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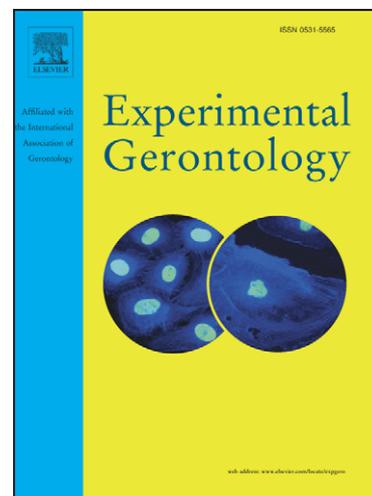
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Age-specific effects of short and long-term caloric restriction on the expression of adiponectin and adiponectin receptors: influence of intensity of food restriction

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Abstract

Hormonal signals from adipose tissue regulate energy homeostasis and may be involved in anti-aging effects of caloric restriction (CR). The adipokine adiponectin is abundantly expressed in adipose tissue and directly sensitizes the body to insulin. The purpose of the current study was to investigate age-dependent effects of different levels of CR (16%, 2 months or 40%, 6 months) on adiponectin and on its receptors AdipoR1 and AdipoR2 in the left ventricle (LV).

In young and senescent rats, 2 months of moderate CR reduced serum leptin. The same diet was sufficient to enhance serum adiponectin, adiponectin expression (visceral fat) and left ventricular AdipoR1 expression in young but not in senescent rats. The higher degree of CR, however, resulted in a mild induction of adiponectin expression in adipose tissue and release into plasma together with increased LV AdipoR1 also in old rats, while these effects were more pronounced in young rats. These changes in adiponectin activation were associated with reduced LV triglyceride content, suggesting an adiponectin-mediated reduced ectopic lipid deposition in nonadipose tissues. Thus, aging is associated with a loss of adiponectin inducibility by moderate CR. This reduction can only partially be overcome by increasing the degree and duration of CR.

Introduction:

Adiponectin (also termed acrp 30, adipoQ, APM-1, GBP28) is an adipocytokine that is specifically and abundantly expressed in adipose tissue and directly sensitizes the body to insulin. Adiponectin structurally belongs to the complement 1q family (review in (Kishore et al., 2004)) and it exists in oligomeric forms: a low-molecular weight (LMW) trimer, a middle-molecular weight (MMW) hexamer, and high-molecular weight (HMW) 12- to 18-mer adiponectin, influencing its biological activity in vivo (Pajvani et al., 2003, Pajvani et al., 2004). 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). AdipoR1 and AdipoR2 are integral membrane proteins with an internal N-terminus and an external C-terminus (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 an adiponectin-binding protein or coreceptor for an as-yet-unidentified signaling receptor (Hug et al., 2004), since it lacks an intracellular domain. AdipoR1 and AdipoR2 serve as receptors for adiponectin and mediate increased activation of the AMP-activated kinase (AMPK), PPAR α activities, fatty-acid oxidation, and glucose uptake by adiponectin (Yamauchi et al., 2003). Both globular and full-length adiponectin activate 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). Blockade of AMPK activation by use of a dominant-negative mutant inhibited these effects of full-length or globular adiponectin,

indicating that stimulation of glucose utilization and fatty-acid oxidation by adiponectin occurs through activation of AMPK (Yamauchi et al., 2002).

Unlike other adipokines such as TNF- α and resistin, which cause insulin resistance, the expression and circulating levels of adiponectin are reduced in diabetic and obese state in animals as well as in humans. Hypoadiponectinemia has also been demonstrated to be independently associated with the metabolic syndrome, stronger than any other inflammatory marker (Matsushita et al., 2006). Exogenous administration of adiponectin on the other hand 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%) were 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 (Polak et al., 2007) or by bariatric surgery (Guldstrand et al., 2003). Furthermore, adiponectin has been suggested to act as a peripheral "starvation" signal promoting the storage of triglycerides preferentially in adipose tissue thereby reducing ectopic lipid deposition in nonadipose tissue (Kim et al., 2007). As a consequence, reduced triglyceride levels in liver, skeletal muscle and heart may convey improved systemic insulin sensitivity and improve metabolic syndrome.

Fat cell derived hormones such as adiponectin or leptin can be envisioned as putative mediators of some of the protective effects of caloric restriction. Although long-term caloric restriction is known to retard the aging process in many organisms including mammals, the basic mechanisms of its efficacy remain unclear and its actions in different organs are

remarkably heterogeneous (Masoro, 2005). CR results also in a metabolic and transcriptional reprogramming of the adipose tissue and may represent a key mechanism influencing a variety of peripheral organs. While there are many data describing the protective effects of CR on whole body insulin sensitivity mainly related to effects on skeletal muscle physiology, much less is known about changes in cardiac metabolism and cardiac function after application of CR. The duration and intensity of this energy intake restriction without essential nutrient deficiency may have a major impact on this process. Since it is very difficult to differentiate basic primary mechanisms from secondary compensations on the basis of long-term effects of caloric restriction, we applied the lowest degree of restriction (- 16% energy intake) and the shortest interval (two months), at which protective actions have been described (Merry, 2002) in the first group of animals, while the second group of animals was treated for 6 months with a food restriction amounting to -40%. Following this treatment, we investigated the age-dependent effects of different degrees and durations of CR on the expression and release of adiponectin from adipose tissue, the expression of its receptors AdipoR1 and AdipoR2 in left ventricular tissue and the storage of triglycerides in peripheral, adiponectin-dependent organs (LV, liver).

