protein stain (Thermo Fisher) or the Coomassie Brilliant Blue G250-based Gelcode Blue (Thermo Fisher) and imaged in an Odyssey scanner (LI-COR).

Native and denaturing immunoblot analysis

Native gel electrophoresis was used to separate adiponectin oligomers in conditioned media or lysates [35]. In total, 15–20 μl of conditioned media were loaded per lane. Ferritin (Sigma) was used as a molecular-mass marker because its hydrodynamic radius is nearly identical with that of hexameric adiponectin [6]. The location of the yellow-coloured ferritin on the nitrocellulose membrane was marked using a pencil. Oxidation states of adiponectin were assessed using non-reducing SDS/PAGE [35]. Membranes were probed using a rabbit antiserum raised against recombinant globular adiponectin expressed and purified as previously described [37] followed by a secondary antibody. M2 monoclonal antibody (Sigma) was used to assess the oligomerization states of FLAG epitope-tagged adiponectin. For all the experiments, the membranes were either developed using ECL® (enhanced chemiluminescence) (Thermo Fisher) or scanned in the Odyssey system (LI-COR).

Statistical Analysis

Data are presented as means $\pm$ S.E.M. Densitometry was performed using ImageJ software. Statistical comparisons between two different groups were performed using Student's paired or unpaired Student's *t* tests with two tails as appropriate. In experiments with more than two groups, the differences were assessed using one-way ANOVA with Tukey's HSD (honestly significant difference) post-hoc test. For studies in which the values from repeated experiments were normalized to that of the control group of each individual experiment as percentages, one-sample, two-tailed, Student's *t* tests were performed to evaluate the statistical significance of measured variables against the expected value of 100. The particular statistical analyses applied to the results in specific experiments are described in the Figure legends. For all the experiments, representative gels are shown and were repeated at least three times unless noted otherwise.

RESULTS

Conversion into trimers from larger oligomers secreted from isolated rat adipocytes

Adiponectin trimers are present in significant amounts in human and rodent plasmas [6,13,15]. To assess the formation of trimeric adiponectin, we determined the relative levels of secreted and intracellular adiponectin oligomers in conditioned media and lysates from isolated rat primary adipocytes. Hexameric and HMW oligomers constituted the most of secreted adiponectin. Trimers appeared 8 h after adipocyte isolation (Figure 1A). Trimers increased disproportionately to hexamers and HMW adiponectin from 8 to 24 h (Figures 1A and 1B). An adiponectin trimer consists of two monomers linked by a disulfide bond and one unpaired monomer, whereas all the monomeric units within hexameric and HMW adiponectin are disulfide-linked [24]. As a result, trimers fractionate as dimers and monomers in non-reducing denaturing PAGE, and hexamers and HMW oligomers fractionate as exclusive dimers. In contrast, all oligomers fractionate as monomers in reducing and denaturing PAGE [24]. Non-reducing denaturing immunoblot analysis of secreted adiponectin showed significant amounts of monomers by 24 h (Figure 1C) consistent with the accumulation of trimers (Figure 1A). There are two potential sources of trimers: secreted from adipocytes or converted from larger oligomers extracellularly. The latter is more consistent with immunoblot analysis of adipocyte lysates showing very low amounts of trimers remaining in cells at later time points (Figure 1D). To determine the origin of trimers, 8 h adipocyte-conditioned media containing mostly hexameric and HMW adiponectin were separated from the cells and incubated for an additional 16 h. Significant amounts of trimers were

