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Age-associated loss in adiponectin-activation by caloric restriction: Lack of compensation by enhanced inducibility of adiponectin-paralogs CTRP2 and CTRP7

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Abstract

Hormonal signals from adipose tissue regulate energy homeostasis but may also be involved in the anti-aging effects of caloric restriction. The purpose of the current study was the investigation of age-dependent effects of caloric restriction on the release of adiponectin, on the expression and activation of adiponectin-related signaling and on parameters of altered insulin sensitivity.

In young and in senescent rats, 2 months moderate caloric restriction reduces serum leptin and insulin (young: -50%; old: -30%) suggesting increased insulin sensitivity. However, the same diet enhances serum adiponectin in young (+60%) but not in senescent (+2%, n=NS) rats. Similarly, adiponectin expression (visceral fat) and muscular AdipoR1/2 expression are induced in young rats but not in senescent rats. The locally produced adiponectin paralogs CTRP2/7 are elevated in muscular tissues of old animals (CTRP2 protein: +40%; CTRP7 protein: +50%) and further induced by caloric restriction but this does not result in an increased activation of their downstream target AMPK.

Thus, aging is associated with a partial loss of adiponectin inducibility following moderate caloric restriction. This loss is not sufficiently compensated by the locally induced adiponectin paralogs CTRP2/7, although caloric restriction results in increased insulin sensitivity in young and in senescent animals. Thus, the improvement in insulin sensitivity appears to be independent of adiponectin induction by caloric restriction in this model.

Introduction

Caloric restriction (CR) extends lifespan in many species including mammals, but the basic mechanisms of its efficacy remain unclear and its actions in different organs are remarkably heterogeneous (Masoro, 2000). Life-long CR attenuates the age-associated increases in mitochondrial radical production (Barja, 2002), in lipid peroxidation (Matsuo et al., 1993) and in protein oxidation (Leeuwenburgh et al., 1997). It was also shown to protect cardiomyocytes from age-associated apoptosis by reducing endogenous DNA damage, by enhancing DNA repair capacity, by reducing the expression of proapoptotic genes and by inducing apoptosis-inhibitory genes (Park and Prolla, 2005). However, caloric restriction also results in dramatic changes in glucose (review in (Kloting and Bluher, 2005)) and lipid metabolism (review in (Barzilai and Gabriely, 2001)). So far, less attention has been drawn to the fact that the protection by CR may also be related to the biology of adipose tissue and the effects of adipose tissue derived hormones. Besides functioning as an important energy storage depot, adipose tissue represents an important and active endocrine organ that controls and monitors whole-body metabolism by secreting a number of bioactive molecules or hormones, the adipokines (Hutley and Prins, 2005).

The most abundant protein within the adipocyte is adiponectin (also called acrp 30, adipoQ, APM-1, GBP28). Unlike many other adipokines, the expression and circulating levels are reduced in diabetic and obese state in animals as well as in humans, and exogenous administration of adiponectin improved insulin sensitivity while lowering blood glucose levels in high fat obese mice (Yamauchi et al., 2001). It has been suggested from animal studies that caloric restriction may accomplish the improved insulin sensitivity through modulation of adiponectin levels. Short-term and long-term caloric restriction (-40%) was reported to result in an increase in adiponectin plasma levels compared to age-matched ad libitum fed rats (Zhu et al., 2004) as well as in mice (Combs et al., 2003). Similarly, some

studies in the human show improved insulin sensitivity together with increased adiponectin plasma concentration following weight reduction by hypocaloric diet (Bobbert et al., 2005; Polak et al., 2007) or by bariatric surgery (Faraj et al., 2003; Guldstrand et al., 2003). However, there is also accumulating evidence arguing against the notion that a caloric restriction-induced increase in adiponectin underlies the CR-related increased insulin sensitivity (Abbasi et al., 2006; Anderlova et al., 2006; Mazzali et al., 2006; Xydakis et al., 2004). Thiazolidinediones on the other hand have repeatedly been shown to effectively enhance insulin sensitivity and this is paralleled by an increase in plasma adiponectin and / or high molecular weight adiponectin (Abbasi et al., 2006; Maeda et al., 2001; Yu et al., 2002).

