Obesity is closely linked to a wide array of pathophysiological conditions, including insulin resistance. The adipose tissue is an important endocrine organ that produces and secretes biologically active molecules, collectively known as adipokines. Obesity is accompanied by a chronic, low-grade inflammation, characterized by a dysregulated production of proinflammatory adipokines, such as tumor necrosis factor-α, interleukin-6, and plasminogen activator inhibitor-1 (PAI-1), and anti-inflammatory adipokines, such as adiponectin. It has been proposed that this dysregulation is a key feature in the pathogenesis of obesity-related insulin resistance. However, mechanisms by which excessive fat accumulation leads to a dysregulation of adipokines have not yet been elucidated.

The receptor for advanced glycation end products (RAGE) is a pattern-recognition receptor and is a multiligand molecule expressed on different cell types, such as adipocytes,
endothelial cells, and macrophages. RAGE is initially identified as the receptor for advanced glycation end products (AGEs), but, in addition to AGEs, RAGE also interacts with multiple members of the proinflammatory S100/calgranulin family and high mobility group box 1 protein. Binding of these ligands to RAGE leads to activation of signaling cascade and induction of nuclear factor-κB, which can subsequently lead to the production of inflammatory mediators. Therefore, the potential role of RAGE in the regulation of inflammation suggests that RAGE might be an important mechanism contributing to obesity-associated dysregulation of adipokines and development of insulin resistance. Nε-(carboxymethyl)lysine (CML) is a major AGE and is an important ligand for RAGE. CML is formed on proteins by nonenzymatic glycation and oxidation reactions. Alternative routes for CML formation have been described, including lipid peroxidation of polyunsaturated fatty acids. In fact, lipid peroxidation is a more important source for CML formation than glycoxidation reactions. Because of the reaction mechanism, CML formation is increased under hyperglycemic and hyperlipidemic conditions. The adipose tissue in obese conditions is characterized by increased levels of fatty acids, lipid peroxidation, and oxidative stress. Therefore, we can deduce that obesity is also a condition in which CML formation is increased under hyperglycemic conditions. Hence, the aim of this study was to investigate the role of CML–RAGE axis in obesity-related inflammation and insulin resistance.

The aim of this study was to investigate the role of CML–RAGE axis in obesity-related inflammation and insulin resistance. In the present study, we showed in humans, in an in vitro model of human adipocytes, and in mice lacking RAGE on a Leprdb−/− background, the importance of the CML–RAGE axis in the development of obesity-related inflammation and insulin resistance.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

CML Accumulation and RAGE Expression Are Increased in Human Adipose Tissue

To evaluate whether CML accumulates in human adipose tissue, we performed immunohistochemistry on biopsies of subcutaneous adipose tissue (SAT) of lean and obese subjects. Main characteristics of the lean (n=9) and obese subjects (n=10) are presented in the Table. Immunohistochemical staining for CML showed that CML was only slightly detectable in SAT of lean subjects, whereas CML was abundantly present in SAT of obese subjects (Figure 1A). Semiquantitative analysis of the CML staining demonstrated a significantly higher CML intensity in SAT of obese subjects when compared with that of lean subjects (Figure 1B). RAGE expression was also slightly higher in obese SAT versus lean SAT although not significant (P=0.189; Figure 1C). However, Western blot analysis demonstrated that RAGE protein levels were significantly higher in SAT of obese subjects versus lean controls (Figure 1D).

To analyze CML localization in adipose tissue and to study CML accumulation in different fat depots further, we also conducted immunohistochemical stainings of SAT and visceral adipose tissue (VAT) obtained from severely obese subjects (n=44; body mass index, >40 kg/m²). Main characteristics of the severely obese subjects are presented in Table I in the online-only Data Supplement. We demonstrated that CML-modified proteins were particularly evident in adipocytes, CD68-positive macrophages, and CD31-positive endothelial cells (Figure IE). In addition, RAGE showed a similar localization as CML (Figure IE). No differences in CML and RAGE localization were observed between SAT and VAT. However, a significant higher CML accumulation (Figure IF) was detected in VAT when compared with SAT of severely obese subjects. In addition, RAGE gene expression levels and RAGE protein levels detected by Western blotting (Figure IG and IH) were significantly higher in VAT when compared with SAT of severely obese subjects.

These data demonstrate that obesity is associated with an accumulation of CML and an increased RAGE gene expression and RAGE protein levels in adipose tissue, with higher CML accumulation and RAGE in VAT than in SAT.

