Published in final edited form as:

Biofactors. 2014 January ; 40(1): 13–26. doi:10.1002/biof.1111.

TRANSCRIPTIONAL IMPACT OF DIETARY METHIONINE RESTRICTION ON SYSTEMIC INFLAMMATION: RELEVANCE TO BIOMARKERS OF METABOLIC DISEASE DURING AGING

Desiree Wanders¹, Sujoy Ghosh², Kirsten P. Stone¹, Nancy T. Van¹, and Thomas W. Gettys¹

¹Laboratories of Nutrient Sensing and Adipocyte Signaling, Baton Rouge, LA

²Computational Biology Pennington Biomedical Research Center, Baton Rouge, LA

Abstract

Calorie restriction (CR) without malnutrition increases lifespan and produces significant improvements in biomarkers of metabolic health. The improvements are attributable in part to effects of CR on energy balance, which limit fat accumulation by restricting energy intake. Normal age-associated increases in adiposity and insulin resistance are associated with development of a systemic pro-inflammatory state, while chronic CR limits fat deposition and expression of inflammatory markers. Dietary methionine restriction (MR) has emerged as an effective CR mimetic because it produces a comparable extension in lifespan. MR also reduces adiposity through a compensatory increase in energy expenditure that effectively limits fat accumulation, but essentially nothing is known about the effects of MR on systemic inflammation. Here we review the relationships between these two interventions and discuss their transcriptional impact. In addition, using tissues from rats after long term consumption of CR or MR diets, transcriptional profiling was used to examine retrospectively the systems biology of 59 networks of molecules annotated to inflammation. Transcriptional effects of both diets occurred primarily in white adipose tissue and liver, and the responses to MR were far more robust than those to CR. The primary transcriptional targets of MR in both liver and white adipose tissue were phagocytes and macrophages, where expression of genes associated with immune cell infiltration and quantity was reduced. These findings support the conclusion that anti-inflammatory responses produced by CR and MR are not strictly dependent upon reduced adiposity, but are significantly influenced by the metabolic mechanisms through which energy balance is altered.

Keywords

obesity; animal models; insulin sensitivity; amino acid sensing

Introduction

Calorie restriction (CR) to 40% of *ad libitum* intake produces a robust and reproducible extension of mean and maximal lifespan across species [1-3]. CR also lessens adipose tissue inflammation [4] and delays death from all causes, including comorbidities associated with chronic inflammatory states such as diabetes and cardiovascular disease [5]. Given the impracticality of adherence to 40% CR in humans [6], a common goal has been to

To whom correspondence should be addressed: Thomas W. Gettys, Ph.D. Laboratory of Nutrient Sensing & Adipocyte Signaling
6400 Perkins Road Pennington Biomedical Research Center Baton Rouge, LA 70808 Phone: 225-763-3165 Fax: 225-763-0274
gettystw@pbrc.edu.

understand underlying mechanisms and identify mimetics that reproduce the beneficial effects of CR without imposition of severe food restriction. These approaches include restriction of dietary fat [7], carbohydrate [8], protein [9], and individual essential amino acids (EAA) [10-13]. Restriction of methionine from normal levels of ~0.6-0.8% to 0.17% has proven particularly effective because it reproduces the life-extending effects of CR without restricting energy intake [10,11]. In addition to longevity, CR and MR share other beneficial outcomes including reduced adiposity, increased insulin sensitivity, and an overall improvement in biomarkers of metabolic health [11,14,15]. The two dietary regimens impact energy balance through different mechanisms, with CR reducing body weight and adiposity by limiting energy intake. In contrast, MR increases energy intake, but it also produces a paradoxical and disproportionate increase in energy expenditure that effectively limits fat deposition [15,16]. Previous studies have established that the reduced adiposity produced by CR is associated with reduced expression of pro-inflammatory markers in peripheral tissues [17], but the role of these effects in the corresponding improvement of metabolic biomarkers and insulin sensitivity remains unclear. Recent studies have shown that MR produces coordinated transcriptional responses in peripheral tissues in conjunction with beneficial effects on lipid metabolism and insulin sensitivity [15,18], but the question of whether chronic MR has anti-inflammatory effects has not been examined. A significant unanswered question is whether decreased adiposity, irrespective of mechanism, produces comparable reductions in systemic inflammation. Using transcriptional profiling of peripheral tissues after long term CR and MR, we examined the systems biology of 58 networks annotated to the inflammatory process. Despite comparable reductions in adiposity with both diets, the anti-inflammatory responses to MR were far more extensive than CR and targeted different inflammatory processes in both liver and white adipose tissue (WAT). Collectively, our findings suggest the interesting possibility that the delays in age-associated inflammation by CR and MR are secondary to the metabolic effects of the diets, rather than a direct result of reduced adiposity. In this review, we will compare the respective effects of CR and MR on systemic inflammation, with special emphasis on potential relationships between the anti-inflammatory effects of the diets and associated improvements in metabolic status.

Aging and Inflammation

The aging process is associated with a progressive development of chronic low-grade inflammation characterized by increased expression of pro-inflammatory genes in peripheral tissues [19,20]. Inflammation is also associated with increased adiposity and thought to contribute to both age and obesity-associated reductions in insulin sensitivity [19,21]. Aging and obesity lead to increased activation of the transcription factor, nuclear factor (NF- κ B), which is responsible for transcriptional activation of many inflammatory mediators, including the inflammatory cytokines, IL-1 β , IL-6, and tumor necrosis factor- α (TNF α) [22-24]. Therefore, NF- κ B is viewed as a major mechanistic target in the developmental progression of chronic inflammatory disease [25-29].

Claudio Franceschi coined the term, “inflammaging” to describe the age-associated development of inflammation [20]. He observed that “inflammaging” is common to age-related inflammatory pathologies such as neurodegeneration, Alzheimer's disease, osteoporosis, atherosclerosis, diabetes, and cancer, and proposed that it was a principal driving force in their etiology [30]. A consensus has emerged that age-associated inflammation involves increased free radical production, which serves to activate signaling molecules and transcription factors that drive the expression of pro-inflammatory molecules [25,31-34]. It is axiomatic that oxygen-utilizing cells produce free radicals, but they defend against oxidative damage through homeostatic systems that produce anti-oxidants. The “Inflammaging hypothesis” proposes that the carefully regulated balance between oxidant

production and detoxification becomes compromised during aging and the associated production of free radicals results in accumulation of oxidative damage [35]. Both reactive oxygen (ROS) and nitrogen (RNS) species are proposed as potential mediators of this inflammatory progression [36], and recent pre-clinical studies provide compelling evidence that aging is associated with increased hypoxia and ROS in visceral fat depots [37].

Obesity and Inflammation

Accumulation of excess adipose tissue accelerates the development of a chronic, systemic pro-inflammatory state, which predisposes to subsequent development of type 2 diabetes, hepatic steatosis, hypertension, atherosclerosis, cancer and asthma [4]. Obesity-induced inflammation is remarkably similar to the inflammation that accompanies aging, including increased production of ROS and inflammatory cytokines [38]. Obesity increases inflammation in adipose tissue by increasing inflammatory cytokines and chemokines, such as TNF α , monocyte chemoattractant protein-1 (MCP-1), and IL-6 [39]. Increased chemokine production contributes to the overall increase in systemic inflammation [40]. Rapid fat accumulation is accompanied by infiltration of liver and adipose tissue by immune cells, primarily macrophages, which contribute to the increase in inflammatory cytokine production within the tissues [41,42]. In addition, monocytes migrate into adipose tissue in response to apoptotic signals from dying adipocytes and undergo transformation into macrophages [42,43]. Collectively, the inflammatory response produced during rapid expansion of adipose tissue includes increased cytokine expression by resident immune cells and adipocytes, and the immune cells that have infiltrated the depot.

Local hypoxia is part of the normal process of adipose tissue expansion [44], and is used as a signaling mechanism within the depot to activate the transcription factor, hypoxia-inducible factor (HIF). The ensuing transcriptional program promotes increased vascularization to support the expanding size of the cells within the depot [45]. The accompanying increase in free fatty acids activates proinflammatory signaling and serves as a chemoattractant for infiltrating macrophages [46,47]. These processes are essential to remodeling the extracellular matrix within the depot to accommodate expansion of adipose tissue. Under certain physiological conditions, the infiltration and activation of macrophages serves as the initial phase of a form of adipose tissue remodeling that is accompanied by increased oxidative capacity of the depot [48]. In this case, the initial inflammatory response within the depot wanes when the oxidative capacity of the tissue reduces fatty acid levels [48]. However, under conditions of a continual positive energy balance, the chronic dysregulation of lipid metabolism reinforces the inflammatory state by providing a continuous supply of proinflammatory signals. It is also worth noting that increased intracellular fatty acids suppress induction of protein kinase A-mediated gene expression in white adipocytes [49]. Given that this signaling pathway is essential to the adaptive increase of oxidative capacity of adipose tissue, the suppressive effect of excess fatty acids limits their own oxidation and exacerbates their contribution to pro-inflammatory signaling within this tissue.

Preclinical studies support the view that obesity accelerates the progression of age-associated systemic inflammation [50]. Aging also exacerbates obesity-induced macrophage infiltration and inflammation in perivascular adipose tissue, leading to increased risk of cardiovascular disease [51]. However, even in the absence of overt obesity, the characteristic increase in systemic markers of inflammation during aging suggests an important role for energy balance-independent mechanisms in the process.