Adiponectin structurally belongs to the complement 1q family (review in (Kishore et al., 2004)) and is known to form homomultimers in plasma. Besides that adiponectin circulates as a full-length or a smaller globular fragment that is much less frequent in human plasma. Both globular and full-length adiponectin activate AMP-activated kinase (AMPK), an enzyme that is switched on by increases in levels of AMP, via increased phosphorylation at Thr-172 of the α -subunit. AMPK functions to restore ATP concentration by stimulating energy-producing processes such as fatty acid oxidation. Activation of AMPK by adiponectin was shown to increase phosphorylation of acetyl-coenzyme A carboxylase (ACC), fatty acid oxidation and glucose uptake via Glut 4, resulting in a decreased triglyceride content and increased insulin sensitivity (Yamauchi et al., 2002).

The effects of adiponectin seem to be mediated by two distinct receptors: the adiponectin receptors AdipoR1, ubiquitously expressed with highest abundance in skeletal muscle, and AdipoR2, most abundantly expressed in liver (Yamauchi et al., 2003). A reduction in the gene expression of these receptors has been described to be associated with diabetes and obesity, while fasting results in renormalization (Tsuchida et al., 2004). In addition, T-cadherin has

been suggested to act as a coreceptor for an as-yet-unidentified signaling receptor through which adiponectin transmits metabolic signals (Hug et al., 2004), since it lacks an intracellular domain.

Meanwhile a family of adiponectin paralogs was discovered and designated as C1q/tumor necrosis factor- α - related proteins (CTRPs) 1-7 (Wong et al., 2004). The CTRPs are predicted to be secreted proteins, but in contrast to adiponectin they do not circulate in high concentration in the serum but may exert their biological effects in a paracrine or autocrine fashion (Wong et al., 2004). Unlike adiponectin their expression is not restricted to the adipose tissue but they seem to be widely expressed (Wong et al., 2004). Recombinant mouse CTRP2, the paralog with the highest similarity to adiponectin, has been shown to induce AMPK phosphorylation, increased glycogen accumulation and fatty acid oxidation *in vitro* (Wong et al., 2004). Therefore, it is conceivable that CTRP2 may exert similar effects as adiponectin via activation of AMPK also *in vivo*.

We hypothesized that a loss in skeletal muscle adiponectin signaling and in systemic adiponectin inducibility contributes to the enhanced insulin resistance of aging skeletal muscle. Therefore, we tested whether a physiological stimulus, such as transient (2 months) moderate (-16%) caloric restriction, can activate systemic adiponectin, locally acting adiponectin paralogs, and adiponectin-related signaling in skeletal muscle of senescent rats to the same extent as in young rats.

Materials and methods

Animals and diet protocol

Male young (4 months) and senescent (24 months) Sprague-Dawley rats were obtained from Charles River (Germany), caged individually with a light/dark cycle of 12 h and had tap water ad libitum. Prior to the application of the diet protocols, daily food intake of the normal, ad libitum offered diet (Altromin^R 1344 / 1850, Altromin GmbH Germany) of each rat was monitored for 14 days and averaged. During the diet protocol of two months duration, rats on control diet (1850 cal/g) received their individual prediet average in order to avoid any degree of diet-induced obesity. Rats subjected to caloric restriction received their prediet average, but of a caloric reduced, fibre-rich diet (Altromin^R 1344 / 1550, 1550 cal/g). Thus, in young rats on control diet (n = 7; 331 ± 6 g body weight at the start of the diet period) the daily energy intake was 50.5 ± 1.2 kcal, in young rats on caloric restriction (n = 12; 334 ± 4 g) this daily intake was 45.2 ± 0.7 kcal. In old rats, the daily energy intake amounted to 45.1 ± 0.9 kcal in control diet (n = 8; 556 ± 6 g) and to 39.7 ± 1.1 kcal in caloric restricted rats (n = 9; 568 ± 6 g). At the end of 8 weeks of diet, rats were sacrificed and blood was obtained by aortic puncture. After the cardiac and skeletal muscle dissection was completed, the epididymal, perirenal, mesenteric and retroperitoneal fat pads were removed and weighed.

RNA Extraction

Total RNA was isolated from skeletal muscle (M. gastrocnemius), left ventricles (LV) and visceral adipose tissue as described before (Chomczynski and Sacchi, 1987). Integrity and quality of the RNA was confirmed by agarose gel electrophoresis and the concentration determined by measuring UV-absorption.