CML Levels Are Increased During Adipocyte Lipid Accumulation and Induce Inflammation

To investigate the role of the CML–RAGE axis in adipocyte lipid accumulation and induce inflammation, we performed an in vitro experiment with human Simpson–Golabi–Behmel syndrome (SGBS) preadipocytes and differentiated SGBS adipocytes as a model for adipogenesis. SGBS preadipocytes were differentiated to mature SGBS adipocytes, which resulted in marked lipid accumulation as detected by Oil Red O staining (Figure 2A). During the course of differentiation, CML levels were significantly increased. In addition, RAGE gene expression and protein levels also showed a significant increase during differentiation of preadipocytes to adipocytes (Figure 2A).

Table. General Characteristics of Lean and Obese Subjects

<table>
<thead>
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<tr>
<td>n</td>
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<td>10</td>
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<tr>
<td>Age, y</td>
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<tr>
<td>BMI, kg/m²</td>
<td>23.4±1.1</td>
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<td>Fasting glucose, mmol/L</td>
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<td>5.9±0.5†</td>
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<tr>
<td>Fasting insulin, mmol/L</td>
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<td>GIR, μmol×kg body weight−1×min−1</td>
<td>37.1±7.9</td>
<td>14.3±7.9*</td>
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</table>

Data are presented as mean±SD, or as percentage. BMI indicates body mass index; and GIR, glucose infusion rate (measure of insulin sensitivity). *P<0.001, †P<0.01.
We next investigated whether the increase of the CML–RAGE axis during adipogenesis is associated with an altered inflammatory profile in preadipocytes and adipocytes. The effect of CML on adipocyte inflammation was examined by incubating SGBS preadipocytes and adipocytes with different modifications of CML-albumin (ie, control-albumin and minimally and highly modified CML-albumin). Increasing grades of CML modification of albumin were determined by UPLC-Tandem MS (ultra performance liquid chromatography-tandem mass spectrometry) and Western blotting (data not demonstrated). Long-term incubation of SGBS preadipocytes with different CML-albumin (for 72 hours) demonstrated a significant increase of RAGE, interleukin-6, and PAI-1 gene expression, whereas adiponectin expression significantly decreased. Incubation of SGBS preadipocytes during short incubation periods had no effect on RAGE, interleukin-6, PAI-1, and adiponectin gene expression (Figure I in the online-only Data Supplement). Interestingly, in SGBS adipocytes, CML increased the RAGE, interleukin-6, and PAI-1 gene expression after a short stimulation with CML, whereas adiponectin expression decreased (Figure 2B). In both SGBS preadipocytes and adipocytes, highly modified CML-albumin had a greater effect on expression levels of inflammatory markers than minimally modified CML-albumin, demonstrating a dose-dependent effect of CML.
Figure 2. (Continued)
To confirm the role of RAGE in the CML-mediated expression of inflammatory markers, preadipocytes and adipocytes were preincubated with anti-RAGE antibody or soluble RAGE (sRAGE). Inhibition of RAGE by anti-RAGE antibody and sRAGE led to the normalization of CML-induced RAGE, PAI-1, interleukin-6, and adiponectin gene expression in SGBS adipocytes (Figure 2C) and preadipocytes (Figure II in the online-only Data Supplement).

Also, the incubation of SGBS preadipocytes and adipocytes with S100 protein, which is another important ligand for RAGE, showed upregulation of RAGE, PAI-1, and interleukin-6 genes and downregulation of adiponectin gene expression (Figure 2D; Figure III in the online-only Data Supplement).

These experiments, therefore, demonstrate in an in vitro model of excessive lipid accumulation that adipogenesis is associated with increased levels of CML-modified proteins, increased RAGE expression, and activation of the CML–RAGE axis leading to changes in expression of adipokines, thus indicating that RAGE plays a central role in dysregulation of adipokines associated with obesity.

Obese, RAGE-Deficient Mice Are Associated With Improved Inflammation, Improved Insulin Resistance, and Increased Plasma CML Levels