Adipose Tissue and Liver Inflammation in Aging and Obesity

Adipose tissue inflammation has been linked to a number of pathological processes including hepatic steatosis, insulin resistance, and cardiovascular disease [52-54]. Studies examining the link between adipose tissue inflammation and disease are often conducted in preclinical models of obesity. However, many of these pathologies, including insulin resistance, are common in older animals that are not obese. For example, older non-obese mice have increased adipose tissue expression of IL-1 β , IL-6, TNF α , and cyclooxygenase-2 compared to young mice [50]. A characteristic feature of aging is that it is associated with an increase in macrophage content in subcutaneous adipose tissue [55]. Wu *et al.* also found evidence of increased inflammation in visceral and subcutaneous adipose tissue with age [19], but they failed to detect an increase in macrophage content within the visceral fat depot. A similar lack of increase in visceral fat macrophage numbers was reported by Lumeng *et al.* [56]. However, the authors showed that chemokine expression from and polarity of existing macrophages within the visceral WAT depot shifted to a more inflammatory state with aging [56]. Interestingly, Wu *et al.* argue that adipocytes, rather than immune cells, are responsible for age-associated inflammation in adipose tissue [19]. Together, these findings point to a more complicated mechanism of age-induced adipose tissue inflammation compared to obesity-induced inflammation, where macrophages are considered the primary mediators of the inflammatory state [41]. Lumeng *et al.* argue that age-associated inflammation in visceral adipose tissue is similar to obesity-induced inflammation in the sense that cells within the stromavascular fraction, including macrophages, are the primary source of inflammatory factors rather than adipocytes [56]. Although studies assessing the mechanisms of obesity- and age-associated development of inflammation have primarily focused on adipose tissue, it is worth noting that the inflammatory status of the liver also increases with age and obesity [57,58]. In fact, consuming a HFD increases hepatic activity of NF- κ B and production of its targets, IL-6, IL-1 β , and TNF α [57]. Hepatic inflammation has been cited as an underlying cause of liver disease and has been linked to hepatic insulin resistance [57,59]. Collectively, these studies make a compelling argument that the characteristic dysregulation of lipid metabolism and insulin sensitivity that occur with obesity and aging may be common mechanisms that play central roles in the co-development of systemic inflammation in both physiological states.

Significance of Effects of Calorie Restriction on Adiposity and Inflammation

The mechanisms engaged by CR that delay onset of age-related pathology across multiple organisms [60-64] have been the subject of intense interest in recent years. The consensus is that calorie restriction delays all causes of death by reducing formation of reactive oxygen species (ROS) and slowing the accumulation of oxidative damage. The “Rate of Living-Free Radical” hypothesis holds that CR reduces oxidative metabolism and the associated flux of electrons through the electron transport chain [35,65], thereby reducing mitochondrial ROS formation by reducing proton motive force. The recent amalgamation of the originally separate theories [66,67] is based on the concept that a negative relationship exists between metabolic rate and lifespan; a conclusion drawn from interspecific comparisons of longevity and metabolic rate. There can be little debate that ROS produce oxidative damage, and there is compelling evidence that CR reduces ROS formation and the accumulation of oxidative damage with age in many tissues [22]. However, the view that CR reduces ROS formation strictly as a result of reduced metabolic rate has been questioned by Brand *et al.* [68,69] who have advanced the novel hypothesis that increased mitochondrial proton leak may be extending lifespan by lowering protonmotive force. According to their “Uncoupling to

Survive” hypothesis, increased proton leak reduces ROS formation and metabolic efficiency.

The health benefits that accrue from reductions in adiposity produced are well established, but the significance of CR-induced fat loss to increased longevity has been questioned [70]. However, the emergence of key evidence linking mitochondrial dysfunction to Metabolic Syndrome [14,38,71-73] and oxidative damage in aging [74] has prompted a re-evaluation of the relationship between fat deposition and the release of deleterious and beneficial bioactive agents (H₂O₂, adiponectin, proinflammatory cytokines, etc) from adipose tissue [14,38,75-77]. Thus, the beneficial effects of reduced ROS/pro-inflammatory cytokines from adipose tissue may provide an important link between reduced adiposity, mitochondrial function, and amelioration of pathologies linked to development of systemic inflammation.

Chung *et al.* [22] proposed a molecular hypothesis of aging in which detrimental age-associated increases in NF- κ B, IL-1 β , IL-6, TNF α , cyclooxygenase-2 (COX2), and inducible nitric oxide synthase (iNOS) are all positively impacted by CR [22]. Ye *et al.* propose that the origin of low-grade inflammation accompanying obesity may stem from metabolites of fatty acids and glucose, including diacylglyceride, ceramide, and ROS, which can act through NF- κ B to promote inflammation [4,78]. CR reduces the concentration of these metabolites, thereby limiting potential ligands for inflammatory kinases and receptors. Suppression of age-associated NF- κ B signaling by CR has been found after both short- and long-term CR [79-81]. CR also enhances the release of anti-inflammatory adipokines from adipose tissue such as adiponectin [82], which produces its beneficial effects in part by reducing TNF α expression and promoting a shift in macrophage polarity towards an anti-inflammatory phenotype [83,84]. Considered together, these findings make a compelling case that adipose tissue is a key target for the cumulative beneficial effects of CR. Reduced adiposity improves insulin sensitivity, reduces systemic inflammation, and improves mitochondrial function in peripheral tissues [38,71], but the significance of reduced fat deposition to increased longevity remains an open question [14,70,85]. However, the emergence of key evidence linking disordered lipid metabolism to mitochondrial dysfunction, insulin resistance, Metabolic Syndrome [38,71-73,86,87], and oxidative damage in aging [74] supports the view that the beneficial effects of CR on lipid metabolism in peripheral tissues may be closely linked to reversing the progression of systemic inflammation. Given the key roles of the liver and adipose tissue in coordinating the integration of systemic lipid metabolism in response to insulin, it seems likely that CR would lessen inflammation in both sites in conjunction with improvements in lipid metabolism.

Anti-inflammatory Effects of Methionine Restriction

Although the positive effects of dietary MR on longevity are well documented [88-91], the effects of MR on systemic inflammation have not been explored in any detail. In a recent evaluation of responses to short-term MR (e.g., 3 mo) in rats [92], decreased mRNA expression of chemokine receptors 2 and 5 (CCR2 and CCR5), chemokine ligand 7 (CCL7) and lipopolysaccharide binding protein in inguinal WAT (IWAT) was reported. A significant increase in expression of macrophage migration inhibitory factor (MIF) was observed in WAT but not liver [92]. Additionally, no changes in hepatic inflammatory genes were reported in this short-term study. In a study of much longer duration in mice, Miller *et al.* [88] found that MR increased serum MIF and hepatic expression of MIF mRNA. Although broader screening of hepatic inflammatory markers was not performed, the authors found that livers from MR mice were significantly more resistant to oxidative cell injury than controls [88]. Short-term studies in mice reported that MR reduced mitochondrial ROS formation of oxidative damage to DNA [93], although it is somewhat

surprising since significant oxidative damage to mitochondrial DNA would be evident in mice this young, regardless of diet. Nevertheless, the consensus that ROS serve as signaling molecules to upregulate expression of inflammatory cytokine subsets [94] predicts that MR, in spite of its effects on *in vivo* energy expenditure [15,16], would decrease expression of inflammatory cytokines in the liver. However, it is unclear whether the effects of MR on hepatic ROS formation reported by Sanz *et al.* [93] would extend to other tissues.

Expression Profiling to Compare Inflammatory Responses to Long-Term CR and MR

Transcriptional responses to CR and aging have been explored for the last 15 years using microarray analysis of multiple tissues from different animal models [95]. The designs and goals have been varied, but in general these studies have sought to either (1) identify transcriptional networks being engaged by CR across multiple tissues or (2) develop a more in depth understanding of the transcriptional responses to CR within individual tissues. In the present study, we have compared the anti-inflammatory responses to sustained CR or MR in peripheral tissues in relation to the impact of the two dietary regimens on relative adiposity after 20 mo on the respective diets. Gene expression profiles were evaluated in liver, quadriceps muscle, inguinal WAT and brown adipose tissue (BAT) using Applied Biosystems rat genome microarrays in combination with a targeted bioinformatics approach to focus on the tissue-specific impact of each diet on the biological process of inflammation. At the beginning of the study (e.g., 5 wks age), all rats weighed ~85 g and their adiposity was ~16%, but after 20 mo on the respective diets, the adiposity of the Control group was $32.7 \pm 0.8\%$ (n=8). The adiposity of the CR ($20.5 \pm 0.9\%$, n=8) and MR groups ($23.3 \pm 0.9\%$, n=8) did not differ and was significantly lower than the adiposity of the Controls.

The microarrays detected 12,000 to 14,000 genes in each tissue for each dietary group, and from these totals, genes annotated to the biological process of inflammation and differentially expressed relative to the Control group were identified. Of the four tissues, only IWAT and liver showed robust signatures for differential expression within the inflammation pathway. From a total of 670 inflammatory genes that were differentially expressed after sustained MR in IWAT, 151 were upregulated while 519 were downregulated. A total of 353 inflammation genes were differentially expressed after sustained CR in IWAT, with 135 upregulated and 219 downregulated. Comparison of the respective gene lists showed an overlap of 269 genes that were differentially affected by both diets (Fig. 1A). A similar pattern emerged for the liver, where 340 inflammation genes were differentially expressed after MR, with 106 upregulated and 234 downregulated. Only 173 hepatic genes were differentially affected after CR, with 64 upregulated and 109 downregulated. In liver, 119 of the differentially affected genes were affected by both diets (Fig. 1B).