Real-time PCR

Reverse transcription (RT) of RNA samples was carried out with Superscript II Reverse Transcriptase (Invitrogen) at 42°C for 30 minutes. We performed RT-PCRs of adiponectin, AdipoR1, AdipoR2, T-cadherin, CTRP1, 2, 3, 6, 7 and 18S rRNA in samples derived from skeletal muscle, rat left ventricles and adipose tissue as appropriate. Primer sequences are provided in Table 1. Standard plasmids were generated by cloning the full length CDS into pCRII TOPO (Invitrogen) and the nucleotide sequence was confirmed by sequencing. Standards were diluted and each standard curve was range-optimized. QPCR reaction (25µl) was performed with a MX3000P (Stratagene), SYBR-Green®-Platinum qPCR MasterMix with internal ROX-standard (Invitrogen). Data were analyzed with Stratagene MxPro-MX3000P v3.20 software. All data are given as nucleotide-copy-numbers/reaction normalized per copy-numbers 18S rRNA/reaction. PCR products were quantified by comparing to a standard curve using pCRII TOPO containing the full length cDNA of adiponectin, AdipoR1, AdipoR2, T-cadherin, CTRP1, 2, 3, 6, 7 or 18S rRNA, respectively.

Western blotting

Muscular and adipose tissue were rapidly homogenized in a buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% Na-deoxycholate, protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail (Sigma). Proteins were quantified using the BCA Protein Assay (Pierce). Fifty µg of protein in 6x Laemmli SDS sample buffer were boiled for 5 minutes and after centrifugation loaded on a SDS-PAGE gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane at 100 V for 60 minutes. The filters were blocked with 0.01% Tween, 2% nonfat dry milk and then incubated with antibodies directed against adiponectin (Biovision, 1:1000), AdipoR1 (Alpha Diagnostics International Inc., 1:1000), AdipoR2 (Alpha Diagnostics International Inc., 1:500), CTRP2, CTRP7 (both Axxora, 1:500), phospho-AMPK (Thr-172) as well as total

alpha AMPK (both Cell Signaling, 1:1000). Prior to this, all antibodies had been tested with specific positive controls or blocking peptides as suggested by the manufacturer and the size of the expected band was verified with the PageRuler™ Prestained Protein Ladder (Fermentas). Vimentin (Sigma, 1:1000) and GAPDH (Abcam, 1:5000) were used to demonstrate equal loading of the gels. After incubation with peroxidase-conjugated secondary antibody, blots were subjected to the enhanced chemiluminescent detection method (Amersham) and exposed to a ECL film.

Plasma analysis of Adiponectin, Leptin, Insulin and Triglycerides

Serum adiponectin, leptin and insulin concentrations were measured by using commercial Enzyme-Linked-Immunosorbent-Assays (Mouse/Rat Adiponectin ELISA-kit, B-Bridge International Inc.; Mouse/Rat Leptin ELISA-Kit, BioVendor; Rat Insulin ELISA, Mercodia AB) according to the manufacturers' instructions. Samples for free fatty acids or triglycerides were collected in tubes containing Paraoxon (Sigma) to inhibit lipoprotein lipase. Plasma and tissue triglycerides were extracted as described before (Bligh and Dyer 1959) and measurement was performed by an enzymatic colorimetric method using a commercial kit (GPO Trinder, Sigma).

Statistical Analysis

All values are expressed as mean $\pm$ SEM. Statistical analysis of differences observed between the groups was obtained by ANOVA. Statistical significance was accepted at the level of $p < 0.05$.

Results

Systemic effects of caloric restriction

In young, growing animals under control diet, body weight increased by 17 ± 1 % within two months, while caloric restriction attenuated this increase to 9 ± 1 % (Fig. 1). In old control rats, however, body weight decreased by 9 ± 1 %, and this decline was augmented to an 18 ± 1 % weight loss in the animals under caloric restriction (Fig. 1). There was no difference in the daily water intake among the rats. Relative weights (organ-weight/body-weight-ratio) of LV, liver, kidney and skeletal muscle were higher in old than in young rats and did not decrease in young or old animals under caloric restriction. Visceral fat mass decreased in young ($13.1\text{g} \pm 0.89$ vs. $8.3\text{g} \pm 0.35$; $p < 0.05$) and in senescent animals ($29.2\text{g} \pm 1.1$ vs. $18.7\text{g} \pm 1.2$; $p < 0.05$) under caloric restriction to a similar extent.

Caloric restriction has been reported to result in an increase in adiponectin plasma levels in rats after caloric restriction compared to age-matched ad libitum fed animals (Zhu et al., 2004). Our moderate caloric restriction significantly enhanced the circulating levels of the adipocyte-derived hormone adiponectin in young rats (Fig. 1 and Table 2). However, it did not modify adiponectin plasma levels in old rats (Fig. 1 and Table 2), illustrating a hitherto unidentified loss of responsivity in adipose tissue with advanced age.