Methods

Animals and diet protocol

In order to differentiate basic primary mechanisms from secondary compensations on the basis of long-term effects of caloric restriction, we first applied the lowest degree of restriction (-16% energy intake) and the shortest interval (two months), at which protective actions have ever been analyzed in the literature in mammals (Merry, 2002). Ad libitum-fed male young and senescent Sprague-Dawley (SD) 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 of each rat was monitored for 14 days and averaged. In those young rats (4 months at the beginning of the study) on control diet (Altromin^R 1344/1800; 1800 cal/g, CD) the daily energy intake was 52.5 ± 3.8 kcal, in young rats on -16% caloric restriction (Altromin^R 1344/1500; 1500 cal/g, CR) this daily intake was 45.2 ± 0.7 kcal. In old rats (22-24 months at the beginning of the study) the daily energy intake amounted to 46.4 ± 5.0 kcal in control diet and to 39.9 ± 0.2 kcal in -16% caloric restricted rats.

In a second group of animals, rats were randomly assigned to either receive control diet or a calorically restricted diet for 6 months. During the diet protocol, rats on control diet (Altromin^R 1344/2500; 2505 cal/g, CD) received their individual prediet average, but not more, in order to avoid any degree of diet-induced obesity. Rats subjected to caloric restriction received also their prediet average, but of a caloric reduced, fibre-rich diet (Altromin^R 1344/1500; 1500 cal/g; CR). Thus, in young rats (4 months at the beginning of the study) on control diet for 6 months the daily energy intake was 64.8 ± 2.2 kcal and in young rats on -40% caloric restriction this daily intake was 37.8 ± 1.9 kcal. In old rats (22-24 months at the beginning of the study) the daily energy intake amounted to 57.9 ± 1.8 kcal in control diet and to 36.6 ± 0.1 kcal in -40% caloric restricted rats. All animals (group 1 and group 2)

were fasted for 12 hours before killing and blood was obtained by aortic puncture. All treatments of animals throughout this study received local institutional animal care and use committee approval.

RNA Extraction

Total RNA was isolated from frozen visceral fat and from left ventricular (LV) tissue by guanidine thiocyanate/cesium chloride centrifugation. Integrity and quality of the RNA was confirmed by agarose gel electrophoresis and the concentration determined by measuring UV-absorption.

RT-PCR

Reverse transcription (RT) of RNA samples was carried out for 30 minutes at 42°C. Real-time PCR and data analysis were performed using the Mx3000P Multiplex Quantitative PCR System (Stratagene). DNA amplification was performed as follows: initial denaturation at 95°C for 10 minutes, 40 cycles of amplification (denaturation at 95°C for 30 seconds, annealing at 60°C for 60 seconds, and extension at 72°C for 60 seconds), followed by a denaturation at 95°C for 60 seconds and a melting curve over the range from 55°C up to 95°C. Fluorescence data were collected at the end of the annealing stage of amplification. We performed Real-time PCR of adiponectin (forward 5'-AATCCTGCCCAGTCATGAAG-3'; reverse 5'-CATCTCCTGGGTCACCCTTA-3'), AdipoR1 (forward 5'-CTTCTACTGCTCCC CACAGC-3'; reverse 5'-GACAAAGCCCTCAGCGATAG-3'), AdipoR2 (forward 5'-TAC-ACACAGAGACGGGCAAC-3'; reverse 5'-GCAGTACACCGTGTGGAAGA-3') and 18S rRNA (18S rRNA Control kit, Yakima Yellow®-Eclipse® Dark Quencher, Eurogentec) in samples derived from rat adipose tissue or left ventricles. Each assay was performed in

duplicate and validation of PCR-runs was assessed by evaluation of the melting curve. All data of mRNA are given as relative units of 18S rRNA concentrations.

Western blotting

Frozen LV tissue was rapidly homogenized in a buffer containing 50 mM Tris HCl, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% sodiumdeoxycholate and protease inhibitor cocktail (Sigma). Proteins were quantified using the BCA Protein Assay (Pierce). 50 μ g of protein were loaded on a 10% SDS-PAGE gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane. The filters were blocked and then incubated with antibodies directed against adiponectin (BioVendor, diluted 1:1000), AdipoR1, AdipoR2 (both Abcam, 1:1000, Vimentin (Sigma, 1:2000) as well as GAPDH (Abcam, 1:5000). After incubation with peroxidase-conjugated secondary antibody, blots were subjected to the enhanced chemiluminescent detection method (Amersham) and exposed to a Kodak MR film.