A more in depth analysis of the common and unique elements of the responses to CR and MR within each tissue was conducted by evaluating the relative impact of the diets on each of 59 individually annotated inflammation pathways within the overall process of inflammation. This approach calculates a negative logP statistic for each pathway based on the Fisher's exact test of proportions. Specifically, the proportion of 'pathway-specific' differentially expressed genes to the total number of differentially expressed genes is compared against the proportion of the total number of pathway-specific genes to the total number of annotated genes in the Ingenuity Knowledge Base, and the null hypothesis that these two proportions are drawn from the same population is tested. If the proportion of 'pathway-specific' differentially expressed genes to the total number of differentially expressed genes is significantly higher than the corresponding background proportion in the Ingenuity Knowledge Base, then that pathway will be deemed to demonstrate a statistically

significant response to diet. It is important to remember that it is not the absolute number of 'pathway-specific' differentially expressed genes but rather the proportion of such genes to the total number of differentially expressed genes that determines statistical significance in this analysis.

Fig. 2A shows the inflammation pathways in IWAT that were significantly impacted by the two dietary regimens and illustrates the greater overall impact of MR compared to CR. Of a total of 59 annotated inflammation pathways, MR produced a significant change (Fisher's $p < 0.05$) in gene expression within 53 of them while CR affected 25 pathways. The greater relative impact of MR is also supported by the fact that this diet uniquely affected 35 inflammatory pathways that were not significantly affected by CR (Fig. 2A). A unique effect of CR was only significant in 6 pathways, and of the 18 pathways affected by both MR and CR, the relative proportion of genes differentially expressed by MR was consistently higher in each of them, leading to more significant p-values (greater negative logP scores) (Fig. 2A). The overall impact of the two diets on liver was less robust but nonetheless highly significant, affecting a total of 29 inflammatory pathways (Fig. 2B). MR affected a total of 22 pathways, 14 of which were unique to MR, while CR affected a total of 15 pathways and 7 of those were unique to CR (Fig. 2B). MR and CR overlapped in affecting 8 pathways in liver, but in contrast to IWAT, the two diets affected similar proportions of genes in 6 of the 8 affected pathways (Fig. 2B). Collectively, this analysis illustrates the significance of the effects of the two diets on inflammatory gene expression and the greater overall impact of MR compared to CR.

The analysis presented in Figs. 2A and 2B illustrates the relative impact of the two diets on specific inflammation pathways but by design, provides no information about the direction of change in expression of affected genes within these pathways. To address this issue, heat maps were generated for each tissue to illustrate the relative change in expression of each gene within pathways that were affected by each diet and both diets. In addition to indicating the up (red) or down (blue) regulation of each gene within the pathways, the heat maps illustrate the extent of overlap of the dietary effects within each pathway. For example, Fig. 3A illustrates that the majority of genes affected by both diets were downregulated, but within these pathways, a significant number of the genes downregulated by MR were unique (e.g., there were no counterparts in the CR treatment, indicated by white). Within pathways affected by one diet or the other, Fig. 3A illustrates the absence of overlap between diets in terms of the affected genes. Interestingly, among all pathways affected by each diet, a small but significant proportion of the genes were significantly upregulated. The upregulated genes include anti-inflammatory cytokines such as adiponectin, IL-10, and IL-4, whose upregulation would reinforce the anti-inflammatory response to each diet. Fig. 3A also illustrates that movement and quantity of phagocytes and macrophages were the primary pathways affected by MR in IWAT. Given that the majority of genes within these pathways were downregulated by MR, it seems reasonable to assume that the primary effect of MR on these pathways was negative. The effect of CR on the infiltration of macrophages pathway was also significant, but in this case at least half the genes annotated to this pathway were upregulated.

The heat maps for liver portray a picture similar to IWAT in the sense that the majority of genes affected by MR and CR were downregulated and the overall anti-inflammatory response to MR was more robust than the response to CR (Fig. 3B). The primary pathways engaged by MR in liver involved accumulation, activation, and morphology of leukocytes and macrophages, while CR affected movement and activation of macrophages (Fig. 3B). Within pathways affected by both diets, 60-70% of genes were downregulated, but as noted in IWAT, a small but significant number of genes were upregulated by both diets (Fig. 3B). Of the 8 pathways affected by both MR and CR (Fig. 2B), MR consistently modified the

expression of at least twice as many genes in each pathway as CR (Fig. 3B). Considered together, these data support the view that MR had a significantly greater effect on inflammatory pathways than CR, and the transcriptional responses were primarily anti-inflammatory.

In addition to microarray analysis, expression of 8 prototypical inflammatory genes was measured in individual tissue samples from each animal and diet by quantitative realtime PCR. The panel of genes included inflammatory cytokines and chemokines (CCL2, IL-1 β , TNF, IL-6, IFN- γ) and immune cell markers (EMR1, ITGAM, ITGAX) that provide readouts of changes in NF- κ B signaling and leukocyte accumulation/activation. NF- κ B signaling is one of the main stress-activated pathways linking obesity and inflammation [96]. The concentration of target mRNAs in each sample was estimated by reverse calibration from standard curves relating mass of target cDNA to cycle threshold as before [97,98]. In addition to liver and IWAT, expression of each gene was evaluated in epididymal (EWAT) and retroperitoneal WAT (RPWAT). Table 1 shows that MR decreased the mRNA expression of 7 of the 8 inflammatory genes in RPWAT, while CR only decreased 2 of the 8 genes. Interestingly, only 1 of the 8 genes was decreased by MR in IWAT, where the only effect of CR was to increase expression of IFN- γ . Three of the 8 genes were decreased by MR in EWAT while CR increased 2 and decreased 1 (Table 1). In contrast, the response to MR in liver involved a significant decrease in 6 of the 8 genes. CR did not produce significant changes in any of the 8 inflammatory genes in liver (Table 1).

Relation Between Development of Metabolic and Anti-inflammatory Responses to MR

A collective comparison of the transcriptional effects of sustained CR and MR shows that MR produced a significantly greater effect on inflammatory genes in WAT and liver than CR, despite the similar reductions in adiposity produced by the dietary regimens. Our previous work [15,16] and that of others [99,100] have clearly shown that the metabolic responses to dietary MR develop soon after introduction of the diet. For example, transcriptional effects in adipose tissue, increases in energy expenditure, reductions in fat deposition, and decreases in insulin all occur within weeks [15,16]. This raises the question of whether improvements in insulin sensitivity are temporally related and linked to the significant anti-inflammatory responses observed after sustained MR. To address this question, expression of prototypical inflammatory markers in WAT and liver were compared after 3, 9 and 20 mo of dietary MR. As reported previously [15], dietary MR reduced serum insulin by 2.1-fold after 3 mo, 3.1-fold after 9 mo, and 7-fold after 20 mo [101], but in the present study, MR failed to reduce expression of any of the inflammatory markers in WAT after 3 mo (Table 2). The expression of 1 of the 8 genes was reduced in liver after 3 mo but none differed from controls after 9 mo. Three of the 8 markers were reduced in RPWAT after 9 mo, while 1 of the 8 were reduced in IWAT and EWAT at this time point (Table 2). As noted above, the reduction in inflammatory gene expression became most evident in RPWAT, liver, and EWAT after 20 mo on the diet (Table 2). Collectively, these data provide little evidence that the anti-inflammatory responses to MR are temporally linked to protection against the progressive development of insulin resistance with age.

Relation Between Anti-obesity and Anti-inflammatory Responses to MR

Given that obesity accentuates the development of inflammation in adipose tissue regardless of age, we evaluated the impact of MR on expression of inflammatory markers in tissues of obesity-prone Osborne-Mendel (OM) rats. In a previous study, we showed that MR for 7 mo after weaning prevented the rapid post weaning expansion of adiposity that occurs in OM rats on the Control diet [15]. The MR-induced reduction in adiposity was accompanied by a

40% reduction in hepatic triglyceride levels from 58 ± 4 nmol/mg in Controls to 41 ± 3 nmol/mg in the MR group. MR also significantly reduced expression of 7 of the 8 genes from our panel of inflammatory markers in the liver (Table 3). However, the diet had little impact on expression of these inflammatory genes among the WAT depots (Table 3). Although somewhat surprising, the potent anti-obesity effects of MR on fat accretion in OM rats had little apparent effect on expression of inflammatory genes in WAT. The primary effect of MR on inflammation in this model was in the liver, where expression of most of the markers was reduced.