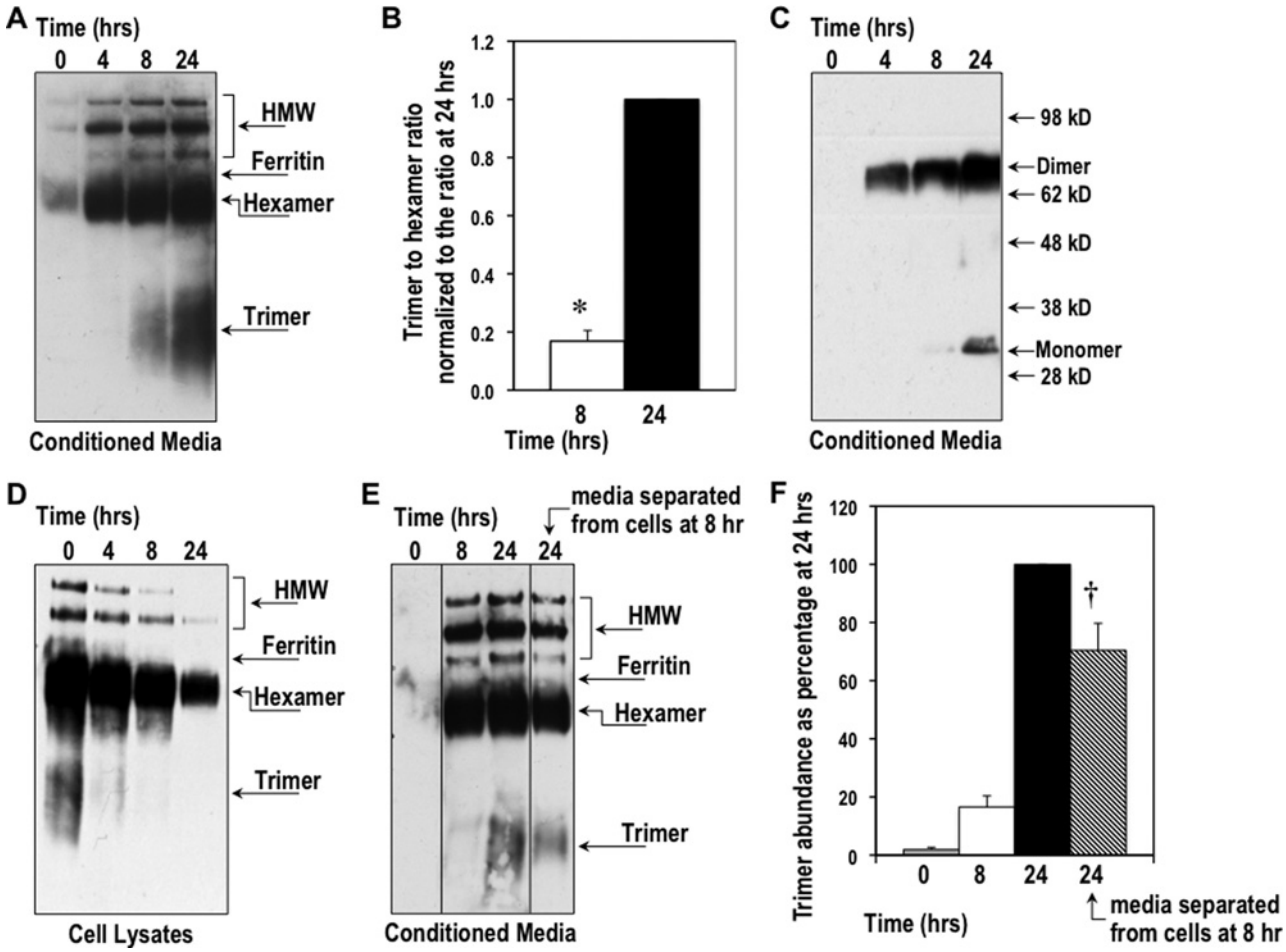

Figure 1 **Conversion into trimers from larger oligomers secreted from isolated rat adipocytes**

Amounts of adiponectin oligomers secreted from rat primary adipocytes at various time points following isolation were determined by (**A**, **B**) native and (**C**) non-reducing denaturing immunoblot analysis as described in the Materials and methods section. To demonstrate the disproportionate increase in trimers compared with hexamers from 8 to 24 h, the trimer to hexamer ratio at the 8-h time point was normalized to that at the 24-h time point for each experiment. Average ratios at the 8- and 24-h time points were shown. Asterisk denotes $P < 0.003$ in one sample, two-tailed, Student's t test against hypothetical mean value of 1 with $n = 3$. Previous non-reducing denaturing analysis showed mature adiponectin trimers to consist of one disulfide-bonded dimer of approximately 50 kDa and one unpaired monomer of approximately 28 kDa [24]. (**D**) shows the results of native immunoblot analysis of adiponectin oligomers in lysates prepared from isolated rat primary adipocyte at various time points following isolation. To determine if at least some of the trimers were converted from larger oligomers, aliquots of rat adipocyte-conditioned media were passed through 0.2 μm filters to remove cells and cell debris at the 8-h time period and then further incubated for 16 h at 37 °C. The amounts of adiponectin oligomers secreted from rat primary adipocytes for 8 and 24 h after isolation and for 8 h after isolation followed by an additional 16 h of incubation in the absence of adipocytes were assessed by native immunoblot analysis and the results are shown in (**E**) and the amount of trimers graphically represented in (**F**). In (**F**), the amounts at 0 and 8-h time points in the presence of adipocytes or at 24 h with the last 16 h in the absence of adipocytes were normalized as percentages to the abundance of trimers 24 h after isolation in each experiment. †$P < 0.03$ in paired two-tailed Student's t test against 8-h post isolation time point with $n = 3$.

observed after the additional incubation period following separation from adipocytes (Figures 1E and 1F), indicating formation of trimers from secreted hexameric or HMW adiponectin in the absence of cells.

Persistent increase in adiponectin trimers in 3T3-L1 adipocyte culture media despite translation arrest

We also examined the formation of adiponectin trimers from differentiated 3T3-L1 adipocytes. Treating 3T3-L1 adipocytes with cycloheximide revealed a persistent presence of intracellular hexamers despite translation arrest (Figure 2A), consistent with pulse–chase studies showing intracellular retention and slow degradation of adiponectin [27] and accumulation of hexamers in human adipose tissue [38]. In contrast, only small amounts of intracellular trimers were detected after 4 h of cycloheximide treatment (Figure 2A). Despite the lack of intracellular trimers, secreted trimers steadily increased up to 16 h (Figure 2B). Hsp70 (heat-shock protein 70), a protein with rapid turnover in

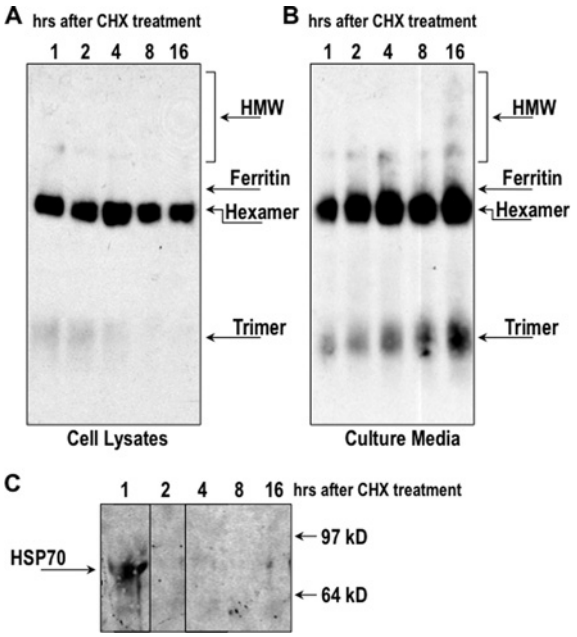

Figure 2 Persistent increase in adiponectin trimers in 3T3-L1 adipocyte culture media despite translation arrest