To define the role of RAGE in inflammation in obesity in vivo further, we developed obese, RAGE-deficient (RAGE−/−Leptin−/−) and obese, wild-type (RAGE+/−Leptin−/−) mice. In the Leptin−/− background, no effect of RAGE on body weight was observed (39±5 and 35±5 g, respectively) at 9 weeks. RAGE−/−Leptin−/− mice exhibited an improved inflammatory profile when compared with RAGE+/−Leptin−/− mice. RAGE deficiency was associated with significant decreased levels of proinflammatory cytokines interleukin-1β, interleukin-12p70, interferon-γ, and tumor necrosis factor-α (P<0.05). Proinflammatory cytokine, interleukin-6 and mKC (mouse keratinocyte-derived chemokine [the mouse analog of human interleukin-8]), levels were also lower in RAGE−/−Leptin−/− mice but were not statistically significant (P=0.131 and 0.200, respectively). The anti-inflammatory cytokines, interleukin-10 and adiponectin (P=0.337 and 0.103), tend to be higher in RAGE−/−Leptin−/− mice when compared with those in RAGE+/−Leptin−/− mice (Figure 3A). In addition, we also calculated an overall inflammation score of all individual inflammatory markers (interleukin-1β, interleukin-12p70, interferon-γ, tumor necrosis factor-α, interleukin-6, mKC [interleukin-8], adiponectin, and interleukin-10) by averaging the z scores of each of the respective markers (z score of an inflammatory marker=[individual’s observed value−mean]/SD). This combined overall inflammation score was significantly lower in the RAGE−/−Leptin−/− mice when compared with that in RAGE+/−Leptin−/− mice (−0.6±0.2 versus 0.7±0.2; P<0.01). Moreover, RAGE deficiency in obese mice led to normalization of the inflammatory markers because the levels of inflammatory markers were comparable with those of RAGE+/−/Leptin−/− mice (Figure 3A). The same pattern of inflammatory cytokines emerged in RAGE deficiency mice when compared with wild-type mice (Figure IV in the online-only Data Supplement).

In addition, RAGE−/−Leptin−/− mice were also associated with altered metabolic phenotype. Glucose tolerance test demonstrated significant differences in response to glucose load between RAGE+/−/Leptin−/− and RAGE−/−/Leptin−/− mice (Figure 3B). RAGE−/−/Leptin−/− mice had an improved glucose metabolism when compared with RAGE+/−/Leptin−/− mice (Figure 3B). After insulin injection, glucose did not significantly decrease in RAGE+/−/Leptin−/− mice (from 24.09±1.32 mmol/L at time 0 minutes to 28.38±0.99 mmol/L at time 120 minutes). In contrast, insulin injection declined blood glucose by 30% in RAGE−/−/Leptin−/− mice (from 26.40±2.31 mmol/L at time 0 minutes to 18.81±2.64 mmol/L at time 90 minutes; Figure 3C). These results, therefore, demonstrate that RAGE deficiency is associated with a general increase in glucose tolerance and insulin sensitivity.

Circulating CML Is Trapped in Adipose Tissue of RAGE/Leptin−/− Mice

Of interest, CML plasma levels were significantly higher in RAGE+/−/Leptin−/− mice when compared with RAGE+/−/Leptin−/− mice (923±144 nmol/L versus 684±78 nmol/L, P<0.05), and in RAGE−/− mice compared with wild-type mice (Figure IV in the online-only Data Supplement), indicating that RAGE also represents a mechanism for the regulation of CML plasma levels.

A possible mechanism whereby RAGE regulates plasma CML levels is a selective uptake of circulating CML in specific tissues or organs. We first investigated the uptake of circulating CML in obesity using Leptin−/− mice. We injected Leptin−/− mice with fluorescently labeled CML-albumin (green fluorescent signal) and visualized the distribution and accumulation of the injected CML-albumin. As a control, we used a fluorescently labeled control-albumin (red fluorescent signal), which was simultaneously injected with CML-albumin in Leptin−/− mice. After injection, plasma clearance of fluorescently labeled CML-albumin was significantly faster than that of control-albumin (t1/2=59±5 minutes...
versus 139±13 minutes; Figure 4A). CML-albumin and control-albumin were distributed via the circulation and were visualized in the liver and kidney (Figure 4B). Importantly, both CML-albumin and control-albumin were colocalized and were restricted to the circulation of these tissues. However, a strong accumulation of CML-albumin was observed in adipose tissue of LeptrDb–/– mice, whereas control-albumin was not detectable in the adipose tissue (Figure 4B). These experiments, therefore, showed that adipose tissue itself is able to trap CML from the circulation actively and selectively, thereby contributing to local CML accumulation in adipose tissue in obesity and decreased plasma CML levels.

To study the localization of the injected CML-albumin in adipose tissue in detail, we injected only green fluorescently labeled CML-albumin in LeptrDb–/– mice and stained the adipose tissue after isolation for CD31 to visualize the endothelial cells (red fluorescently labeled anti-CD31 antibody). Adipocytes showed blue autofluorescence. Fluorescently labeled CML-albumin was present at the site of administration, namely the vasculature of the adipose tissue, but more importantly, CML-albumin was also detected in the cytosol and at the membrane of adipocytes (Figure 4C).