Discussion

Restriction of dietary methionine by 80% (e.g. from normal levels of 0.86% to 0.17%) extends lifespan by ~25% in rats [10,11] and mice [88,89] by delaying all causes of death. Soon after its introduction, MR also produces significant changes in energy balance and insulin sensitivity. The effects on energy homeostasis include increased energy expenditure (EE) and reduced fat deposition despite a paradoxical increase in energy intake [15,16]. The metabolic effects of dietary MR are accompanied by transcriptional responses in liver and adipose tissue that reduce circulating and tissue lipids, and modify endocrine function of adipose tissue [15,16,101]. More importantly, after 8 wks of dietary MR, tissue-specific insulin sensitivity, measured by hyperinsulinemic-euglycemic clamps, was 3-fold higher in liver, heart, skeletal muscle, and adipose tissue (unpublished observations). These short-term improvements in insulin sensitivity and metabolic biomarkers are also retained after long-term consumption of the MR diet [15,101]. Therefore, an important unanswered question is whether the metabolic responses to MR are an essential component of the overall mechanism of enhanced longevity.

Several investigators have made the argument that aging constitutes a special case of chronic low grade inflammation characterized by activation of resident immune cells and infiltration of adipose tissue by immune cells [22], which upon activation, cause cellular damage by increased formation of reactive oxygen and nitrogen species. The molecular inflammation hypothesis of aging is based in part on the ability of chronic CR to ameliorate the development of redox-sensitive inflammation [22,102]. It follows from these observations that the life-prolonging action of CR is dependent upon its ability to delay and lessen the accumulation of molecular damage from the progression of age-associated inflammatory processes. Given that MR produces a similar increase in longevity and associated improvement in metabolic biomarkers, the goals of the present work were to (1) compare the effects of chronic MR and CR on systemic inflammation, and (2) examine the temporal development of MR's effects on inflammatory markers in relation to its effects on adiposity and metabolic biomarkers. Using an unbiased microarray approach to address the first objective, we found that both dietary regimens induced significant anti-inflammatory transcriptional programs in inguinal WAT and liver. However, MR altered the expression of twice the number of genes as CR in both tissues, supporting the conclusion that the anti-inflammatory response to MR was significantly more robust than the response to CR. It should also be noted that both diets affected a significant number of the same genes in both tissues (~30-40%), supporting the view that the diets engage common transcriptional control mechanisms in each tissue. Whether these are responsive to changes in fat deposition or signaling associated with energy balance remain to be established.

A more detailed bioinformatics analysis of responses to both diets, based on inflammation pathways annotated under the broad heading of inflammation, identified affected pathways based on overlap between differentially regulated genes and genes within each pathway. The similarities and differences are highlighted graphically in Figs 2 and 3, but the implication of the differences is that distinct populations of immune cell types were being affected by MR

and CR. For example, inflammatory pathways affected by CR in both tissues involved infiltration, movement and activation of macrophages while the effects of MR in both tissues were targeted to leukocytes and phagocytes. A closer examination of the 670 genes affected by MR in IWAT shows that 519 of them were downregulated, suggesting an overall negative effect of MR on leukocytes and phagocytes in adipose tissue. In liver, 234 of the 340 genes affected by MR were downregulated, whereas only 109 of the 173 affected by CR were downregulated. A significant limitation of these studies is that the RNA used for transcriptional analysis was prepared from the entire WAT depot so the RNA is representative of the cell types present within the depot when harvested. In other words, this analysis does not distinguish between the transcriptional responses in immune cells that are resident within the stromovascular fraction within the depot and immune cells that have infiltrated the depot in response to metabolite or chemotactic signals. It seems likely that both processes are involved and more rigorous approaches involving cell fractionation, imaging and cell-sorting based methods will be needed to resolve their relative contributions.

Our initial transcriptional analysis supports the view that 20 mo of dietary MR significantly reduced the expression of inflammatory markers in WAT and liver, but it is unclear when this response occurred. One possibility is that aging produces a steady, gradual increase in expression of inflammatory markers in concert with the gradual accumulation of fat, increase in insulin resistance, and overall metabolic dysregulation that progresses with age. Given the rapid metabolic impact of MR after its introduction [15], the anti-inflammatory effects of the diet may be age-independent and develop quickly after initiation of the diet. To further explore this question we used quantitative real time PCR to examine the expression of a panel of inflammatory markers among three distinct white adipose tissue depots (e.g., RPWAT, EWAT, IWAT) and liver after 3, 9 and 20 mo of dietary MR. The panel of genes included inflammatory cytokines and chemokines (CCL2, IL-1 β , TNF, IL-6, IFN- γ) and immune cell markers (EMR1, ITGAM, ITGAX) that provide readouts of changes in NF- κ B signaling and leukocyte accumulation/activation. The rationale for focusing on these pathways is two-fold. First, NF- κ B signaling is one of the main stress-activated pathways linking obesity and inflammation [96]. Second, loss of function approaches [103] and pharmacological inhibition of the NF- κ B activator, IKK ϵ , reduced inflammation, macrophage infiltration, and suppressed proinflammatory cytokine production from adipose-resident macrophages [104]. Using this panel of markers, we found both a duration- and depot-specific anti-inflammatory effect of MR. RPWAT and liver were highly affected by MR after 20 mo, but after 9 mo, only a modest effect on marker gene expression was detected in RPWAT. Moreover, 3 mo of MR produced essentially no effect on marker gene expression among any of these tissues. Therefore, the significant reductions in adiposity, tissue and serum lipid levels, and increases in insulin sensitivity that occur after 3 and 9 mo of MR [15] occur well before the anti-inflammatory effects of MR become evident. In a study of the transcriptional responses to MR after 3 mo, Perrone *et al.* [92] found that MR decreased IWAT expression of chemokine receptors (e.g., CCR2 and CCR5), chemokine ligand 7 (Ccl7) and LPS binding protein (Lbp), and increased expression of 5 genes annotated to inflammation. In liver, the authors reported downregulation of 5 genes annotated as Immune Response, which include chemokine ligand 9 (CXCL9) and 12 [92]. These findings are consistent with the conclusion that MR has minimal effects on inflammation after 3 mo. Overall, the findings from the current study support the view that MR prevents the normal increase in expression of inflammatory genes that occurs with increasing age rather than producing a gradual decrease in expression of these markers after its introduction. Stated another way, MR “clamps” the inflammatory status of tissues, while in tissues of Control animals, the expression of inflammatory markers increases over time. Current views hold that age- and obesity-associated increases in adipose tissue inflammation reflect the activation of existing and recruited immune cells within the depot. Our studies do

not allow us to assess whether resident or recruited immune cells are the primary targets of MR, but after 20 mo on the diet, it is clear that the primary annotated effects involve accumulation, activation, movement, migration and quantity of immune cells within the affected tissue. Thus, our findings are consistent with the suggestion that MR inhibits the recruitment of new immune cells to the tissue and activation of existing cells within the depot. A variation of this hypothesis was suggested by Higami *et al.* [105], who proposed that CR had little effect on the activity of resident immune cells, but functioned instead by limiting age-associated recruitment of macrophages into adipose tissue [105].

The significance of these musings relate to the eventual deployment of dietary MR in obese humans with metabolic disease [18] and some degree of pre-existing metabolic inflammation. In this setting, the significant question is whether MR is capable of reversing pre-existing, obesity-enhanced inflammatory status. Our current and previous findings with obesity-prone OM rats administered a high fat MR diet provide convincing evidence that MR is effective in preventing the development of obesity and reducing tissue lipids [15]. Despite a nearly 2-fold difference in adiposity between Control and MR rats, expression of our panel of inflammatory markers among WAT depots did not differ between treatments. In contrast, MR reduced hepatic expression of 7 of the 8 inflammatory genes while reducing hepatic triglycerides by 40%. A shortcoming of these studies is that they are missing the negative control group (e.g., low fat diet) that is needed to assess the effect of adiposity, independent of MR, on inflammation in this model. In a limited study assessing CR in the context of a high fat diet, Park *et al.* [17] found that CR reduced expression of inflammatory markers in WAT and liver. Thus, the lack of a decrease in WAT inflammatory genes may be unique to the OM model. Notwithstanding this limitation, the robust anti-inflammatory response to MR in the liver suggests that dietary MR could be effective in ameliorating significant components of the systemic inflammation in patients with metabolic disease.

Conclusions and Perspectives

The present studies provide compelling evidence that sustained, long-term consumption of a methionine-restricted diet produces a robust remodeling of inflammatory gene expression in liver and WAT. Over 60% of affected genes in liver are downregulated while the number in IWAT is over 75%. These transcriptional changes appear temporally unrelated to improvements in metabolic biomarkers and occur well after the diet reduces fat deposition, suggesting that the anti-inflammatory effects of MR are not responsible for improved insulin sensitivity. This conclusion is not meant to imply that MR would not be useful in lessening the heightened inflammation characteristic of adults with metabolic disease, where diet-induced reductions in tissue lipids and hepatic inflammation would diminish or perhaps reverse disease progression and extend healthspan as a result. Thus, the broader implication of our findings is that the life-extending properties of MR in pre-clinical models derive from the ability of the diet to prevent the normal increase in inflammatory status and associated molecular damage that occurs with age. An important remaining challenge is to translate the therapeutic potential of dietary MR to the clinic and evaluate the efficacy of this dietary approach in the treatment of metabolic disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by ADA 1-12-BS-58 (TWG) and NIH DK-096311 (TWG). This work also made use of the Genomics and Cell Biology & Bioimaging core facilities supported by NIH P20-GM103528 (TWG) and NIH P30 DK072476. DW is supported by NIH Institutional Training Grant: 5T32DK064584-10.