On the other hand, the restriction induced the anticipated decline in plasma leptin in young and in old rats (Fig. 1 and Table 2). Plasma insulin, obtained 12-18 h after offering the last meal, was lower in young control rats and caloric restriction lowered plasma insulin, both in old and in young rats (Table 2). The moderate caloric restriction for 2 months resulted in a significant decrease of plasma triglycerides in senescent animals (Table 2), although these triglyceride levels remained significantly higher compared to young animals (Table 2). Skeletal muscle content of triglycerides was increased in senescent animals and showed only a minor decrease after 2 months of caloric restriction ($7.61\text{ mg/g wt} \pm 0.99$ vs. $5.81\text{ mg/g wt} \pm$

1.13). Skeletal muscle content of triglycerides in young animals on the other hand was significantly lowered by caloric restriction ($4.34 \text{ mg/g wt} \pm 1.93$ vs. $2.45 \text{ mg/g wt} \pm 0.94$, $p < 0.01$). A comparable tissue content of triglycerides and a similar pattern after short-term caloric restriction was observed in the LV as well as in liver tissue (not shown).

Gene expression of adiponectin, adiponectin receptors and adiponectin paralogs

In young rats, caloric restriction enhanced the mRNA and protein expression of adiponectin in visceral fat, while this adaptation was not observed in old rats (Fig. 2). The commonly used housekeeping gene GAPDH demonstrated a significant downregulation in senescent visceral fat (Fig. 2) but not in muscular tissue (Fig. 5). Therefore, vimentin was used to normalize the data from adipose tissue.

Adiponectin influences cellular gene expression and signal transduction by binding to the receptors AdipoR1 and AdipoR2. The expression of AdipoR1 was higher than the expression of AdipoR2 in skeletal muscle (Fig. 3) or in LV (not shown) and mRNA of both receptors was increased after 2 months caloric restriction in young rats (Fig. 3) as described previously in mice (Tsuchida et al., 2004). However, in senescent animals caloric restriction did not result in a change in the expression of AdipoR1 or AdipoR2 mRNA (Fig. 3). Western blot analyses revealed an induction of AdipoR1 in young (123.2 ± 19.3 vs. 203 ± 9.2 ; $p < 0.05$) but not in old rats (129.6 ± 25.3 vs. 133 ± 19.7 ; $p = \text{NS}$) under caloric restriction (Fig. 3), while AdipoR2 protein (Fig. 3) was not different among the groups (young: 79.1 ± 5.2 vs. 83 ± 4.9 ; $p = \text{NS}$ and old: 76.9 ± 3.6 vs. 77.1 ± 6.1 ; $p = \text{NS}$). The mRNA expression of the putative adiponectin receptor T-cadherin was not altered in any of the groups (not shown).

While adiponectin is almost exclusively expressed in adipose tissue, the adiponectin paralogs are expressed widely. Rat muscular tissues (LV and skeletal muscle) expressed significant amounts of CTRP1, 2, 6, and 7, similar to what has been described in mouse tissue before (Wong et al., 2004). While aging was associated with a significant decrease in the expression

(Fig. 2) and release of adiponectin (Fig. 1) from adipose tissue, some adiponectin paralogs appeared to be increased in senescent skeletal muscle (Figs. 4+5) and myocardium (not shown). CTRP1 and CTRP6 mRNA expression were not significantly influenced by caloric restriction or age (Fig. 4), while CTRP2 and CTRP7 mRNA were significantly induced in senescent skeletal muscle (Fig. 4) and myocardium (not shown) under caloric restriction. Western blot analyses demonstrated a significantly higher CTRP2 and CTRP7 protein expression in senescent animals than in young animals on control diet but no further increase in CTRP2/7 protein expression in old animals under caloric restriction (Fig. 5).

Activation of AMPK in young and senescent rats under caloric restriction

Adiponectin and CTRP2 have both been shown to induce phosphorylation of AMPK. To investigate the effects of an increased adiponectin expression in young animals under caloric restriction and an increased CTRP2/7 expression concomitantly with a diminished adiponectin expression in old animals we analyzed activation of AMPK in these animals. As shown in figure 6, total AMPK- α protein expression in skeletal muscle was not influenced by caloric restriction or age. On the other hand, caloric restriction results in a robust phosphorylation of AMPK- α at Thr-172 in young rats that was totally absent in senescent animals (Fig. 6). Apparently, CTRP2 and CTRP7, locally produced in skeletal muscle and LV tissue, are insufficient to compensate for the loss of adiponectin from adipose tissue.