3T3-L1 adipocytes were treated with 20 μg/ml cycloheximide (CHX) and levels of adiponectin oligomers in (**A**) conditioned media and (**B**) lysates at various time points after addition of cycloheximide were analysed by native immunoblot procedure described in the Materials and methods section. (**C**) To confirm the efficacy of the cycloheximide treatment, amounts of Hsp70 protein in lysates at different time points after addition of cycloheximide were assessed by immunoblot analysis using an antibody against Hsp70 (Cell Signaling) following fractionation in reducing and denaturing SDS/PAGE.

non-stressed cells [39], was not detected 2 h post-cycloheximide treatment (Figure 2C). The results indicate that trimers continuously increased in media without newly synthesized protein.

Direct demonstration of extracellular adiponectin oligomer conversion from hexamers to trimers

To determine whether adiponectin trimers are formed extracellularly, two experimental approaches were employed. First, HMW and hexamer adiponectin purified from bovine serum were enclosed in a 12–14 kDa MWCO dialysis apparatus floating on top of 3T3-L1 adipocyte cultures to prevent mixing between purified adiponectin oligomers and adiponectin present in the calf serum added to cell culture media. Secondly, purified recombinant FLAG epitope-tagged individual oligomers were incubated with 3T3-L1 adipocyte-conditioned media after the cells were removed. Purified bovine adiponectin hexamers converted into trimers and HMW adiponectin following incubation with 3T3-L1 adipocytes (Figure 3A, left-hand panel). In contrast, purified bovine HMW adiponectin (mostly 18mers) did not undergo conversion into trimers or hexamers (Figure 3A, right-hand panel). Similarly, small amounts of FLAG epitope-tagged trimers were observed after FLAG-tagged hexamers were incubated with conditioned media collected from 3T3-L1 adipocyte cultures for 24 h (Figure 3B, left-hand panel). FLAG-tagged HMW or

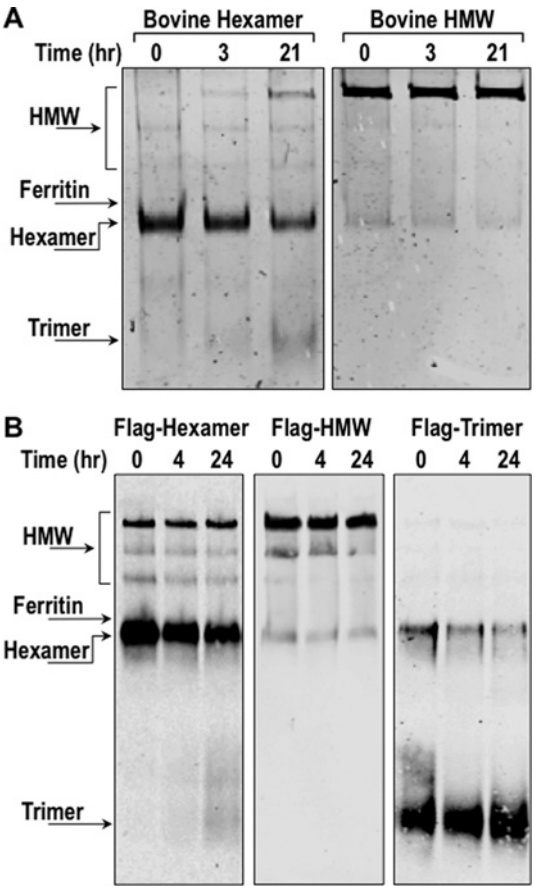

Figure 3 Direct demonstration of extracellular adiponectin oligomer conversion from hexamers to trimers

Bovine serum and FLAG epitope-tagged adiponectin oligomers were purified as described in the Materials and methods. Section (**A**) Hexamer (left-hand panel) and HMW (right-hand panel) adiponectin purified from bovine serum (2 μg/ml in PBS) were placed inside a 12–14 kDa MWCO dialysis apparatus constructed from microcentrifuge tubes and incubated with 3T3-L1 adipocytes at 37 °C for 0, 3 and 21 h. (**B**) FLAG-tagged hexamers (left-hand panel), HMW adiponectin (middle panel) and trimers (right-hand panel) were incubated at 37 °C with conditioned media separated from differentiated 3T3-L1 adipocytes at a concentration of 2 μg/ml for 0, 4 and 24 h. Samples removed from reaction mixtures at specific time points were fractionated on native acrylamide gels and amounts of adiponectin oligomers were assessed by immunoblotting as described in the Materials and methods section.

trimer adiponectin did not convert into other oligomers (Figure 3B, middle and right-hand panels).