References

1. Deyl Z, Juricova M, Stuchlikova E. The effect of nutritional regimes upon collagen concentration and survival of rats. *Adv. Exp. Med. Biol.* 1975; 53:359–369. [PubMed: 1119345]
2. McCay CM, Crowell MF, Maynard LA. The effect of retarded growth upon the length of life span and upon the ultimate body size. 1935. *Nutrition.* 1989; 5:155–171. [PubMed: 2520283]
3. Kemnitz JW. Calorie restriction and aging in nonhuman primates. *ILAR J.* 2011; 52:66–77. [PubMed: 21411859]
4. Ye J, Keller JN. Regulation of energy metabolism by inflammation: a feedback response in obesity and calorie restriction. *Aging (Albany, NY).* 2010; 2:361–368. [PubMed: 20606248]
5. Omodei D, Fontana L. Calorie restriction and prevention of age-associated chronic disease. *FEBS Lett.* 2011; 585:1537–1542. [PubMed: 21402069]
6. Redman LM, Heilbronn LK, Martin CK, de JL, Williamson DA, DeLany JP, Ravussin E. Metabolic and behavioral compensations in response to caloric restriction: implications for the maintenance of weight loss. *PLoS. ONE.* 2009; 4:e4377. [PubMed: 19198647]
7. Walker KZ, O'Dea K. Is a low fat diet the optimal way to cut energy intake over the long-term in overweight people? *Nutr Metab Cardiovasc. Dis.* 2001; 11:244–248. [PubMed: 11831109]
8. Bravata DM, Sanders L, Huang J, Krumholz HM, Olkin I, Gardner CD, Bravata DM. Efficacy and safety of low-carbohydrate diets: a systematic review. *JAMA.* 2003; 289:1837–1850. [PubMed: 12684364]
9. Pamplona R, Barja G. Mitochondrial oxidative stress, aging and caloric restriction: The protein and methionine connection. *Biochim. Biophys. Acta.* 2006; 1757:496–506. [PubMed: 16574059]
10. Orentreich N, Matias JR, DeFelice A, Zimmerman JA. Low methionine ingestion by rats extends life span. *J Nutr.* 1993; 123:269–274. [PubMed: 8429371]
11. Richie JP Jr, Leutzinger Y, Parthasarathy S, Malloy V, Orentreich N, Zimmerman JA. Methionine restriction increases blood glutathione and longevity in F344 rats. *FASEB J.* 1994; 8:1302–1307. [PubMed: 8001743]
12. Anthony TG, McDaniel BJ, Byerley RL, McGrath BC, Cavener DR, McNurlan MA, Wek RC. Preservation of liver protein synthesis during dietary leucine deprivation occurs at the expense of skeletal muscle mass in mice deleted for eIF2 kinase GCN2. *J. Biol. Chem.* 2004; 279:36553–36561. [PubMed: 15213227]
13. Theuer RC. Effect of essential amino acid restriction on the growth of female C57BL mice and their implanted BW10232 adenocarcinomas. *J Nutr.* 1971; 101:223–232. [PubMed: 5540443]
14. Barzilai N, Gabriely I. The role of fat depletion in the biological benefits of caloric restriction. *J Nutr.* 2001; 131:903S–906S. [PubMed: 11238783]
15. Hasek BE, Stewart LK, Henagan TM, Boudreau A, Lenard NR, Black C, Shin J, Huypens P, Malloy V, Plaisance EP, Krajcik RA, Orentreich N, Gettys TW. Dietary methionine restriction enhances metabolic flexibility and increases uncoupled respiration in both fed and fasted states. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2010; 299:R728–R739. [PubMed: 20538896]
16. Plaisance EP, Henagan TM, Echlin H, Boudreau A, Hill KL, Lenard NR, Hasek BE, Orentreich N, Gettys TW. Role of β -adrenergic receptors in the hyperphagic and hypermetabolic responses to dietary methionine restriction. *Am J Physiol Regul Integr Comp Physiol.* 2010; 299:R740–R750. [PubMed: 20554934]
17. Park S, Park NY, Valacchi G, Lim Y. Calorie restriction with a high-fat diet effectively attenuated inflammatory response and oxidative stress-related markers in obese tissues of the high diet fed rats. *Mediators Inflamm.* 2012 doi:10.1155/2012/984643.
18. Plaisance EP, Greenway FL, Boudreau A, Hill KL, Johnson WD, Krajcik RA, Perrone CE, Orentreich N, Cefalu WT, Gettys TW. Dietary methionine restriction increases fat oxidation in obese adults with metabolic syndrome. *J Clin Endocrinol Metab.* 2011; 96:E836–E840. [PubMed: 21346062]
19. Wu D, Ren Z, Pae M, Guo W, Cui X, Merrill AH, Meydani SN. Aging up-regulates expression of inflammatory mediators in mouse adipose tissue. *J Immunol.* 2007; 179:4829–4839. [PubMed: 17878382]

20. Franceschi C, Bonafe M, Valensin S, Olivieri F, De LM, Ottaviani E, De BG. Inflamm-aging. An evolutionary perspective on immunosenescence. *Ann. N. Y. Acad. Sci.* 2000; 908:244–254. [PubMed: 10911963]
21. Paolisso G, Rizzo MR, Mazziotti G, Tagliamonte MR, Gambardella A, Rotondi M, Carella C, Giugliano D, Varricchio M, D'Onofrio F. Advancing age and insulin resistance: role of plasma tumor necrosis factor-alpha. *Am J Physiol.* 1998; 275:E294–E299. [PubMed: 9688632]
22. Chung HY, Kim HJ, Kim JW, Yu BP. The inflammation hypothesis of aging: molecular modulation by calorie restriction. *Ann. N. Y. Acad. Sci.* 2001; 928:327–335. [PubMed: 11795524]
23. Shea LM, Beehler C, Schwartz M, Shenkar R, Tuder R, Abraham E. Hyperoxia activates NF-kappaB and increases TNF-alpha and IFN-gamma gene expression in mouse pulmonary lymphocytes. *J Immunol.* 1996; 157:3902–3908. [PubMed: 8892621]
24. Tak PP, Firestein GS. NF-kappaB: a key role in inflammatory diseases. *J Clin Invest.* 2001; 107:7–11. [PubMed: 11134171]
25. Sarkar D, Fisher PB. Molecular mechanisms of aging-associated inflammation. *Cancer Lett.* 2006; 236:13–23. [PubMed: 15978720]
26. Helenius M, Hanninen M, Lehtinen SK, Salminen A. Aging-induced up-regulation of nuclear binding activities of oxidative stress responsive NF-kB transcription factor in mouse cardiac muscle. *J Mol. Cell Cardiol.* 1996; 28:487–498. [PubMed: 9011632]
27. Helenius M, Hanninen M, Lehtinen SK, Salminen A. Changes associated with aging and replicative senescence in the regulation of transcription factor nuclear factor-kappa B. *Biochem. J.* 1996; 318:603–608. [PubMed: 8809053]
28. Korhonen P, Helenius M, Salminen A. Age-related changes in the regulation of transcription factor NF-kappa B in rat brain. *Neurosci. Lett.* 1997; 225:61–64. [PubMed: 9143018]
29. Salminen A, Huuskonen J, Ojala J, Kauppinen A, Kaarniranta K, Suuronen T. Activation of innate immunity system during aging: NF-kB signaling is the molecular culprit of inflamm-aging. *Ageing Res. Rev.* 2008; 7:83–105. [PubMed: 17964225]
30. Franceschi C, Bonafe M. Centenarians as a model for healthy aging. *Biochem. Soc. Trans.* 2003; 31:457–461. [PubMed: 12653662]
31. Ahmad A, Banerjee S, Wang Z, Kong D, Majumdar AP, Sarkar FH. Aging and inflammation: etiological culprits of cancer. *Curr. Aging Sci.* 2009; 2:174–186. [PubMed: 19997527]
32. Bruunsgaard H, Pedersen M, Pedersen BK. Aging and proinflammatory cytokines. *Curr. Opin. Hematol.* 2001; 8:131–136. [PubMed: 11303144]
33. Chung JH, Seo AY, Chung SW, Kim MK, Leeuwenburgh C, Yu BP, Chung HY. Molecular mechanism of PPAR in the regulation of age-related inflammation. *Ageing Res. Rev.* 2008; 7:126–136. [PubMed: 18313368]
34. Dice JF. Cellular and molecular mechanisms of aging. *Physiol Rev.* 1993; 73:149–159. [PubMed: 8419964]
35. Harman D. Aging: a theory based on free radical and radiation chemistry. *J Gerontol.* 1956; 11:298–300. [PubMed: 13332224]
36. Ogawa K, Suzuki K, Okutsu M, Yamazaki K, Shinkai S. The association of elevated reactive oxygen species levels from neutrophils with low-grade inflammation in the elderly. *Immun. Ageing.* 2008; 5:13. doi: 10.1186/1742-4933-5-13. [PubMed: 18950479]
37. Zhang L, Ebenezer PJ, Dasuri K, Fernandez-Kim SO, Francis J, Mariappan N, Gao Z, Ye J, Bruce-Keller AJ, Keller JN. Aging is associated with hypoxia and oxidative stress in adipose tissue: implications for adipose function. *Am J Physiol Endocrinol. Metab.* 2011; 301:E599–E607. [PubMed: 21586698]
38. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin. Invest.* 2004; 114:1752–1761. [PubMed: 15599400]
39. Greenberg AS, Obin MS. Obesity and the role of adipose tissue in inflammation and metabolism. *Am J Clin Nutr.* 2006; 83:461S–465S. [PubMed: 16470013]
40. Bullo M, Garcia-Lorda P, Megias I, Salas-Salvado J. Systemic inflammation, adipose tissue tumor necrosis factor, and leptin expression. *Obes. Res.* 2003; 11:525–531. [PubMed: 12690081]

41. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest.* 2003; 112:1796–1808. [PubMed: 14679176]
42. Oh DY, Morinaga H, Talukdar S, Bae EJ, Olefsky JM. Increased macrophage migration into adipose tissue in obese mice. *Diabetes.* 2012; 61:346–354. [PubMed: 22190646]
43. Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg AS, Obin MS. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J. Lipid Res.* 2005; 46:2347–2355. [PubMed: 16150820]
44. Ye J, Gao Z, Yin J, He Q. Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. *Am. J Physiol Endocrinol. Metab.* 2007; 293:E1118–E1128. [PubMed: 17666485]
45. Cao Y. Angiogenesis modulates adipogenesis and obesity. *J Clin. Invest.* 2007; 117:2362–2368. [PubMed: 17786229]
46. Kosteli A, Sugaru E, Haemmerle G, Martin JF, Lei J, Zechner R, Ferrante AW Jr. Weight loss and lipolysis promote a dynamic immune response in murine adipose tissue. *J Clin. Invest.* 2010; 120:3466–3479. [PubMed: 20877011]
47. Kosteli A, Sugaru E, Haemmerle G, Martin JF, Lei J, Zechner R, Ferrante AW Jr. Weight loss and lipolysis promote a dynamic immune response in murine adipose tissue. *J Clin. Invest.* 2010; 120:3466–3479. [PubMed: 20877011]
48. Granneman JG, Li P, Zhu Z, Lu Y. Metabolic and cellular plasticity in white adipose tissue I: effects of beta3-adrenergic receptor activation. *Am. J Physiol Endocrinol. Metab.* 2005; 289:E608–E616. [PubMed: 15941787]
49. Mottillo EP, Granneman JG. Intracellular fatty acids suppress beta-adrenergic induction of PKA-targeted gene expression in white adipocytes. *Am. J. Physiol Endocrinol. Metab.* 2011; 301:E122–E131. [PubMed: 21505145]
50. Wu D, Ren Z, Pae M, Han SN, Meydani SN. Diet-induced obesity has a differential effect on adipose tissue and macrophage inflammatory responses of young and old mice. *Biofactors.* 2013 DOI: 10.1002/biof.1075.
51. Bailey-Downs LC, Tucsek Z, Toth P, Sosnowska D, Gautam T, Sonntag WE, Csiszar A, Ungvari Z. Aging Exacerbates Obesity-Induced Oxidative Stress and Inflammation in Perivascular Adipose Tissue in Mice: A Paracrine Mechanism Contributing to Vascular Redox Dysregulation and Inflammation. *J. Gerontol. A Biol. Sci. Med. Sci.* 2012 doi: 10.1093/gerona/gls238.
52. Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, Kitazawa S, Miyachi H, Maeda S, Egashira K, Kasuga M. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest.* 2006; 116:1494–1505. [PubMed: 16691291]
53. Sevastianova K, Sutinen J, Kannisto K, Hamsten A, Ristola M, Yki-Jarvinen H. Adipose tissue inflammation and liver fat in patients with highly active antiretroviral therapy-associated lipodystrophy. *Am J Physiol Endocrinol. Metab.* 2008; 295:E85–E91. [PubMed: 18430964]
54. Berg AH, Scherer PE. Adipose tissue, inflammation, and cardiovascular disease. *Circ. Res.* 2005; 96:939–949. [PubMed: 15890981]
55. Ortega, M. d.; V; Xu, X.; Koska, J.; Francisco, AM.; Scalise, M.; Ferrante, AW., Jr.; Krakoff, J. Macrophage content in subcutaneous adipose tissue: associations with adiposity, age, inflammatory markers, and whole-body insulin action in healthy Pima Indians. *Diabetes.* 2009; 58:385–393. [PubMed: 19008342]
56. Lumeng CN, Liu J, Geletka L, Delaney C, Delproposto J, Desai A, Oatmen K, Martinez-Santibanez G, Julius A, Garg S, Yung RL. Aging is associated with an increase in T cells and inflammatory macrophages in visceral adipose tissue. *J. Immunol.* 2011; 187:6208–6216. [PubMed: 22075699]
57. Cai D, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J, Shoelson SE. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat. Med.* 2005; 11:183–190. [PubMed: 15685173]
58. Lee JS, Ward WO, Ren H, Vallanat B, Darlington GJ, Han ES, Laguna JC, DeFord JH, Papaconstantinou J, Selman C, Corton JC. Meta-analysis of gene expression in the mouse liver

- reveals biomarkers associated with inflammation increased early during aging. *Mech. Ageing Dev.* 2012; 133:467–478. [PubMed: 22704917]
59. Elsharkawy AM, Mann DA. Nuclear factor-kappaB and the hepatic inflammation-fibrosis-cancer axis. *Hepatology.* 2007; 46:590–597. [PubMed: 17661407]
 60. McCay CM, Crowel MF, Maynard LA. The effect of retarded growth upon the length of the life span and upon the ultimate body size. *J. Nutr.* 1935; 10:63–79.
 61. Weindruch R, Sohal RS. Seminars in medicine of the Beth Israel Deaconess Medical Center. Caloric intake and aging. *N. Engl. J Med.* 1997; 337:986–994. [PubMed: 9309105]
 62. Anderson RM, Latorre-Esteves M, Neves AR, Lavu S, Medvedik O, Taylor C, Howitz KT, Santos H, Sinclair DA. Yeast life-span extension by calorie restriction is independent of NAD fluctuation. *Science.* 2003; 302:2124–2126. [PubMed: 14605207]
 63. Houthoofd K, Braeckman BP, Johnson TE, Vanfleteren JR. Life extension via dietary restriction is independent of the Ins/IGF-1 signalling pathway in *Caenorhabditis elegans*. *Exp. Gerontol.* 2003; 38:947–954. [PubMed: 12954481]
 64. Partridge L, Piper MD, Mair W. Dietary restriction in *Drosophila*. *Mech. Ageing Dev.* 2005; 126:938–950. [PubMed: 15935441]
 65. Pearl, R. *The Rate of Living.* University of London Press; London: 1928.
 66. Sohal RS, Allen RG. Relationship between metabolic rate, free radicals, differentiation and aging: a unified theory. *Basic Life Sci.* 1985; 35:75–104. [PubMed: 4062824]
 67. Sohal RS, Weindruch R. Oxidative stress, caloric restriction, and aging. *Science.* 1996; 273:59–63. [PubMed: 8658196]
 68. Brand MD. Uncoupling to survive? The role of mitochondrial inefficiency in ageing. *Exp. Gerontol.* 2000; 35:811–820. [PubMed: 11053672]
 69. Speakman JR, Talbot DA, Selman C, Snart S, McLaren JS, Redman P, Krol E, Jackson DM, Johnson MS, Brand MD. Uncoupled and surviving: individual mice with high metabolism have greater mitochondrial uncoupling and live longer. *Aging Cell.* 2004; 3:87–95. [PubMed: 15153176]
 70. Harrison DE, Archer JR, Astle CM. Effects of food restriction on aging: separation of food intake and adiposity. *PNAS.* 1984; 81:1835–1838. [PubMed: 6608731]
 71. Lowell BB, Shulman GI. Mitochondrial dysfunction and type 2 diabetes. *Science.* 2005; 307:384–387. [PubMed: 15662004]
 72. Petersen KF, Dufour S, Befroy D, Garcia R, Shulman GI. Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. *N. Engl. J Med.* 2004; 350:664–671. [PubMed: 14960743]
 73. Fernandez-Real JM, Ricart W. Insulin resistance and chronic cardiovascular inflammatory syndrome. *Endocr. Rev.* 2003; 24:278–301. [PubMed: 12788800]
 74. Petersen KF, Befroy D, Dufour S, Dziura J, Ariyan C, Rothman DL, DiPietro L, Cline GW, Shulman GI. Mitochondrial dysfunction in the elderly: possible role in insulin resistance. *Science.* 2003; 300:1140–1142. [PubMed: 12750520]
 75. Lin Y, Berg AH, Iyengar P, Lam TK, Giacca A, Combs TP, Rajala MW, Du X, Rollman B, Li W, Hawkins M, Barzilai N, Rhodes CJ, Fantus IG, Brownlee M, Scherer PE. The hyperglycemia-induced inflammatory response in adipocytes: the role of reactive oxygen species. *J Biol. Chem.* 2005; 280:4617–4626. [PubMed: 15536073]
 76. Gupta G, Cases JA, She L, Ma XH, Yang XM, Hu M, Wu J, Rossetti L, Barzilai N. Ability of insulin to modulate hepatic glucose production in aging rats is impaired by fat accumulation. *Am. J Physiol Endocrinol. Metab.* 2000; 278:E985–E991. [PubMed: 10826999]
 77. Lin Y, Rajala MW, Berger JP, Moller DE, Barzilai N, Scherer PE. Hyperglycemia-induced production of acute phase reactants in adipose tissue. *J Biol. Chem.* 2001; 276:42077–42083. [PubMed: 11546817]
 78. Boland MP, O'Neill LA. Ceramide activates NFkappaB by inducing the processing of p105. *J. Biol. Chem.* 1998; 273:15494–15500. [PubMed: 9624136]
 79. Yu BP. Aging and oxidative stress: modulation by dietary restriction. *Free Radic. Biol. Med.* 1996; 21:651–668. [PubMed: 8891668]