Analyses of Plasma Adiponectin, Plasma Leptin and Tissue Triglycerides

Serum adiponectin and leptin 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) according to the manufacturers' instructions. 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 16% and 40% caloric restriction

In the moderate, short-term restriction group, the body weight of young animals under control diet increased by $17 \pm 1\%$ within two months, while 16% caloric restriction attenuated this increase to $9 \pm 1\%$ (Fig. 1A). In the old control rats, however, body weight decreased by $9 \pm 1\%$ and this decline was augmented to an $18 \pm 1\%$ weight loss in the animals under 16% caloric restriction for 2 months (Fig. 1A). There was no difference in the daily water intake among the rats. Ventricular weights were higher in old control rats than in young ones, also after normalization per body weight (Tab. 1). However, the moderate caloric restriction of two months duration did not alter ventricular weight/body weight ratios within the age-matched groups (Tab. 1)

In the young, growing animals under control diet, body weight increased by $10 \pm 1\%$ within six months, while 40% caloric restriction resulted in a decrease by $14 \pm 2\%$ (Fig. 1C). In the old control rats, body weight was decreased by $10 \pm 2\%$ after 6 months, and this decline was augmented to $38 \pm 4\%$ weight loss in the animals under caloric restriction (Fig. 1C). None of the young animals died during the study (not shown), while survival was significantly increased in old animals under 40% caloric restriction within the first 4 months of the diet compared to old animals under control diet (Fig. 1D). Ventricular weights were higher in old control rats than in young ones (Tab. 2) and were significantly decreased under 40% caloric restriction in young as well as in old animals (Tab. 2).

Effects of caloric restriction on the expression and release of adiponectin

The moderate caloric restriction significantly enhanced the circulating levels of the adipocyte-derived hormone adiponectin in young rats (Fig. 2A), but it did not modify adiponectin plasma levels in the old rats (Fig. 2A), illustrating a hitherto unidentified loss of reactivity in adipose tissue with advanced age. On the other hand, the restriction induced the anticipated

decline in plasma leptin only in the old rats, while this decline did not reach significance in the young rats (Fig. 2B). 40% caloric restriction resulted in a dramatic increase in adiponectin plasma levels in young animals (Fig. 2C) as has been observed previously with caloric restriction or fasting in animals (Zhu et al., 2004). Old control animals on the other hand demonstrated an adiponectin deficit and even the strong stimulation with 40% caloric restriction resulted only in a mild increase of adiponectin plasma concentrations in these animals (Fig. 2C). These differences in adiponectin plasma levels were also reflected by adiponectin mRNA and protein expression in the visceral adipose tissue: 16% and 40% of caloric restriction resulted in a strong induction of adiponectin expression in young animals (Fig. 3). Old control animals on the other hand demonstrated an adiponectin deficit. While 16% of caloric restriction were not sufficient to stimulate the visceral adipose tissue of senescent animals (Fig. 3A), the strong stimulation with 40% caloric restriction resulted in a modest increase of adiponectin mRNA and protein expression in these animals (Fig. 3B). Leptin plasma levels were reduced in both groups under 40% caloric restriction (Fig. 2D).

Effects of caloric restriction on the expression of adiponectin receptors

Adiponectin influences cellular gene expression and signal transduction by binding to the receptors AdipoR1 and AdipoR2. The expression of AdipoR1 mRNA was higher than the expression of AdipoR2 mRNA in left ventricular tissue (Fig. 4). While AdipoR2 expression was not altered following moderate or strong caloric restriction in any of the groups, the higher degree of caloric restriction (-40%) resulted in a strong induction of AdipoR1 mRNA and protein in young and in old animals (Fig. 4A), that was not observed in the old hearts following 16% of CR (Fig. 4B). The corresponding values from AdipoR1 Western blot analyses of the LV after 16% CR are: in young rats 147.6 ± 14.9 vs. 292.1 ± 18.1 ; $p < 0.05$; in old rats 135.6 ± 15.8 vs. 141.0 ± 17.2 ; $p = \text{NS}$ and after 40% CR: in young rats: 105.1 ± 10.2 vs. 196.3 ± 12.7 ; $p < 0.05$; in old rats: 98.2 ± 9.8 vs. 184 ± 11.1 ; $p < 0.05$. Left ventricular AdipoR2

protein expression was not different among any of the groups (not shown). We observed no significant age-dependent decrease in the expression of LV adiponectin receptors as described in the skeletal muscle of mice suffering from diabetes or obesity (Tsuchida et al., 2004).

Effects of caloric restriction on the tissue triglyceride content

Recently, adiponectin has been suggested to act as a peripheral "starvation" signal promoting the storage of triglycerides preferentially in adipose tissue thereby reducing triglyceride levels in the liver and muscle (Kim et al., 2007). Left ventricular content of triglycerides was increased in senescent animals and did not decrease after 2 months of moderate caloric restriction ($4.87 \text{ mg/g wt} \pm 1.19$ vs. $4.74 \text{ mg/g wt} \pm 1.23$) while it was significantly lowered by the same caloric restriction in young animals ($2.90 \text{ mg/g wt} \pm 1.29$ vs. $1.64 \text{ mg/g wt} \pm 0.63$, $p < 0.01$). Similar changes were observed in the liver of these animals ($5.51 \text{ mg/g wt} \pm 0.89$ vs. $4.99 \text{ mg/g wt} \pm 0.53$ in senescent animals; $4.46 \text{ mg/g wt} \pm 0.43$ vs. $3.07 \text{ mg/g wt} \pm 0.30$ in young animals, $p < 0.05$). The higher degree and longer lasting caloric restriction (-40%, 6 months) resulted in a significant decrease in left ventricular triglyceride content in young ($2.61 \text{ mg/g wt} \pm 0.36$ vs. $1.70 \text{ mg/g wt} \pm 0.28$, $p < 0.05$) and in senescent animals ($4.69 \text{ mg/g wt} \pm 0.70$ vs. $2.76 \text{ mg/g wt} \pm 0.32$). Similar changes were observed in the liver of these animals (not shown).