Breakdown of adiponectin hexamers coupled with appearance of trimers in 3T3-L1 and isolated rat adipocyte cultures

Reduction of the disulfide bond near the N-terminus of adiponectin is known to convert hexamers into trimers [6,16], suggesting that disulfide cleavage is potentially a mechanism for conversion into the trimeric isoform. The observation that only small amounts of trimers converted from hexamers in conditioned media in the absence of cells (Figure 3) is consistent

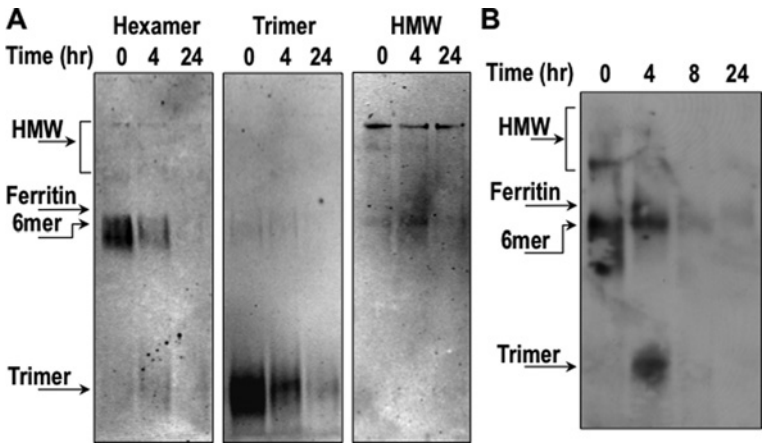

Figure 4 Breakdown of adiponectin hexamers coupled with the appearance of trimers in 3T3-L1 and isolated rat adipocyte cultures

(**A**) FLAG-tagged adiponectin enriched in hexamers (left-hand panel), trimers (the middle panel) and HMW isoforms (right-hand panel) were added to serum-free media and incubated with differentiated 3T3-L1 adipocytes at 37 °C for various lengths of time as indicated. (**B**) FLAG-tagged adiponectin enriched in hexamers was incubated with isolated rat primary adipocytes for the indicated lengths of time and amounts of adiponectin oligomers remaining at each time point were measured using immunoblot analysis against the FLAG epitope. Experiments were performed in duplicate.

with disulfide bond reduction and cleavage as the mechanism. As hexamers accept electrons from reducing agents and become trimers, these reducing agents are themselves oxidized. Without replenishment of reducing equivalents from cells, consumption of reducing agents is expected to limit conversion. Thus, we examined the conversion of adiponectin oligomers in the presence of 3T3-L1 adipocytes. When added to 3T3-L1 adipocyte cultures, nearly all the FLAG-tagged hexamers disappeared by 24 h and only small amounts remained by 4 h (Figure 4A, left-hand panel). These hexamers could have been taken up into the cells, proteolytically cleaved and/or converted into trimers. At the 4-h time point after addition of FLAG-tagged hexamers, FLAG-tagged trimers were visible in adipocyte cultures (Figure 4A, left-hand panel). This result is consistent with those in Figure 3 showing conversion from hexamers into trimers. In the presence of cells, FLAG-tagged trimers almost completely disappeared from the cultures after 24 h of incubation (Figure 4A, middle panel), indicating rapid proteolysis and/or uptake into cells. In contrast, FLAG-tagged HMW adiponectin remained stable over 24 h (Figure 4A, right-hand panel).

To assess whether the conversion from hexamer into trimer can occur in primary adipocyte culture, we performed similar experiments in the presence of isolated rat adipocytes. Again, most of the FLAG epitope-tagged adiponectin hexamers rapidly disappeared after 4 h of incubation with isolated rat adipocytes, whereas significant amounts of FLAG-tagged trimers appeared at a 4 h incubation with primary rat adipocytes, suggesting conversion from hexamers into trimers (Figure 4B).

Conversion of adiponectin hexamers in 3T3-L1 adipocyte cultures

In order to distinguish the conversion of hexamers seen in Figure 4 from degradation or uptake into cells, we enclosed purified FLAG-tagged adiponectin in the dialysis membrane prior to incubation with cells. To avoid contact between FLAG-tagged adiponectin and cultured cells, we placed FLAG-tagged hexamers inside the dialysis apparatus (12–14 kDa MWCO) and floated them on top of the 3T3-L1 adipocyte cultures as shown in Figure 3(A). Significant amounts of adiponectin trimers were converted from hexamers after 24 h of incubation in 3T3-L1 adipocytes (Figure 5A). Incubation of FLAG-tagged adiponectin in media alone in the absence of 3T3-L1 adipocytes did not result in significant conversion into trimers (Figure 5A). The extent of conversion into trimers as a ratio of trimer to hexamer in the dialysis apparatus was significantly lower at 4 h but higher at 24 h than that in the adipocyte-conditioned media without cells (Figure 5B). These results suggest that the comparatively low level of hexamer-to-trimer conversion in conditioned media (Figure 3B) is due to the lack of replenishment of the limiting agent(s) secreted by cells. The decrease in extent of conversion into trimers at 4 h was probably due to slow diffusion of these agents across the dialysis membrane.

Disulfide reduction as potential mechanism for conversion into trimers

The conserved cysteine residue near the N-terminus of adiponectin plays an important role in adiponectin oligomerization [12,13,16,24]. To assess if disulfide bond reduction or proteolytic degradation is the mechanism for conversion into trimers, we examined conversion into trimers in the dialysis apparatus with a 6–8 kDa MWCO membrane in the presence of 3T3-L1 adipocytes. As shown in Figure 6(A). FLAG-tagged adiponectin enclosed within the 6–8 kDa MWCO dialysis membrane underwent conversion into trimers. Examination of the PMAP database [40] did not reveal proteases smaller than 8 kDa, only peptidases. Furthermore,