80. Kim HJ, Kim KW, Yu BP, Chung HY. The effect of age on cyclooxygenase-2 gene expression: NF-kappaB activation and IkappaBalpha degradation. *Free Radic. Biol. Med.* 2000; 28:683–692. [PubMed: 10754263]
81. Jung KJ, Lee EK, Kim JY, Zou Y, Sung B, Heo HS, Kim MK, Lee J, Kim ND, Yu BP, Chung HY. Effect of short term calorie restriction on pro-inflammatory NF-kB and AP-1 in aged rat kidney. *Inflamm. Res.* 2009; 58:143–150. [PubMed: 19199090]
82. Zhu M, Miura J, Lu LX, Bernier M, DeCabo R, Lane MA, Roth GS, Ingram DK. Circulating adiponectin levels increase in rats on caloric restriction: the potential for insulin sensitization. *Exp. Gerontol.* 2004; 39:1049–1059. [PubMed: 15236764]
83. Zhao T, Hou M, Xia M, Wang Q, Zhu H, Xiao Y, Tang Z, Ma J, Ling W. Globular adiponectin decreases leptin-induced tumor necrosis factor-alpha expression by murine macrophages: involvement of cAMP-PKA and MAPK pathways. *Cell Immunol.* 2005; 238:19–30. [PubMed: 16438946]
84. Ohashi K, Parker JL, Ouchi N, Higuchi A, Vita JA, Gokce N, Pedersen AA, Kalthoff C, Tullin S, Sams A, Summer R, Walsh K. Adiponectin promotes macrophage polarization toward an anti-inflammatory phenotype. *J Biol. Chem.* 2010; 285:6153–6160. [PubMed: 20028977]
85. Gabriely I, Ma XH, Yang XM, Atzmon G, Rajala MW, Berg AH, Scherer P, Rossetti L, Barzilai N. Removal of visceral fat prevents insulin resistance and glucose intolerance of aging: an adipokine-mediated process? *Diabetes.* 2002; 51:2951–2958. [PubMed: 12351432]
86. Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O, Bain J, Stevens R, Dyck JR, Newgard CB, Lopaschuk GD, Muoio DM. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab.* 2008; 7:45–56. [PubMed: 18177724]
87. An J, Muoio DM, Shiota M, Fujimoto Y, Cline GW, Shulman GI, Koves TR, Stevens R, Millington D, Newgard CB. Hepatic expression of malonyl-CoA decarboxylase reverses muscle, liver and whole-animal insulin resistance. *Nat. Med.* 2004; 10:268–274. [PubMed: 14770177]
88. Miller RA, Buehner G, Chang Y, Harper JM, Sigler R, Smith-Wheelock M. Methionine-deficient diet extends mouse lifespan, slows immune and lens aging, alters glucose, T4, IGF-I and insulin levels, and increases hepatocyte MIF levels and stress resistance. *Aging Cell.* 2005; 4:119–125. [PubMed: 15924568]
89. Sun L, Sadighi Akha AA, Miller RA, Harper JM. Life-span extension in mice by preweaning food restriction and by methionine restriction in middle age. *J. Gerontol. A Biol. Sci. Med. Sci.* 2009; 64:711–722. [PubMed: 19414512]
90. Sung B, Park S, Yu BP, Chung HY. Modulation of PPAR in aging, inflammation, and calorie restriction. *J Gerontol A Biol Sci Med Sci.* 2004; 59:997–1006. [PubMed: 15528772]
91. Takata T, Ishikawa F. Human Sir2-related protein SIRT1 associates with the bHLH repressors HES1 and HEY2 and is involved in HES1- and HEY2-mediated transcriptional repression. *Biochem Biophys Res Commun.* 2003; 301:250–257. [PubMed: 12535671]
92. Perrone CE, Mattocks DA, Plummer JD, Chittur SV, Mohny R, Vignola K, Orentreich DS, Orentreich N. Genomic and Metabolic Responses to Methionine-Restricted and Methionine-Restricted, Cysteine-Supplemented Diets in Fischer 344 Rat Inguinal Adipose Tissue, Liver and Quadriceps Muscle. *J Nutrigenet. Nutrigenomics.* 2012; 5:132–157. [PubMed: 23052097]
93. Sanz A, Caro P, Ayala V, Portero-Otin M, Pamplona R, Barja G. Methionine restriction decreases mitochondrial oxygen radical generation and leak as well as oxidative damage to mitochondrial DNA and proteins. *FASEB J.* 2006; 20:1064–1073. [PubMed: 16770005]
94. Naik E, Dixit VM. Mitochondrial reactive oxygen species drive proinflammatory cytokine production. *J Exp. Med.* 2011; 208:417–420. [PubMed: 21357740]
95. Swindell WR. Genes and gene expression modules associated with caloric restriction and aging in the laboratory mouse. *BMC. Genomics.* 2009; 10:585. doi: 10.1186/1471-2164-10-585. [PubMed: 19968875]
96. Baker RG, Hayden MS, Ghosh S. NF-kappaB, inflammation, and metabolic disease. *Cell Metab.* 2011; 13:11–22. [PubMed: 21195345]

97. Prpic V, Watson PM, Frampton IC, Sabol MA, Jezek GE, Gettys TW. Differential mechanisms and development of leptin resistance in A/J vs C57BL/6J mice during diet-induced obesity. *Endocrinology*. 2003; 144:1155–1163. [PubMed: 12639896]
98. Zhang Y, Huypens P, Adamson AW, Chang JS, Henagan TM, Lenard NR, Burk D, Klein J, Perwitz N, Shin J, Fasshauer M, Kralli A, Gettys TW. Alternative mRNA splicing produces a novel biologically active short isoform of PGC-1 α . *J. Biol. Chem*. 2009; 284:32813–32826. [PubMed: 19773550]
99. Perrone CE, Mattocks DA, Hristopoulos G, Plummer JD, Krajcik RA, Orentreich N. Methionine restriction effects on 11 β -HSD1 activity and lipogenic/lipolytic balance in F344 rat adipose tissue. *J. Lipid Res*. 2008; 49:12–23. [PubMed: 17909224]
100. Perrone CE, Mattocks DA, Jarvis-Morar M, Plummer JD, Orentreich N. Methionine restriction effects on mitochondrial biogenesis and aerobic capacity in white adipose tissue, liver, and skeletal muscle of F344 rats. *Metabolism*. 2009; 59:1000–1011. [PubMed: 20045141]
101. Malloy VL, Krajcik RA, Bailey SJ, Hristopoulos G, Plummer JD, Orentreich N. Methionine restriction decreases visceral fat mass and preserves insulin action in aging male Fischer 344 rats independent of energy restriction. *Aging Cell*. 2006; 5:305–314. [PubMed: 16800846]
102. Kim HJ, Jung KJ, Yu BP, Cho CG, Choi JS, Chung HY. Modulation of redox-sensitive transcription factors by calorie restriction during aging. *Mech. Ageing Dev*. 2002; 123:1589–1595. [PubMed: 12470896]
103. Chiang SH, Bazuine M, Lumeng CN, Geletka LM, Mowers J, White NM, Ma JT, Zhou J, Qi N, Westcott D, Delproposto JB, Blackwell TS, Yull FE, Saltiel AR. The protein kinase IKK ϵ regulates energy balance in obese mice. *Cell*. 2009; 138:961–975. [PubMed: 19737522]
104. Reilly SM, Chiang SH, Decker SJ, Chang L, Uhm M, Larsen MJ, Rubin JR, Mowers J, White NM, Hochberg I, Downes M, Yu RT, Liddle C, Evans RM, Oh D, Li P, Olefsky JM, Saltiel AR. An inhibitor of the protein kinases TBK1 and IKK- ϵ improves obesity-related metabolic dysfunctions in mice. *Nat. Med*. 2013; 19:313–321. [PubMed: 23396211]
105. Higami Y, Barger JL, Page GP, Allison DB, Smith SR, Prolla TA, Weindruch R. Energy restriction lowers the expression of genes linked to inflammation, the cytoskeleton, the extracellular matrix, and angiogenesis in mouse adipose tissue. *J Nutr*. 2006; 136:343–352. [PubMed: 16424110]

Figure 1.

Differential regulation of gene expression by dietary methionine restriction (MR) or calorie restriction for 20 mo in liver and inguinal WAT as assessed by microarray. **A** – Venn diagram showing numbers of genes that are regulated by both diets or uniquely regulated by each diet in inguinal white adipose tissue. **B** – Venn diagram showing numbers of genes that are regulated by both diets or uniquely regulated by each diet in liver.