To investigate the role of RAGE in the trapping of CML, we repeated this CML trapping experiment in our unique mouse model (ie, RAGE–/–/LeptrDb–/– and RAGE+/+/LeptrDb–/– mice). RAGE–/–/LeptrDb–/– and RAGE+/+/LeptrDb–/– mice were injected with fluorescently labeled CML-albumin (red fluorescently signal). A significant accumulation of CML-albumin was observed in adipose tissue of RAGE+/+/LeptrDb–/– mice, whereas the fluorescently labeled CML-albumin was completely absent in adipose tissue of RAGE–/–/LeptrDb–/– mice (Figure 4D). This indicates that circulating CML is trapped in the adipose tissue via a RAGE-dependent mechanism. From that observation, we can deduce that RAGE-mediated trapping of CML in adipose tissue contributes to the local accumulation of CML in adipose tissue in RAGE+/+/LeptrDb–/– mice, which is associated with a decrease of plasma CML levels.

**CML Plasma Levels Are Decreased in Obesity in Humans**

To investigate whether obesity is associated with decreased plasma CML in humans, CML plasma levels were measured in
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lean and obese subjects. As demonstrated in Figure 5A, plasma protein–bound CML concentrations were significantly lower in obese when compared with those in lean subjects. Additional analyses demonstrated strong inverse correlations between protein-bound CML plasma concentrations and body mass index (Figure 5B). These data indicate that the CML accumulation observed in obese adipose tissue is accompanied by lower circulating protein–bound CML levels, suggesting that a CML trapping mechanism is operating in humans. This decrease of plasma protein–bound CML levels in obesity and the correlation with body mass index were confirmed in severely obese subjects (Figure V in the online-only Data Supplement).

We also evaluated the effect of weight loss on plasma CML levels. Plasma samples from severely obese subjects undergoing bariatric surgery were taken before and 6 months after surgery. During this period, they lost 16±9 kg on average. Their mean body mass index was 41.7±3.1 kg/m² before surgery and 36.3±4.4 kg/m² after 6 months. As demonstrated in Figure 5C, weight loss by bariatric surgery was associated with an increase of CML plasma levels.

CML Plasma Levels Are Associated With Insulin Resistance in Humans

The association of decreased plasma levels of CML in obesity with insulin resistance, as determined by a hyperinsulinemic-euglycemic clamp, was investigated in obese subjects (n=10) and lean controls (n=9). Obese subjects had a significantly lower insulin sensitivity when compared with lean subjects (glucose infusion rate, 16.1±9.6 versus 37.4±8.3 μmol×kg body weight⁻¹×minutes⁻¹; P<0.001). A strong correlation was found between CML plasma concentrations and insulin sensitivity (r=0.669; P<0.01; Figure 5D), indicating that low CML plasma levels in obesity are associated with high insulin resistance.

Discussion

Here we show, for the first time, a role of the CML–RAGE axis in obesity-associated dysregulation of adipokines and in the development of obesity-related insulin resistance. First, we demonstrated in humans and in an in vitro model of adipogenesis that obesity is associated with increased CML accumulation and RAGE expression. Second, we found that the activation of this CML–RAGE axis resulted in a dysregulated expression of pro- and anti-inflammatory cytokines. We found that the RAGE–/–/LeptrDb–/– mice were characterized by improved inflammatory profile and improved insulin sensitivity. Third, we also found a RAGE-mediated trapping of CML in adipose tissue in RAGE+/+LeptrDb–/– mice. In line with this concept, we also found decreased CML plasma levels in obese subjects, and this decrease in CML was associated with decreased insulin sensitivity in obese subjects. Taken together, this study demonstrates a novel function of RAGE in regulating CML levels in obesity, and that the activation of the CML–RAGE axis plays a major role in the dysregulation of adipokines and the development of obesity-associated insulin resistance.

Although CML has been regarded as a traditional AGE formed from glucose, increasing numbers of reports have emphasized that CML is mainly formed from lipid peroxidation reactions and can, therefore, be considered as an advanced lipoxidation end product.14,18 The increased oxidation of fatty