Discussion

The structurally related members of the C1q and TNF superfamily are involved in processes as diverse as host defense, apoptosis, inflammation, differentiation, hibernation or insulin-resistant obesity (review in (Kishore et al., 2004). Adiponectin, which belongs to this growing family of proteins, is an adipokine that is expressed specifically and abundantly in adipose tissue and directly sensitizes the body to insulin. Among others, adiponectin induces AMPK activation in muscle resulting in increased glucose uptake and glycogen accumulation and in addition, it leads to decreased fatty acid synthesis but increased β -oxidation of fatty acids. While adiponectin is almost exclusively expressed by differentiated adipocytes, the recently discovered adiponectin paralogs CTRP1-7 are widely expressed and may exert their biological effects in a paracrine or autocrine fashion (Wong et al., 2004). Functional analyses suggest that CTRP2 mediates similar effects on glucose or fatty acid metabolism compared to adiponectin (Wong et al., 2004), while there are no such data available for the adiponectin paralog CTRP7. CTRP2 has been shown to enhance glycogen accumulation and fatty acid oxidation in C2C12 myotubes by activating the AMPK signaling pathway (Wong et al., 2004). However, no CTRP specific receptor mediating these effects has been described so far.

In the present study, we tried to investigate the age-dependent influence of moderate, short-term caloric restriction on the fat cell-derived hormone adiponectin and adiponectin-related signal transduction as well as its recently discovered paralogs CTRP1-7 in muscular tissue.

Two months of -16% caloric restriction attenuate the adolescence-associated gain in body weight in young animals (Fig. 1). Aging is associated with a decrease of body weight in most rat strains at 18 months of age. In senescent rats under the restricted diet, this decrease in body weight is further augmented compared to age-matched controls (Fig. 1). As described previously in Fischer 344 rats by others (Mooradian et al., 2000) serum leptin concentration is

significantly increased in senescent rats. The moderate caloric restriction results in a decrease in serum leptin that is more pronounced in old than in young rats (Fig. 1). Differences in the response of immature or mature rats to leptin treatment have been described before (Qian et al., 1998) suggesting that normal rats become resistant to leptin as they age. Insulin resistance on the other hand is a key feature of the metabolic syndrome and defined as a state that requires the pancreas to secrete additional insulin to obtain the physiological effects achieved by lower amount of insulin in the normal, non insulin resistant state. However, there are major differences in the development of insulin resistance or type 2 diabetes among the different rat strains. Fischer 344 but not Sprague-Dawley rats for instance are known as a strain that develops insulin resistance at relatively young age (Levy et al., 2002). In the present study, plasma insulin is higher in old control rats than in young control rats suggesting the development of insulin resistance with advanced age in these Sprague-Dawley rats. Plasma insulin is lowered under caloric restriction in old and in young rats. Apparently, also senescent rats respond to the insulin-sensitizing effects of caloric restriction. Therefore, we further investigated whether alterations in adiponectin release may underlie the observed increase in insulin sensitivity following caloric restriction.

Adiponectin is considered a key hormone to control body metabolism as it was shown to increase fatty acid oxidation or glucose uptake, to decrease triglyceride content and to increase insulin sensitivity (Yamauchi et al., 2002). Therefore, adiponectin expression in adipose tissue and release into plasma may have a major impact on the protective and insulin-sensitizing effects of caloric restriction. Furthermore, weight loss has been shown to result in an increase of adiponectin expression in visceral adipose tissue in lean and obese rats (Milan et al., 2002) and was reported to result in an increase in adiponectin plasma levels in rats after caloric restriction compared to age-matched ad libitum fed animals (Zhu et al., 2004). Our moderate caloric restriction significantly enhanced the circulating levels of the adipocyte-

derived hormone adiponectin in young rats (Fig. 1 and Table 2). However, it did not modify adiponectin plasma levels in old rats (Fig. 1 and Table 2), illustrating a hitherto unidentified loss of responsivity in adipose tissue with advanced age. Combs et al. reported that chronic caloric restriction (-40%) results in an increase in adiponectin plasma levels in young as well as in senescent mice (Combs et al., 2003). Compared to our study, the duration and the extent of caloric restriction were significantly higher and may explain the difference in responsivity observed in senescent animals. However, there are also some studies arguing against the notion that the increase in adiponectin underlies the increased insulin sensitivity following caloric restriction (Abbasi et al., 2006; Anderlova et al., 2006; Mazzali et al., 2006; Xydakis et al., 2004) or physical activity (Hulver et al., 2002; Ryan et al., 2003). Therefore, changes in adiponectin expression or secretion may not be instrumental in improving insulin resistance in these therapeutic settings, while the majority of patients in response to thiazolidinedione treatment on the other hand demonstrate improved insulin sensitivity together with an increase in plasma adiponectin (Abbasi et al., 2006; Maeda et al., 2001; Yu et al., 2002).