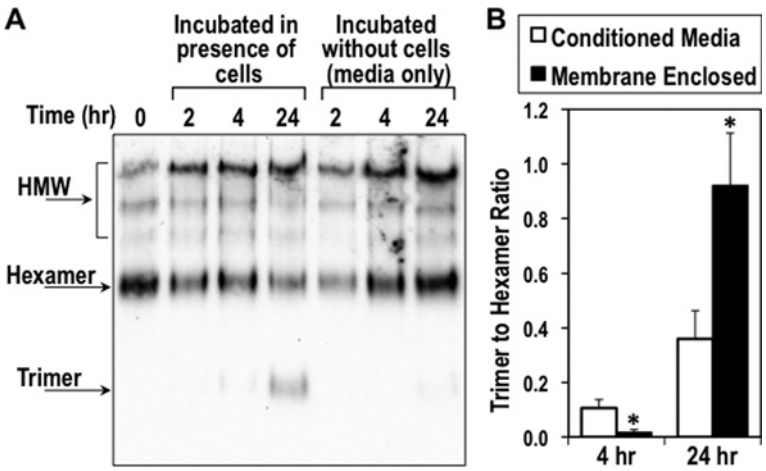

Figure 5 Comparison of conversion into trimers in the absence or presence of 3T3-L1 adipocytes

(**A**) FLAG-tagged adiponectin enriched in hexamers was incubated in the presence of differentiated 3T3-L1 adipocytes but kept inside the dialysis apparatus as described in the Materials and methods section to prevent the uptake of oligomers by cells. Identical reactions were also carried out in the absence of adipocytes as negative controls. Samples were removed at specific time points and amounts of FLAG-tagged adiponectin oligomers at each time point were measured using immunoblot analysis. (**B**) Comparison of trimer to hexamer ratio at 4 and 24 h in conditioned media in the absence of adipocytes compared with when starting material was enclosed in the dialysis apparatus (12–14 kDa MWCO) in the presence of adipocytes. * Denotes $P < 0.05$ in Student's unpaired, two-tailed Student's t test with $n = 8$ and 3 for experiments in conditioned media and the dialysis apparatus, respectively.

appearance of trimers within the dialysis apparatus following incubation with adipocytes was associated with the conversion of disulfide-bonded adiponectin dimers to monomers (Figure 6B). To determine whether extracellular thiol groups contribute to oligomer conversion, 3T3-L1 adipocyte-conditioned media were treated with thiol-inactivating agents NEM or diamide prior to incubation with FLAG-tagged adiponectin hexamers. NEM or diamide treatment reduced the amount of converted trimers (Figures 6C and 6D), suggesting that thiol groups are necessary for conversion activity in 3T3-L1 adipocyte-conditioned media.

Differential sensitivity of HMW and hexameric adiponectin to reduction by glutathione

If disulfide reduction is the mechanism by which adiponectin hexamers are converted into trimers, it should occur under redox conditions found in the extracellular environment (-137mV for the GSH/GSSG redox couple) [41]. We incubated purified hexamers and HMW adiponectin in glutathione buffers containing both GSSG and GSH species. At -137 mV, a significant portion of adiponectin hexamers converted into trimers, concomitant with reduction from disulfide-bonded dimers to monomers (Figure 7A). In contrast, HMW adiponectin remained stable at -137mV with no visible conversion into hexamers or trimers and was resistant to reduction of disulfide-bonded dimers to monomers (Figure 7B). The reactions had essentially reached equilibrium by 48 h; therefore the lack of HMW adiponectin conversion at -137 mV was not an artefact of slow kinetics *in vitro* (Figure 7B). As a positive control, portions of HMW adiponectin converted into hexamers and trimers by disulfide reduction after 48 h of incubation in 28.6 mM GSH without GSSG (Figure 7B).

Acceleration of adiponectin conversion by reduced NADPH and inhibition by auranofin

The results in Figure 7 suggest that adiponectin oligomer conversion utilizes a redox-based mechanism involving thiols. Specific oxidoreductases, including thioredoxin reductase, that utilize NADPH as electron donors in intracellular antioxidant defence, act extracellularly to affect cell behaviour by transferring reducing equivalents [42,43]. We performed the FLAG-tagged hexamer conversion reactions in 3T3-L1 adipocyte-conditioned media with the addition of the non-thiol electron carrier NADPH, its oxidized form, $NADP^+$, or auranofin, an inhibitor of thioredoxin and glutathione reductases [44]. NADPH, but not $NADP^+$, enhanced the conversion into trimers (Figures 8A and 8B). Auranofin inhibited conversion into trimers (Figures 8C and 8D).

DISCUSSION

In order to test the hypothesis that adiponectin oligomer conversions can take place near the cell surface, we sought evidence of conversion in both 3T3-L1 and primary rat adipocyte culture media. We observed that adiponectin hexamers converted into trimers when incubated in conditioned media from 3T3-L1 adipocytes and isolated rat primary adipocytes, both of which are the targets of adiponectin action [45,46]. The mechanism is probably redox-mediated, because (1) conversion into trimers was associated with reduction of disulfide-bonded dimers to monomers (Figures 6A and 6B), (2) conversion was inhibited in conditioned