Figure 2. Differential regulation of inflammation pathways by dietary methionine restriction (MR, blue bars) or calorie restriction (CR, red bars) for 20 mo in liver and inguinal WAT as

assessed by microarray. **A** – Inflammation pathway enrichment analysis conducted using Ingenuity to identify pathways affected by MR and CR, MR alone or CR alone in inguinal white adipose tissue. The key to the inflammation pathways is as follows: 1 - inflammatory response, 2 - cell movement of phagocytes, 3 - quantity of phagocytes, 4 - activation of leukocytes, 5 - migration of phagocytes, 6 - accumulation of leukocytes, 7 - accumulation of myeloid cells, 8 - cell-mediated response, 9 - inflammation of organ, 10 - cell movement of neutrophils, 11 - cell movement of macrophages, 12 - response of phagocytes, 13 - recruitment of phagocytes, 14 - quantity of neutrophils, 15 - recruitment of neutrophils, 16 - chemotaxis of leukocytes, 17 - chemotaxis of phagocytes, 18 - chemotaxis of antigen presenting cells, 19 - adhesion of phagocytes, 20 - activation of phagocytes, 21 - immune response of cells, 22 - activation of lymphocytes, 23 - activation of mononuclear leukocytes, 24 - inflammation of large intestine, 25 - infiltration by neutrophils, 26 - quantity of macrophages, 27 – colitis, 28 - inflammation of intestine, 29 - accumulation of granulocytes, 30 - immune response of phagocytes, 31 - activation of T lymphocytes, 32 - degranulation of cells, 33 - accumulation of mononuclear leukocytes, 34 - antibody response, 35 - activation of antigen presenting cells, 36 - accumulation of phagocytes, 37 - activation of myeloid cells, 38 - binding of phagocytes, 39 - inflammation of respiratory system component, 40 - transmigration of phagocytes, 41 - phagocytosis of cells, 42 - morphology of phagocytes, 43 - chemotaxis of myeloid cells, 44 - inflammation of lung, 45 - degranulation of phagocytes, 46 - migration of dendritic cells, 47 - inflammation of liver, 48 - TH1 immune response, 49 – phagocytosis, 50 - chemotaxis of macrophages, 51 - adhesion of mast cells, 52 - adhesion of neutrophils, 53 - accumulation of lymphocytes, 54 - aggregation of blood platelets, 55 - infiltration by macrophages, 56 - migration of bone marrow-derived dendritic cells, 57 - chemotaxis of dendritic cells, 58 - cell movement of monocytes, 59 - accumulation of eosinophils. **B** – Inflammation pathway enrichment analysis conducted using Ingenuity to identify pathways affected by MR and CR, MR alone or CR alone in liver. Significance of pathway enrichment is determined by Fisher's exact test and represented as the negative logarithm of the p-value (Negative logP).

Figure 3. Heat maps showing differential regulation of inflammation pathways by dietary methionine restriction (MR) or calorie restriction for 20 mo in liver and inguinal WAT as assessed by microarray. **A** – Heat map of inflammation pathways that are differentially regulated by both CR and MR, CR but not MR, and MR but not CR in inguinal white adipose tissue. **B** – Heat map of inflammation pathways that are differentially regulated by both CR and MR, CR but not MR, and MR but not CR in liver. Downregulated genes are shown in blue and upregulated genes are shown in red, with deeper shades representing higher degree of differential expression.

Inflammatory gene mRNA expression changes among tissues when dietary methionine restriction (MR) or 40% calorie restriction (CR) was initiated at weaning and measured after 20 mo on the respective diets.¹

Table 1

Gene Symbol	RPWAT ²		IWAT		EWAT		LIVER	
	MR	CR	MR	CR	MR	CR	MR	CR
CCL2	-2.9	-2.6	-1.8	-1.8	2.0	1.8	-2.2	1.9
IL-1β	-3.9	-1.8	1.2	1.1	2.9	2.8	-1.9	-1.7
TNF	-2.6	-1.3	-1.1	1.3	-1.0	1.7	-2.6	1.0
IL-6	-4.7	-4.0	1.7	1.8	3.1	3.6	-1.9	8.1
IFN-γ	-4.7	6.6	-1.1	2.3	-1.1	1.3	-4.0	-1.7
EMR1	-3.3	-2.6	-1.7	-2.3	-1.4	-1.4	-1.8	-1.5
ITGAM	-5.8	-1.6	-1.8	-1.6	-2.0	-1.6	-3.4	-1.6
ITGAX	-9.3	-2.4	-2.1	-2.5	-8.7	-3.3	-4.2	-3.0

¹ Rats were provided the Control diet, MR diet, or the Control diet at 40% *ad libitum* intake and euthanized after 20 mo on the respective diets.

² Retroperitoneal (RPWAT), inguinal (IWAT), and epididymal (EWAT) white adipose tissue and liver were harvested and mRNA levels of each gene in each tissue were measured by quantitative real time PCR. Expression levels of each gene in each tissue were compared using analysis of variance and Bonferroni corrections and fold changes in expression were calculated as the ratio of mean expression levels in the MR or CR groups to the Control group. Mean fold-changes denoted with blue or red shading differ from controls at P<0.05 and are representative of 8 rats per diet.

Table 2

Inflammatory gene mRNA expression changes among tissues after 3, 9, or 20 mo of dietary methionine restriction (MR)¹

Gene Symbol	RPWAT ²						IWAT						EWAT						LIVER					
	3	9	20	3	9	20	3	9	20	3	9	20	3	9	20	3	9	20	3	9	20	3	9	20
CCL2	-2.0	-1.3	-2.9	-2.1	-1.7	-1.8	-2.4	-2.9	2.0	-1.6	-1.8	-2.2	-2.4	-2.9	2.0	-1.6	-1.8	-2.2	-2.4	-2.9	2.0	-1.6	-1.8	-2.2
IL-1 β	-1.6	-3.5	-3.9	-2.7	2.3	1.2	-2.1	-1.5	2.9	-1.6	-1.4	-1.9	-2.1	-1.5	2.9	-1.6	-1.4	-1.9	-2.1	-1.5	2.9	-1.6	-1.4	-1.9
TNF	-1.4	-1.9	-2.6	-1.9	2.2	-1.1	-1.7	-1.5	-1.0	-1.4	-1.1	-2.6	-1.7	-1.5	-1.0	-1.4	-1.1	-2.6	-1.7	-1.5	-1.0	-1.4	-1.1	-2.6
IL-6	-2.1	-3.6	-4.7	-3.9	2.9	1.7	-1.5	-3.5	3.1	-2.6	-2.2	-1.9	-1.5	-3.5	3.1	-2.6	-2.2	-1.9	-1.5	-3.5	3.1	-2.6	-2.2	-1.9
IFN- γ	-2.3	-2.2	-4.7	-3.8	3.2	-1.1	-2.0	-2.0	-1.1	-2.2	-3.1	-4.0	-2.0	-2.0	-1.1	-2.2	-3.1	-4.0	-2.0	-2.0	-1.1	-2.2	-3.1	-4.0
EMR1	1.0	-1.4	-3.3	-1.9	-1.1	-1.7	-1.5	-1.2	-1.4	-1.1	-1.1	-1.8	-1.5	-1.2	-1.4	-1.1	-1.1	-1.8	-1.5	-1.2	-1.4	-1.1	-1.1	-1.8
ITGAM	-1.1	-1.9	-5.8	-2.2	-1.2	-1.8	-1.5	-1.4	-2.0	-1.7	-2.3	-3.4	-1.5	-1.4	-2.0	-1.7	-2.3	-3.4	-1.5	-1.4	-2.0	-1.7	-2.3	-3.4
ITGAX	-3.6	-4.1	-9.3	-2.2	-4.3	-2.1	-3.1	-4.0	-8.7	1.0	-1.9	-4.2	-3.1	-4.0	-8.7	1.0	-1.9	-4.2	-3.1	-4.0	-8.7	1.0	-1.9	-4.2

¹ Rats were provided a Control or MR diet after weaning and euthanized after 3, 9, or 20 mo on the respective diets.

² Retroperitoneal (RPWAT), inguinal (IWAT), and epididymal (EWAT) white adipose tissue and liver were harvested and mRNA levels of each gene in each tissue at each time point were measured by quantitative real time PCR. Expression levels of each gene in each tissue were compared using analysis of variance and Bonferroni corrections and fold changes in expression were calculated as the ratio of mean expression levels in the MR group to the Control group. Mean fold-changes denoted with blue shading differ from controls at P<0.05 and are representative of 8 rats per diet per time point.

Table 3

Inflammatory gene mRNA expression changes among tissues when dietary methionine restriction (MR) was formulated into a high fat diet, initiated at weaning in Osborne-Mendel rats and measured 8 mo thereafter.

Gene Symbol	RPWAT ^I	EWAT	IWAT	LIVER
CCL2	-1.1	-2.4	1.0	-7.0
IL-1 β	-1.2	-5.1	-1.1	-2.7
TNF	1.1	1.0	1.4	-3.3
IL-6	-5.0	-4.8	1.5	-4.0
IFN- γ	-1.9	1.2	1.2	-1.2
EMR1	-1.4	-2.1	1.0	-2.5
ITGAM	2.6	-1.1	1.5	-3.3
ITGAX	6.0	2.1	2.8	-5.0

^I Retroperitoneal (RPWAT), inguinal (IWAT), and epididymal (EWAT) white adipose tissue and liver were harvested and mRNA levels of each gene in each tissue were measured by quantitative real time PCR. Expression levels of each gene in each tissue were compared using analysis of variance and Bonferroni corrections and fold changes in expression were calculated as the ratio of mean expression levels in the MR group to the Control group.. Mean fold-changes denoted with blue shading differ from controls at P<0.05 and are representative of 8 rats per diet.