In the current study, adiponectin serum concentration is increased in young animals under caloric restriction but remains unaltered in senescent animals (Fig. 1), although the proportionate decrease in visceral fat mass is similar in young and in old animals. Similarly, adiponectin mRNA and protein expression are induced in visceral fat of young rats but not in senescent rats (Fig. 2) illustrating a so far unknown loss of responsivity of the visceral fat with advanced age.

The expression of both adiponectin receptors has been shown to be increased in fasted animals (Tsuchida et al., 2004), in humans after 4 weeks of physical activity (Bluher et al., 2006) but decreased in refed or obese mice (Inukai et al., 2005; Tsuchida et al., 2004). Whereas the skeletal (Fig.3) and cardiac (not shown) muscle of young animals demonstrate a diet-induced induction of both receptors as described before in mice (Tsuchida et al., 2004) no

increase is observed in senescent animals (Fig. 3). Therefore, the adiponectin deficit may be further amplified in the periphery by the absence of induction of the adiponectin receptors AdipoR1 and AdipoR2 in the senescent skeletal muscle following caloric restriction (Fig. 3). The expression of both AdipoR1 and AdipoR2 is also significantly reduced in muscle and adipose tissue of *ob/ob* mice together with hyperinsulinemia and impairment of adiponectin-mediated activation of AMPK (Tsuchida et al., 2004) - a state that one could call adiponectin resistance. However, senescent animals have not been investigated so far and there are also reports in the literature arguing against a direct responsiveness of AdipoR1/2 to changes in the nutritional status (Beylot et al., 2006). Our study supports the idea that senescent animals display a significant deficit in adiponectin and adiponectin-induced signal transduction. One can speculate whether a higher degree caloric restriction together with a longer duration may be sufficient to improve the release of adiponectin from adipose tissue in senescent animals. However, the degree of restriction applied in the current study was sufficient to improve insulin resistance in young and in old rats supporting observations that adiponectin is dispensable in improving insulin resistance in some therapeutic interventions.

The expression of adiponectin and various CTRPs is similar in skeletal and cardiac muscle: both tissues express only traces of adiponectin but significant amounts of CTRP1, 2, 6 and 7 mRNA (Fig. 4). The adiponectin paralogs CTRP2/7 are higher in old animals and caloric restriction results in a further induction of CTRP2/7 mRNA (Fig. 4) in both muscular tissues but does not result in a further increase in CTRP2/7 protein (Fig. 5) in old rats. Although CTRPs are predicted to be secreted proteins they do not seem to circulate in high concentration but unlike adiponectin have been suggested to exert their effects in an autocrine or paracrine way (Wong et al., 2004).

The major molecular mechanism underlying the insulin-sensitizing action of adiponectin was shown to be mediated by activation of AMPK (Yamauchi et al., 2002). Similar to the effects

of adiponectin, CTRP2 has been shown to induce a rapid phosphorylation of α -AMPK on Thr-172, its downstream target acetyl-CoA carboxylase (ACC) on Ser-79 and it also phosphorylated p44/42 MAPK in C2C12 myotubes *in vitro* (Wong et al., 2004). In muscle, this AMPK activation leads to increased glucose uptake and glycogen accumulation, while phosphorylation of ACC results in increased fatty acid oxidation. Therefore, we asked whether the upregulation of CTRP2 in cardiac and skeletal muscle of senescent rats under caloric restriction might in part compensate for the adiponectin deficiency due to the lack of responsivity of senescent adipose tissue. Although CTRP2 has been shown to activate AMPK we observed only in muscular tissues of young rats but not in senescent animals a significant increase in AMPK phosphorylation after 2 months of caloric restriction. The induction of fat-derived, circulating adiponectin by caloric restriction in young animals may lead to an activation of AMPK and subsequently to increased fatty acid oxidation, glucose uptake resulting in decreased triglyceride content in skeletal and cardiac muscle. The present study shows that the local induction of CTRP2 and CTRP7 in old animals is not sufficient to activate AMPK to the same extent as in young animals, suggesting that adiponectin is not dispensable for AMPK activation in senescent rat skeletal and cardiac muscle. Therefore, in this rat model CTRP2 or other CTRPs are unable to substitute for adiponectin function *in vivo*. This is also supported by the fact that tissue triglycerides are decreased by caloric restriction in young animals but not in senescent. However, besides adiponectin other adipokines have been described to strongly activate AMPK and therefore, we cannot exclude that further deficits of these putative mediators may also have contributed to the diminished AMPK activation in aged animals. Similarly, besides AMPK other pathways have been shown to be activated during caloric restriction and improve insulin sensitivity: Akt2 was shown to be essential for the full effect of caloric restriction on insulin-stimulated glucose uptake in skeletal muscle (McCurdy and Cartee, 2005). Therefore, the functional relevance of the age-associated reduction of circulating adiponectin together with a decrease in adiponectin