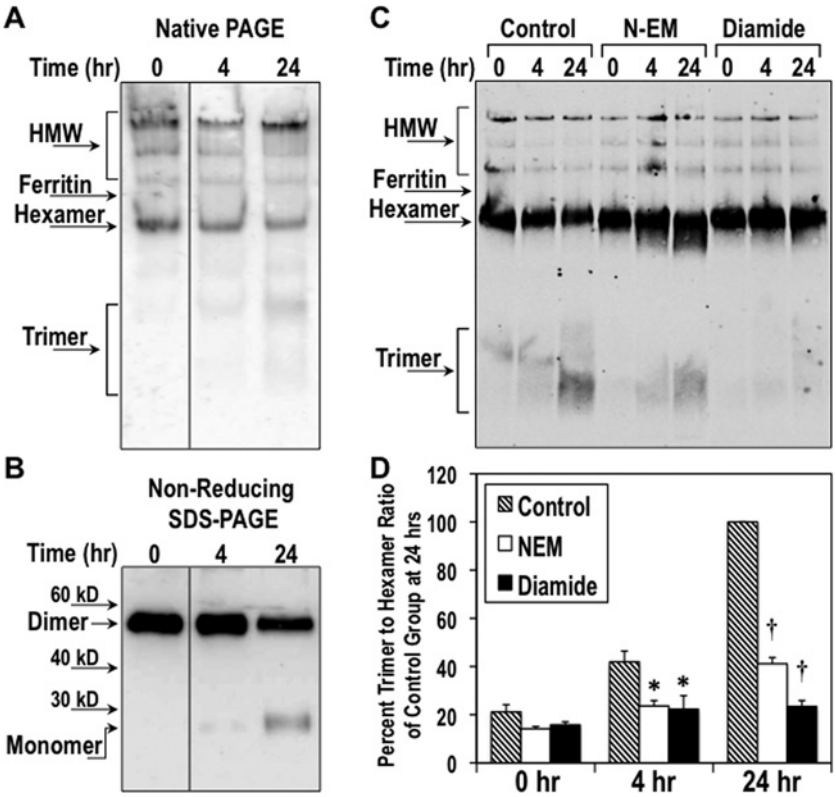

Figure 6 **Disulfide reduction as mechanism of conversion into trimers**

(**A**, **B**) Conversion into trimers within the 6–8 kDa MWCO dialysis apparatus. FLAG-tagged adiponectin enriched in hexamers and dissolved in PBS was added to the inside of microcentrifuge caps, enclosed in 6–8 kDa MWCO dialysis membranes, and floated on top of 3T3-L1 adipocyte cultures in six-well plates as described in the Materials and methods section. Oligomerization and disulfide-bonding states of enclosed adiponectin were determined using (**A**) native and (**B**) non-reducing denaturing immunoblot analyses. (**C**, **D**) Sensitivity of conversion process to thiol-reactive agents. The adiponectin conversion activity of conditioned media from differentiated 3T3-L1 adipocytes was tested after treatment with 10 mM NEM or 10 mM diamide to assess the requirement for free thiols using (**C**) native immunoblot procedures. The conversion activity was assayed using FLAG-epitope-tagged adiponectin enriched in hexamers. For all the groups within a single experiment, the ratios of trimer to hexamer abundance at each time point were normalized to that of the control group after 24-h incubation and expressed as percentages of the trimer/hexamer ratio of the control group from the same experiment at 24 h time point. Average trimer-to-hexamer ratio percentages from three experiments were plotted in (**D**) as histograms. $*P < 0.05$ compared with the control group at the same time point in one-way repeated measures ANOVA using Tukey's HSD post-hoc test. $\dagger P < 0.005$ in one-sample, two-tailed Student's t test against the expected value of 100 ($n = 3$).

media pre-treated with thiol-reactive agents (Figures 6C and 6D) and (3) addition of NADPH-accelerated conversion (Figure 8). To our knowledge, the present study is the first to describe extracellular conversion of adiponectin oligomers. The only other instance of extracellular adiponectin processing involved generation of globular adiponectin by leucocyte elastase [47]. Our results suggest that interconversion of adiponectin oligomers occurs in the extracellular space. Adiponectin accumulates near the plasma membrane in the liver and skeletal muscle [48] and co-localizes with dystrophin in skeletal muscle (T.-S. Tsao, S. Zarnegar and H. Lodish, unpublished work), suggesting that the signalling activities of adiponectin may be context-dependent according to the extracellular redox state.

In Figure 7(A), it appears that the most of adiponectin had vanished in the rightmost lane, suggesting that adiponectin may have collapsed further beyond the trimer. However, there was no Coomassie Blue-stainable material below the trimer band in that lane or in the rightmost lane in Figure 7(B), indicating no further conversion beyond trimers. The reason behind the apparent discrepancy is most probably the differential staining of the oligomers by the Coomassie Brilliant Blue G250 dye. We have observed previously that the staining intensity of adiponectin trimers appeared to be lower than that of the 18mer HMW species in Coomassie dyes (Figure 1 of [35] and Figures 2 and 3 of [36]). Adiponectin is highly negatively charged at neutral pH (calculated pI approximately 5.4) and binds strongly to anion exchange columns. As a result it may not react strongly with the anionic Coomassie dye under native conditions. The surface anionic charges of the trimers should be shielded in the HMW species, allowing Coomassie dye to interact strongly via hydrophobic interactions [49]. Consistent with this interpretation, there was no difference in the amount of adiponectin stained by