Discussion

Caloric restriction is a reproducible and simple experimental manipulation known to extend maximum lifespan and it has also been shown to retard a variety of age-associated pathophysiological changes (Masoro, 2005). Aging is associated with insulin resistance and alterations in body fat distribution (Enzi et al., 1986). Among others, CR retards these age-associated changes in insulin sensitivity, possible through decreasing obesity (Barzilai and Gabriely, 2001). Accordingly, surgical removal of visceral fat has been shown to result in a comparable improvement of insulin resistance (Barzilai et al., 1999). The fat cell derived hormone adiponectin has been suggested to play a key role in this process by different mechanisms such as increased glucose uptake, altered fatty acid combustion (Yamauchi et al., 2002) or decreased ectopic lipid deposition (Kim et al., 2007). Similarly, some studies in the human show improved insulin sensitivity together with increased adiponectin plasma concentration following weight reduction by hypocaloric diet (Polak et al., 2007) or by bariatric surgery (Guldstrand et al., 2003).

However, the longevity enhancing action of caloric restriction appears to depend on the degree of restriction, up to a reduction down to 35% of ad libitum caloric intake, and a duration of two months is regarded as minimum required for metabolic equilibrium (Merry, 2002). A reduction down to 84%, as applied in the short-term low degree study here, is below the threshold range of restriction with documented survival benefit in rodents (Merry, 2002). A comparative analysis on effects of fasting, short-term or long-term CR on gene expression of white adipose tissue in young mice showed that only a few genes were altered by fasting or short-term CR, making these transcript phenotypes almost undistinguishable from control animals (Higami et al., 2004). Long-term CR (9 months) on the other hand resulted in major changes in gene expression including genes involved in amino acid, lipid, carbohydrate or mitochondrial energy metabolism as well as genes associated with altered insulin sensitivity

(Higami et al., 2004). Long-term CR also reduced the size and cytoplasmic lipid content of adipocytes and up-regulated genes involved in adipocyte differentiation (Higami et al., 2004). Unfortunately, age-associated transcriptional differences in the response to fasting, short-term or long-term CR were not investigated in that study (Higami et al., 2004). The present study shows that the intensity and duration of caloric restriction determines its age-dependent effects on adipose tissue and cardiac muscle. While moderate CR was sufficient to induce potentially protective effects only in young animals, the higher intensity and duration of CR evoked similar, although somewhat weaker effects also in the old animals. Therefore, therapeutics that influence insulin sensitivity or mimic some of the effects of CR may have tremendously different impact on patients depending on their age.

Two months of -16% caloric restriction attenuated the adolescence-associated gain in body weight in young animals (Fig. 1A) while the higher degree of CR (6 months, -40%) resulted in a weight loss also in young animals (Fig. 1C). This was also associated with an improved survival in senescent rats (Fig. 1D) after 4-6 months, which was not achieved by the moderate, short-term CR (Fig. 1B). Adiponectin is a key hormone to control body metabolism as it was shown to increase fatty acid oxidation and 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 increased adiponectin plasma levels in rats after caloric restriction compared to age-matched ad libitum fed animals (Zhu et al., 2004). Both types of CR significantly enhanced the circulating levels of the adipocyte-derived hormone adiponectin in young rats (Fig. 2 A+C). However, the moderate restriction did not modify adiponectin plasma levels in old rats (Fig. 2A). The higher intensity and longer

duration of CR further potentiated the release of adiponectin from adipose tissue in young animals, while it resulted only in a modest induction in old animals, which is still lower than in young controls (Fig. 2C). This illustrates a loss of responsivity in adipose tissue with advanced age, although 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). However, this loss of responsivity did not include alterations in leptin plasma changes following CR (Fig 2B+D). As described previously in Fischer 344 rats by others (Mooradian et al., 2000) serum leptin concentration was increased in senescent rats. Both types of caloric restriction resulted in a decrease in serum leptin that was more pronounced following the high degree CR in young but not in old animals (Fig. 2B+D). Similar to adiponectin plasma levels, adiponectin mRNA and protein expression were induced in visceral fat of young rats (Fig. 3) following both types of diet. Old animals, however, critically depend on a stronger stimulation (40% CR for 6 months) to upregulate their adiponectin mRNA and protein expression in visceral adipose tissue (Fig. 3B).

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 mice (Tsuchida et al., 2004). Whereas the cardiac muscle of young animals demonstrated a diet-induced induction of AdipoR1 receptor (Fig. 4) as described before in the skeletal muscle of mice (Tsuchida et al., 2004), no increase is observed in senescent animals following the moderate restriction (Fig. 4). 6 months of stronger CR resulted in a comparable induction of AdipoR1 in the cardiac muscle of young and old animals. Cardiac AdipoR2 was not altered in any group (Fig.4). This indicates a remarkable difference to the effects of CR in the LV compared to skeletal muscle, since we observed an induction of AdipoR1 and AdipoR2 in young animals in this organ after CR before (Rohrbach et al., 2007). Although AdipoR1 or AdipoR2 are mainly expressed in