expression in adipose tissue and locally induced adiponectin paralogs (CTRP2 and CTRP7) remains to be further elucidated.

Although adiponectin expression and release into the plasma do not respond to the stimulatory effect of caloric restriction in senescent rats the treatment results in a significant decrease of plasma triglycerides as described before in senescent Fischer 344 rats (Jiang et al., 2005). Even so these plasma triglyceride levels remain significantly higher compared to young animals and in addition, muscle (skeletal and cardiac) or liver content of triglycerides does not decrease after 2 months of caloric restriction in these old rats. Others have also shown that 1 week of moderate caloric restriction in young rats did not affect the triglyceride content while after 3 weeks muscular but not liver triglyceride content was reduced (Barazzoni et al., 2005b). No data were obtained from senescent animals in that study (Barazzoni et al., 2005b). Besides adiponectin other adipokines or hormones can be envisioned as regulators of lipid and energy metabolism reacting to caloric restriction. Ghrelin for example has been shown to induce tissue-specific changes in lipid metabolism favouring triglyceride deposition in liver over skeletal muscle (Barazzoni et al., 2005a). It remains to be elucidated why this moderate, short-term caloric restriction applied here was sufficient to stimulate only some metabolic pathways in senescent animals but insufficient to activate others such as AMPK and whether a higher degree and longer duration of caloric restriction may be sufficient to result in further improvement also in senescent animals.

In summary, aging is associated with a so far unidentified partial loss of responsivity of adipose tissue following moderate, short-term caloric restriction. Although we observed a lack of adiponectin increase and muscular AMPK activation in response to the diet in old animals, insulin levels decreased in young and in old animals. The locally induced adiponectin paralogs CTRP2 and CTRP7 on the other hand are not capable to compensate for

this loss of adiponectin and AMPK activation in senescent rats and may therefore have additional functions in this model.

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Figure legends**Figure 1: Effects of caloric restriction on body weight and Adiponectin or Leptin release**

Body weight is lower in young rats under 16% caloric restriction ($n = 12$; hatched columns) compared to age-matched animals on control diet ($n = 7$) and it is further reduced in senescent rats ($n = 9$; hatched columns) compared to age-matched controls ($n = 8$). Caloric restriction significantly enhanced the circulating levels of adiponectin in young rats, however it did not modify adiponectin plasma levels in the old rats. On the other hand, the restriction induced a decline in plasma leptin in young and in old rats. Data are mean $\pm$ SEM, ***: $p < 0.001$.

Figure 2: Effects of caloric restriction on Adiponectin mRNA and protein expression

Left panel: Real-time RT-PCR revealed a significant induction of adiponectin mRNA in visceral adipose tissue in young rats under caloric restriction but not in old rats. Numbers of animals as described in figure 1. Middle panel: Densitometry of protein data normalized to Vimentin. Homogenates of visceral fat from young and old rats with control diet or caloric restriction (hatched columns) probed with antibodies detecting Adiponectin (number of animals as described in Fig. 1). Data are mean $\pm$ SEM, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Right panel: Western blot: Homogenates of visceral fat probed with antibodies against Adiponectin, Vimentin and GAPDH (representative examples). C = rats with control diet; R = rats with -16% caloric restriction.

Figure 3: Effects of caloric restriction on AdipoR1 and AdipoR2 mRNA and protein expression

Left and middle panel: RT-PCR analysis revealed an induction of AdipoR1 and AdipoR2 mRNA in young animals under caloric restriction ($n = 12$; hatched columns) compared to age-matched animals on control diet ($n = 7$) without affecting the expressional level in senescent

animals (n = 9; hatched columns) compared to age-matched controls (n = 8). Shown are the results of quantitative real-time PCR, each run in triplicate. Data are mean $\pm$ SEM, *: p< 0.05. Right panel: Representative examples of AdipoR1 and AdipoR2 Western blots. C = rats with control diet; R = rats with -16% caloric restriction.