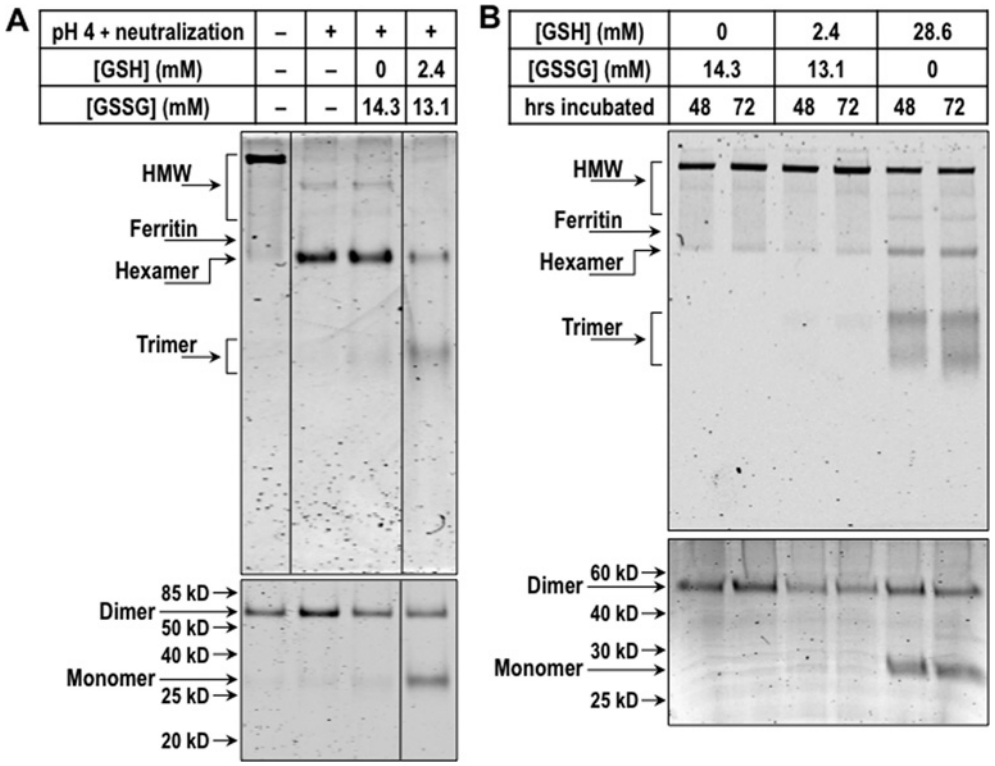

Figure 7 **Differential sensitivity of (A) adiponectin hexamers and (B) HMW adiponectin to reduction by glutathione**
Adiponectin hexamers were prepared from purified calf serum HMW adiponectin by adding glycine to lower the pH of the solution to 4 followed by re-neutralization back to pH 7. HMW and hexameric adiponectin were treated at 37 °C with different concentrations of GSH and GSSG as indicated. Redox reaction mixtures with hexamers were incubated for 6 h, whereas those with HMW adiponectin were incubated for 48 and 72 h. The leftmost lane in part B contains the purified bovine HMW adiponectin that had not been treated with glycine to lower pH. The oligomerization state was analysed using native gel electrophoresis. The oxidation states of the cysteine residue near the N-terminus of adiponectin were determined by separation of the monomeric (reduced) and dimeric (oxidized) adiponectin by non-reducing denaturing SDS/PAGE following addition of 10 mM NEM to protect free thiols. In reactions with 2.4 mM GSH and 13.1 mM GSSG, the reduction potential was calculated to be −137 mV by applying the Nernst equation using a standard reduction potential of −0.24 V.

Coomassie Blue under denaturing conditions (Figure 7A, bottom panel).

In the present study, levels of HMW adiponectin were seen to increase over time in a subset of the experiments (Figures 3A, 5A, 6A and 8A). We previously observed that adiponectin hexamers purified from bovine serum rapidly redistributed to HMW and trimeric adiponectin via disulfide rearrangement *in vitro* in the absence of other proteins [35]. It is possible that hexamers could spontaneously undergo conversion into both trimers and HMW adiponectin in conditioned media. However, unlike conversion into trimers, conversion into HMW adiponectin does not always seem to occur (Figures 3B and 6C). The reason for the differences in HMW adiponectin appearance is presently unclear.

The sensitivity of adiponectin oligomer conversion into thiol-reactive agents suggests extracellular redox-active molecules are involved in conversion. The greater extent of conversion in the presence of cells than in conditioned media alone (Figure 5B) suggests the putative extracellular redox system responsible for conversion is actively maintained and replenished by the cells. Oligomer conversion taking place inside a dialysis apparatus with 6–8 kDa MWCO (Figure 6A) suggests that a secreted factor smaller than 8 kDa is a component of the putative extracellular redox system. Several small thiol-based redox-active molecules are present in the extracellular environment or circulation, including glutathione, thioredoxin, glutaredoxin and cysteine [43]. GSH is found in human plasma at low micromolar concentrations and at higher abundance than GSSG [50,51]. In addition, biologically active thioredoxin reductase is secreted into human plasma [42]. Enhanced conversion into trimers in the conditioned media by NADPH and inhibition by auranofin (Figure 8) is consistent with the involvement of thioredoxin reductase or possibly glutathione reductase. Currently, how different redox-active molecules interact with each other extracellularly is poorly understood. Whether small redox-sensitive molecules including thioredoxin or glutaredoxin directly interact with adiponectin to mediate conversion remains to be answered.

The targets of the thiol-reactive agents in conditioned media could be cysteine proteases, but that is unlikely. Adiponectin hexamers consist of pairs of trimers held together with disulfide bonds near the N-terminus. Proteolytic cleavage resulting in removal of disulfide-bonding cysteine residues would have