cardiomyocytes (Fujioka et al., 2006) other cell types among them macrophages, endothelial cells or smooth muscle cells (Chinetti et al., 2004) have also been shown to express the adiponectin receptors. Therefore, we can not exclude that these cellular sources may have contributed to the increased AdipoR1 mRNA and protein expression in our LV lysates. One can speculate that the adiponectin deficit in old animals may be further amplified in peripheral organs including the heart by the absence of inducibility of the adiponectin receptors AdipoR1 and AdipoR2 in the senescent heart following moderate caloric restriction (Fig. 4). Thus, potential cardioprotective effects of the adiponectin axis ranging from improved myocardial glucose and lipid metabolism or protection against ischemia/reperfusion injury (Tao et al., 2007) to improved vascular function by modulating the endothelial inflammatory response (Goldstein and Scalia, 2004) may be blunted in senescent hearts. The expression of both AdipoR1 and AdipoR2 was also shown to be significantly reduced in skeletal 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). Although there are no data available in the literature describing changes in the expression of adiponectin receptors in cardiac or skeletal muscle, it has been described that very short-term (3 days) CR differentially triggers mitochondrial adaptive mechanisms in the skeletal muscle depending on the intensity of food restriction (Johnson et al., 2006). Thus, adipose tissue (Higami et al., 2004) and muscular tissue (Johnson et al., 2006) both show a graduate response to the stimulatory effects of different types of CR. Unfortunately, both studies (Higami et al., 2004, Johnson et al., 2006) did not investigate senescence-associated alterations in this response.

Adiponectin acts as a "starvation" signal in peripheral organs such as liver, skeletal muscle or cardiac muscle by promoting the storage of triglycerides preferentially in adipose tissue while the ectopic lipid deposition in nonadipose tissues is reduced (Kim et al., 2007). Similar to the results obtained for the adiponectin expression and release into the plasma, senescent rats did not show a significant decrease of cardiac (and liver) triglycerides in response to the stimulatory effects of moderate caloric restriction as described before in kidneys from senescent Fischer 344 rats (Jiang et al., 2005), while the stronger stimulus (6 months, -40%) was sufficient in old and in young animals. 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., 2005). No data were obtained from senescent animals in that study (Barazzoni et al., 2005). However, besides adiponectin other adipokines or hormones can be envisioned as regulators of lipid and energy metabolism reacting to caloric restriction. Ghrelin, which was not investigated in the current study, for example has been shown to induce tissue-specific changes in lipid metabolism favouring triglyceride deposition in liver over skeletal muscle (Barazzoni et al., 2005). Alterations in the type of energy substrate or the rates of ATP production have consequences on LV contractile function and stress responsivity of the heart. Novel mechanisms for heart disease in obesity, called "lipotoxic cardiomyopathy", whereby lipid accumulation within the myocardium is directly cardiotoxic and causes LV dysfunction were described in rodents and subsequently also reported in patients with congestive failure (Szczepaniak et al., 2007, Unger, 2002). Recent studies in healthy individuals and patients with heart failure reveal that myocardial lipid content increases with the degree of obesity and contributes to the progressive deterioration in LV function observed in obese patients (Szczepaniak et al., 2007). Changes in the expression and release of adipose-derived cytokines such as adiponectin may contribute to this ectopic storage of triglycerides (Kim et al., 2007) in the LV with subsequent functional deterioration.

So far, there are only few studies investigating the age-specific effects of caloric restriction on cardiac function and cardiac metabolism including triglyceride storage within the cardiomyocyte. Ischemic preconditioning has no beneficial effect on the postischemic functional recovery of senescent human myocardium (Bartling et al., 2003), while it has also been shown to reduce postischemic dysfunction in the hearts from young-adult and food-restricted but not in ad libitum-fed senescent rats (Abete et al., 2002). Therefore, mechanisms activated by caloric restriction itself or by CR mimetics may represent powerful tools to improve cardiac metabolism and to preserve cardiac functions also in aged human myocardium. The fat cell derived hormone adiponectin has been shown to mediate various cardioprotective effects and can therefore be envisioned as one putative mediator of the protective effects of caloric restriction on the heart. Our study provides data that senescent animals display a significant adiponectin deficit and only high-degree caloric restriction may improve the release of adiponectin from adipose tissue although on a very low level. This observation may also have an impact on therapeutical strategies or outcome of elderly patients suffering from diseases such as type 2 diabetes, obesity, metabolic syndrome or cardiac dysfunction. CR is feasible in laboratory animals and has proven to be a valuable research tool, but it is not applicable in the aging population as a therapeutic or preventive intervention because of the enhanced risks for sarcopenia, osteoporosis and depression. Nevertheless, cardiometabolic pathways activated during CR might yield targets for pharmacological mimetics of selected components within the spectrum of CR actions.

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Figure Legends**Figure 1: Effects of caloric restriction on body weight and survival**

1A: The gain in body weight is reduced in young rats under 16% caloric restriction (n = 12; hatched columns) compared to age-matched animals on control diet (n = 7). The reduction in body weight in old control animals (n = 7) is further increased (n = 9; hatched columns) by caloric restriction for 2 months.

1B. Survival is not improved in old animals under 16% caloric restriction (depicted in grey, CR) compared to old animals under control diet (depicted in black; CD) after 2 months. **: p < 0.01; ***: p < 0.001 vs. respective control.

1C: Body weight is reduced in young rats under 40% caloric restriction (n = 6; hatched columns) compared to age-matched animals on control diet (n = 6) and body weight is further reduced in senescent rats under 40% caloric restriction (n = 4; hatched columns) compared to age-matched controls (n = 6) after 6 months.

1D. Survival is significantly increased in old animals under 40% caloric restriction (depicted in grey; CR) within the first 4 months compared to old animals under control diet (depicted in black; CD).