Figure 4: Effects of caloric restriction on the expression of CTRP1, 2, 6 and 7 mRNA

Rat skeletal and cardiac muscle express significant amounts of CTRP1, 2, 6 and 7. Real-time RT-PCR demonstrated a significant increase in CTRP2 and CTRP7 mRNA only in senescent animals under caloric restriction. Numbers of animals as described in figure 1. Data are mean $\pm$ SEM, *: p< 0.05, **: p< 0.01.

Figure 5: Effects of caloric restriction on CTRP2 and CTRP7 protein expression

Left panel: Densitometry of protein data normalized to GAPDH. Homogenates muscle tissue from young and old rats with control diet or caloric restriction (hatched columns) probed with CTRP2 antibody (number of animals as described in Fig. 1). Middle panel: Densitometry of protein data normalized to GAPDH. Homogenates of skeletal muscle probed with an antibody detecting CTRP7. Data are mean $\pm$ SEM, *: p< 0.05. Right panel: Representative examples of Western blots. C = rats with control diet; R = rats with -16% caloric restriction.

Figure 6: Effects of caloric restriction on AMPK activation

Densitometry of protein data from Western blot. Total AMPK- α is not influenced by age or caloric restriction while phosphorylation of AMPK- α at Thr-172 is increased in young rats under 16% caloric restriction (n = 12; hatched columns) compared to age-matched animals on control diet (n = 7) while it is low in senescent rats under control diet (n = 8) and not altered by caloric restriction (n = 9; hatched columns). Data are mean $\pm$ SEM, p< 0.01, ***: p< 0.001.

Homogenates of skeletal muscle probed with antibodies against AMPK- α , phospho-AMPK- α and GAPDH (representative examples). C = rats with control diet; R = rats with -16% caloric restriction.

Table 1: Primer sequences

Gene	GenBank accession #	Forward Primer	Reverse Primer
adiponectin	<u>NM_144744</u>	5'-AATCCTGCCCAGT CATGAAG-3'	5'-CATCTCCTGGGTCA CCCTTA-3'
AdipoR1	<u>NM_207587</u>	5'-CTTCTACTGCTCCC CACAGC-3'	5'-GACAAAGCCCTCAG CGATAG-3'
AdipoR2	<u>NM_001037979</u>	5'-TACACACAGAGAC GGGCAAC-3'	5'-GCAGTACACCGTGT GGAAGA-3'
T-cadherin	<u>AF494095</u>	5'-AACAAAGGCGAACT ACAACCTG-3'	5'-AGAGAGAAGAGCA GCAGCAAG-3'
CTRP 1	<u>NM_019959</u>	5'-ACGAGTTCAGGAG GAACAACA-3'	5'-GCAGGTAGGTCTCC TTCTGGT-3'
CTRP 2	<u>XM_573071</u>	5'-GGACCTAAGGGCA AGAAAGG-3'	5'-GTAGTGGCCTCCCT CATTCA-3'
CTRP 3	<u>NM_030888</u>	5'-TCCACAAGCTGGA GGACTG-3'	5'-TCGCCTTTGTCTCCT TTCTC-3'
CTRP 6	<u>BC101907</u>	5'-AGGTCAGGCCGTA CATCAAC-3'	5'-CAGAGAAGGCCGA GTAATGC-3'
CTRP 7	<u>XM_223507</u>	5'-GGGATGGTAGAGA TGGCAGA-3'	5'-CCCTTGATCTCCTC GGTCTC-3'

Table 2: Plasma concentrations after 2 months – 16% caloric restriction

	diet	young rats	n	old rats	n
Adiponectin (ng/ml)	C	4.9 ± 0.3	7	5.5 ± 0.5	8
	R	7.8 ± 0.2 ***	12	5.6 ± 0.4	9
Leptin (pg/ml)	C	22.5 ± 3.7		30.2 ± 6.3	
	R	14.9 ± 1.7 *		12.2 ± 2.9 ***	
Insulin (ng/ml)	C	0.86 ± 0.21		1.71 ± 0.36 ^a	
	R	0.41 ± 0.17 *		1.13 ± 0.29 *	
Triglycerides (mmol/l)	C	0.12 ± 0.07		1.66 ± 0.26 ^b	
	R	0.10 ± 0.04		0.70 ± 0.17 **	

C= rat with control diet; R = rat with caloric restriction, ^a p < 0.01; ^b p < 0.05 vs. young control, *: p < 0.05; **: p < 0.01; ***: p < 0.001 vs. respective control

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Figure 1

Figure 2

Figure 3

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Figure 5

Figure 6