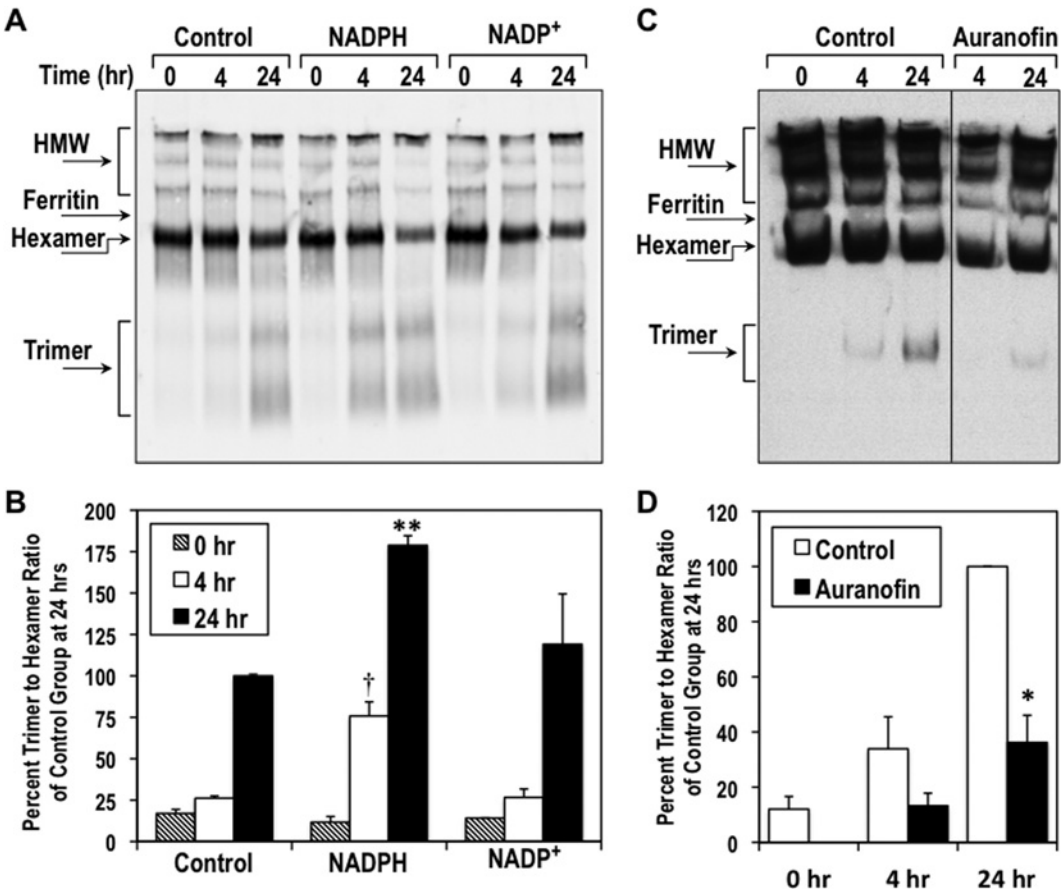

Figure 8 Acceleration of adiponectin conversion by NADPH and inhibition by auranofin

The hexameric adiponectin conversion activity of conditioned media from differentiated 3T3-L1 adipocytes was tested in the presence of (**A**, **B**) 100 μM NADPH or NADP$^+$ or (**C**, **D**) 15–20 nM auranofin. The conversion activity was assayed using FLAG-epitope-tagged adiponectin enriched in hexamers and the representative native immunoblot analyses results are shown in (**A**) and (**C**). To assess the extent of conversion in all the groups within a single experiment, the ratios of trimer to hexamer abundance at each time point were normalized to that of the control group at 24-h time point and expressed as percentages. Average trimer-to-hexamer ratio percentages from three experiments each were plotted in (**B**) and (**D**) as histograms. †$P < 0.05$ compared with the control and the NADP$^+$ groups at the same time point in one-way ANOVA with Tukey's HSD post-hoc test. *$P < 0.03$ and **$P < 0.005$ in one-sample two-tailed Student's t tests against the expected value of 100 ($n = 3$).

also removed the N-terminal FLAG epitope, making the recombinant protein undetectable using antibodies against the FLAG epitope.

Conversion of hexamers to trimers inside the dialysis apparatus shown in Figure 3(A), Figure 5 and Figure 6(A) was robust but only apparent at the 24-h time point. There may be a lag period for adiponectin conversion as putative redox agents slowly cross the dialysis membrane. The half-time for removal of 5 mM DTT (dithiothreitol) by dialysis was approximately 2 h (results not shown), suggesting that larger molecules would take even longer to cross the dialysis membrane.

Extracellular conversion of adiponectin oligomers may represent another layer of regulation of adiponectin action. A more reducing extracellular environment may favour conversion of hexamers into trimers while a more oxidized extracellular environment may inhibit conversion. Cells, including 3T3-L1 adipocytes, exert homoeostatic control on the cysteine/cystine redox state to maintain it at a relatively constant level [29,52]. Deviation from the normal redox state could alter a variety of cellular processes, including proliferation, inflammation and apoptosis [29]. The plasma GSH/GSSG ratio is decreased in older individuals and in Type 2 diabetic patients [30], and plasma cysteine/cystine redox state is more oxidized in mice fed a high-fat diet [52]. These data raise the possibility that the conversion of adiponectin hexamers into trimers could be inhibited in people or animals with insulin resistance.

Different, and often contradictory, physiological actions have been attributed to the isoforms of adiponectin. Our current findings may resolve some of these conflicts. If the extracellular redox environment differed in various studies in the literature, the conversion of one oligomeric form into another may account for differences in the results. This might not have been obvious

in previous studies as our data showed that the converted trimers are rapidly cleared by cells (Figure 4).

Currently, the physiological role of the hexamer is unclear. Hexamers are not as metabolically active as either the HMW or the trimer isoforms, yet they constitute a major adiponectin species in circulation [5,15,19,24]. Results from the current study suggest that hexamers could be a source of trimers if the extracellular environment contains the necessary redox system with appropriate potential for oligomer conversion.

AUTHOR CONTRIBUTION

All the authors contributed to the study design and data interpretation and analysis. In addition, Martha Nuñez, David B. Briggs, Bethany L. Laskowski, Jimmy J. Chhun and Joseph K. Eleid acquired data and reviewed and approved the manuscript. Michael J. Quon reviewed, edited and approved the manuscript. Jeong-a Kim and Tsu-Shuen Tsao acquired data and drafted, edited and approved the manuscript.

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