Figure 2: Effects of caloric restriction on plasma concentrations of adiponectin and leptin

2A+B: Two months of 16% caloric restriction raise adiponectin serum levels in young rats while the decrease in leptin does not reach statistical significance. Serum adiponectin, however, is not increased by the restriction in senescent rats, while leptin levels are depressed by 60%.

2C+D: After 6 months of 40% caloric restriction adiponectin serum levels in young rats are elevated while data from senescent rats suggest a adiponectin deficiency and low reactivity of

adipose tissue to the stimulatory effect of caloric restriction. Serum leptin is decreased in both groups after 6 months of caloric restriction (40%).

*: $p < 0.05$; ***: $p < 0.001$ vs. respective control.

Figure 3: Effects of caloric restriction on adiponectin mRNA and protein expression in visceral fat

A: Data from 2 months 16% CR; B: Data from 6 months, 40% CR.

3A+B: Left panel: RT-PCR analysis of adiponectin mRNA in visceral adipose tissue from young animals under caloric restriction compared to age-matched animals (number of animals as described in Fig. 1). Shown are the results of quantitative real-time PCR, each run in triplicate. Middle panel: Densitometry of protein data. Homogenates of visceral adipose tissue from young and old rats with control diet or caloric restriction (hatched columns) probed with an antibody detecting adiponectin (number of animals as described in Fig. 1). Blots were also probed with Vimentin as a loading control. Right panel: Western blot: Homogenates of visceral fat probed with antibodies against Adiponectin and Vimentin (representative examples). CD = rats with control diet; CR = rats with caloric restriction. Data are mean \pm SEM, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Figure 4: Effects of caloric restriction on AdipoR1 and AdipoR2 mRNA and protein expression in the LV

A: Data from 2 months 16% CR; B: Data from 6 months, 40% CR.

4A+B: Left and middle panel: RT-PCR analyses of AdipoR1 and AdipoR2 mRNA in left ventricular tissue from young animals under caloric restriction compared to age-matched animals (number of animals as described in Fig. 1). Shown are the results of quantitative real-time PCR, each run in triplicate. Right panel: Representative examples of AdipoR1 and

AdipoR2 Western blots. CD = rats with control diet; CR = rats with caloric restriction. Data are mean \pm SEM, **: $p < 0.01$.

ACCEPTED MANUSCRIPT

Table 1: Effects of 16% caloric restriction on LV and body weight

	diet	young rats	n	old rats	n
Left ventricle (g)	CD	0.82 ± 0.03	7	1.37 ± 0.06 ^a	7
	CR	0.74 ± 0.01	12	1.29 ± 0.03 ^a	9
Body weight before diet (g)	CD	332 ± 6	7	556 ± 6 ^a	8
	CR	334 ± 4	12	568 ± 6 ^a	11
Body weight after diet (g)	CD	396 ± 7	7	509 ± 11 ^a	7
	CR	362 ± 4 *	12	466 ± 5 ^{***, b}	9
LV/BW (‰)	CD	2.13 ± 0.06	7	2.72 ± 0.17 ^a	7
	CR	2.03 ± 0.02	12	2.76 ± 0.07 ^a	9

CD= rat with control diet; CR = rat with caloric restriction, ^a p < 0.001; ^b p < 0.01 vs. young control or young calorically-restricted animal as appropriate, * p < 0.05; *** p < 0.001 vs. respective age-matched control

Table 2: Effects of 40% caloric restriction on LV and body weight

	diet	young rats	n	old rats	n
Left ventricle (g)	CD	1.19 ± 0.03	6	1.75 ± 0.06 ^b	6
	CR	1.02 ± 0.01 **	6	1.14 ± 0.05 **	4
Body weight before diet (g)	CD	457 ± 17	6	547 ± 20 ^c	8
	CR	445 ± 5	6	574 ± 24 ^c	8
Body weight after diet (g)	CD	500 ± 15	6	494 ± 21	6
	CR	382 ± 9 ***	6	351 ± 13 ***	4
LV/BW (‰)	CD	2.39 ± 0.04	6	3.54 ± 0.08 ^a	6
	CR	2.67 ± 0.04**	6	3.27 ± 0.02 ^b	4

CD= rat with control diet; CR = rat with caloric restriction, ^a p < 0.001; ^b p < 0.01, ^c p < 0.05 vs. young control or young calorically-restricted animal as appropriate, * p < 0.05; *** p < 0.001 vs. respective age-matched control

Figure 1

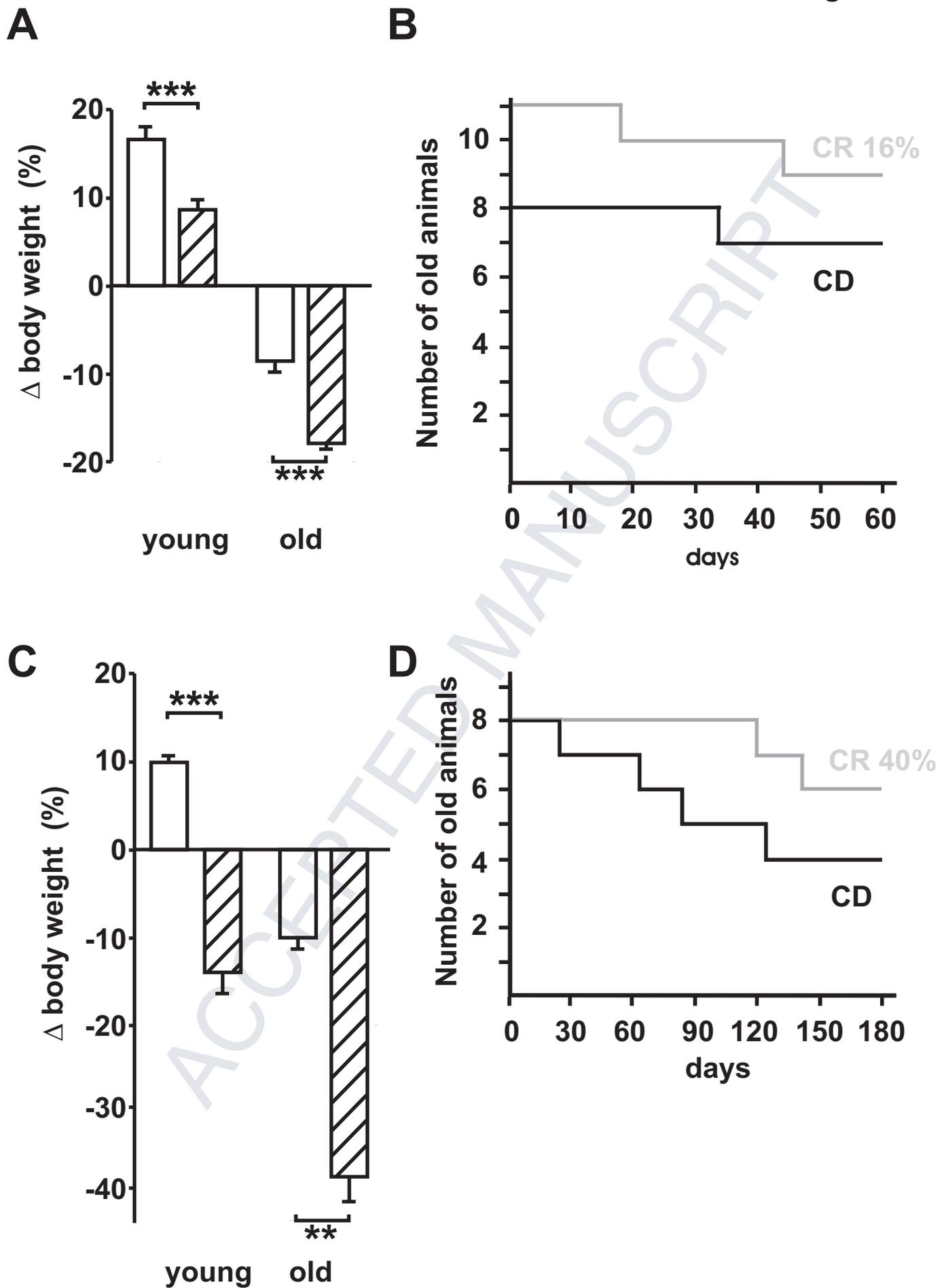
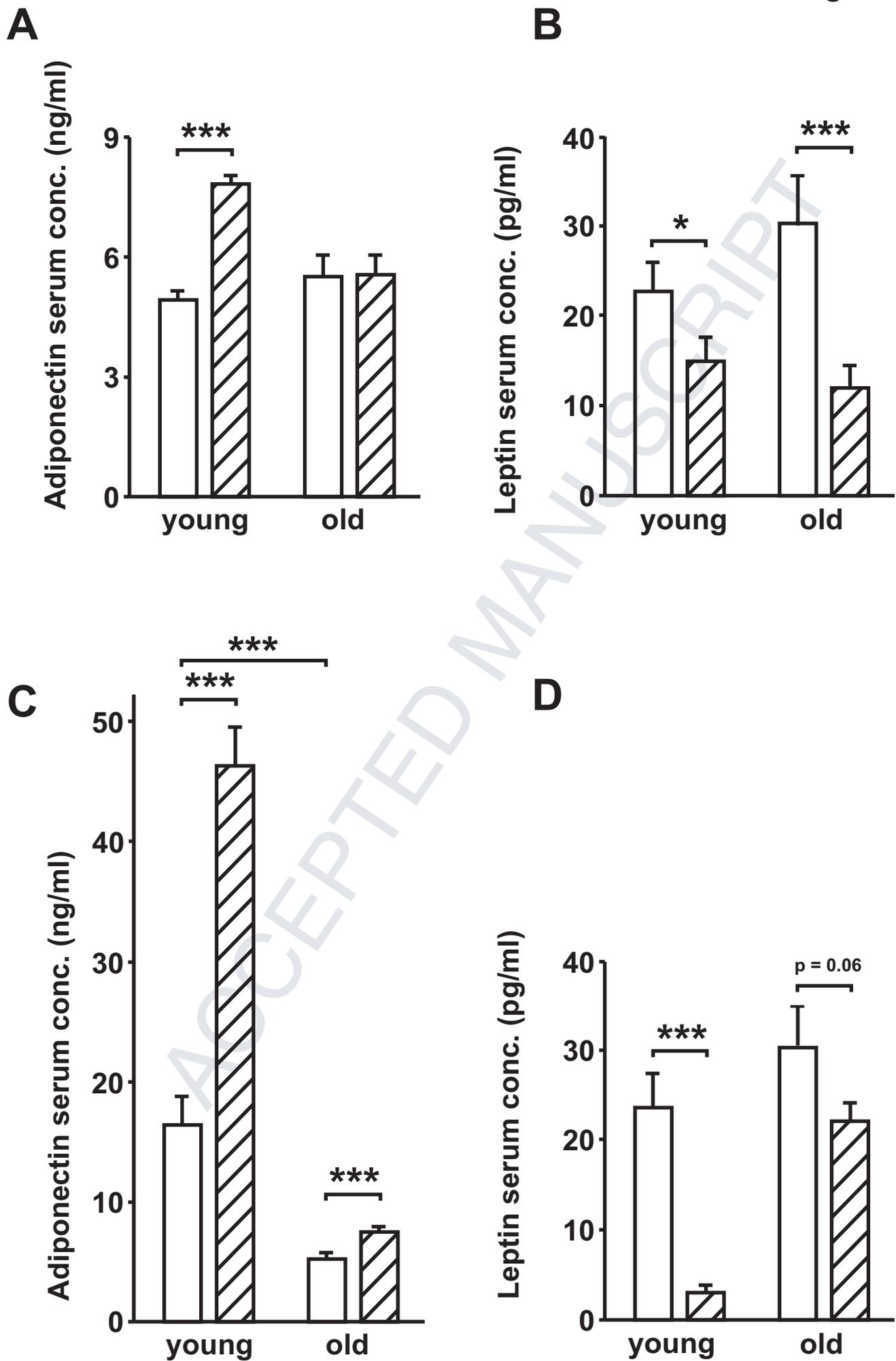
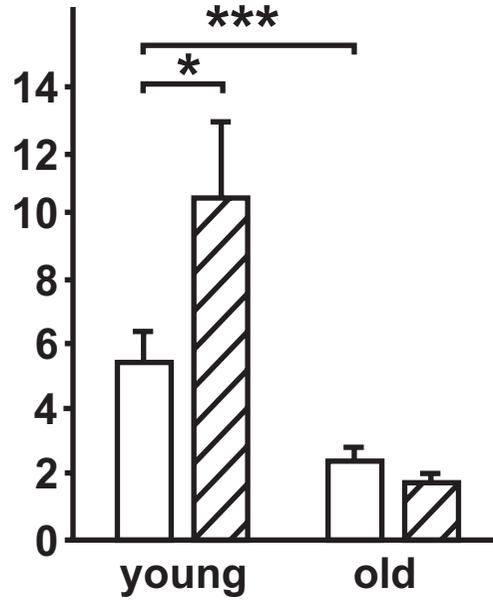
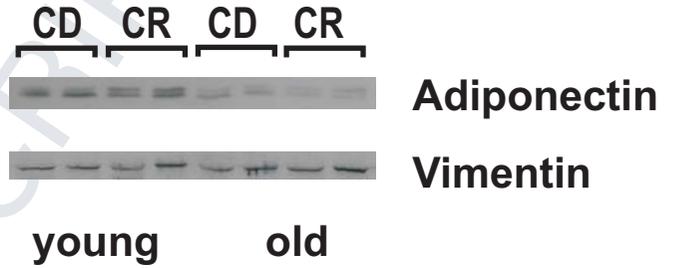
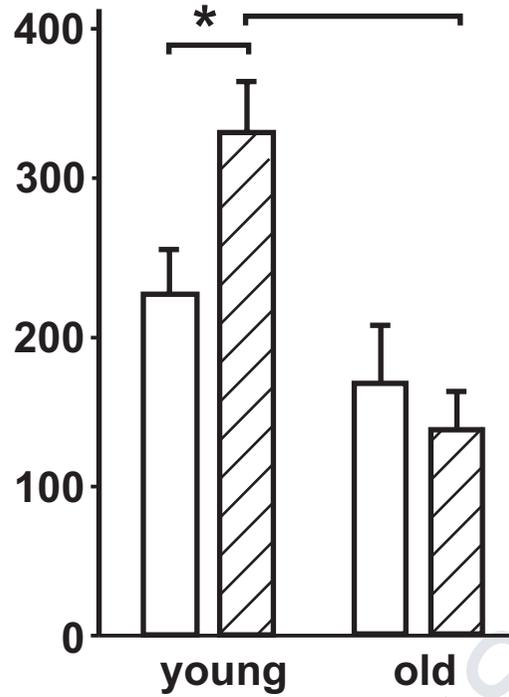
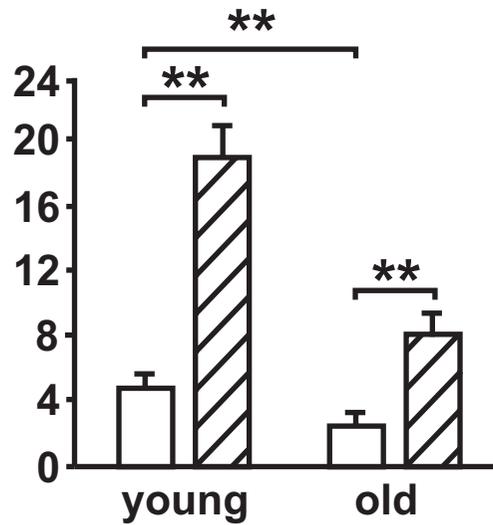


Figure 2



Aadiponectin copies mRNA /
copies 18S rRNA

adiponectin protein (d. u.)

**B**adiponectin copies mRNA /
copies 18S rRNA

adiponectin protein (d. u.)