Figure 4. Trapping of Nε-(carboxymethyl)lysine (CML) in adipose tissue is mediated by receptor for advanced glycation end product (RAGE). A, Clearance of CML-albumin from circulation was significantly faster than the clearance from control-albumin. *P<0.001. B, Trapping of CML-albumin (green fluorescent signal) and control-albumin (red fluorescent signal) in adipose tissue, liver, and kidney was studied in LeptrDb–/– mice using 2-photon microscopy. This demonstrated that only CML-albumin could be detected in adipose tissue, whereas in liver and kidney both CML-albumin and control-albumin were present. C, (i) High vascularization of the adipose tissue (adipocytes showed blue autofluorescence) was observed after staining the adipose tissue with an antibody against endothelial marker CD31 (red fluorescence); (ii and iii) overlay images showed that CML-albumin (green fluorescent signal) is found at site of administration (ie, in the circulation), but more importantly CML-albumin was found in the cytoplasm and at the membrane of adipocytes (see arrows). D, Fluorescently labeled CML-albumin (red fluorescent signal) was detected in adipocytes of RAGE+/+LeptrDb–/– mice, whereas no CML-albumin was trapped in RAGE–/–LeptrDb–/– mice (see arrows).
Acids and formation of lipid peroxidation products in obese conditions prompted us to hypothesize that CML formation is increased in obesity. Our in vitro data confirmed that lipid accumulation during differentiation of adipocytes was associated with increased endogenous CML formation. In addition, we demonstrated in human adipose tissue that CML accumulation is higher in adipose tissue of obese subjects when compared with that in adipose tissue of lean subjects. In VAT, we found more CML accumulation than in SAT of obese subjects. This study is the first study investigating CML accumulation in human adipose tissue. Other studies have already demonstrated that development of fatty livers was associated with increased formation of CML in lipid-laden hepatocytes. In addition, CML accumulation was observed in the muscle tissue of obese subjects, which was correlated with weight gain. These data and our study reveal that obesity increases levels of CML, probably through increased lipid peroxidation. However, we cannot fully rule out potential roles of other pathways in the formation of CML. Previous work by others suggested potential roles for the myeloperoxidase family of enzymes in generation of CML.

CML exerts biological effects via altered gene expression mediated by RAGE. CML-activation of RAGE triggers multiple signaling cascades, resulting in activation and translocation of nuclear transcription factors (nuclear factor-κB) and transcription of target genes, including inflammatory cytokines. It has been demonstrated that a positive autoregulatory loop exists on RAGE activation by CML, which in turn induces RAGE expression and subsequent RAGE-mediated perpetuated nuclear factor-κB activation. This indicates that ligation of RAGE results in a constantly growing and renewable pool of RAGE, thereby amplifying the inflammatory response. Therefore, we can conceive that the CML-rich environment seen in the obese adipose tissue may be accompanied by the upregulation of RAGE. In our study, we indeed observed that RAGE expression accompanies CML accumulation and is also upregulated during differentiation of adipocytes. Moreover, RAGE gene expression was higher in adipose tissue of obese subjects when compared with that in adipose tissue of lean subjects and was higher in VAT when compared with SAT of severely obese subjects. The simultaneous increase of CML and RAGE in obesity and the colocalization of RAGE and CML in the human adipose tissue support a key role for CML–RAGE axis in obesity-associated inflammation.

Only limited reports have investigated the role of AGEs in adipocyte inflammation, and no study to date has investigated the role of CML-RAGE in human adipocyte inflammation. Unno et al already showed that glycolaldehyde-modified albumin increased the expression of leptin in mouse adipocytes. Moreover, PAI-1 expression in rat adipocytes was upregulated on incubation with nondefined AGEs. Our data reveal that the incubation of preadipocytes and adipocytes with CML increased the expression of inflammatory markers, RAGE, PAI-1, and interleukin-6, whereas the expression of adiponectin was decreased on CML incubation. In both preadipocytes and adipocytes, we demonstrated that CML has functional consequences at the level of adipokine dysregulation. However, in adipocytes, a rapid CML-mediated upregulation of RAGE, PAI-1, interleukin-6, and downregulation of adiponectin was observed, whereas a delayed effect of CML on expression of inflammatory markers was detected in preadipocytes. Differences in basal RAGE gene expression between preadipocytes and adipocytes may underlie this effect. In preadipocytes and adipocytes, we showed that strategies to inhibit CML-RAGE interaction or to decoy CML, via administration of anti-RAGE antibody or soluble RAGE, respectively, reduced the CML-mediated inflammatory responses. Our current findings are the first to demonstrate a direct role of RAGE in human adipocyte inflammation in vitro. In addition, our data delineate that CML as ligand for RAGE plays fundamental role in RAGE-mediated adipocyte inflammation.

![Figure 5](http://atvb.ahajournals.org/)

Figure 5. Nε-(Carboxymethyl)lysine (CML) plasma levels are decreased in obesity and associated with insulin sensitivity. A, CML plasma levels were significantly lower in obese subjects when compared with those in lean controls. B, CML plasma levels were inversely related with body mass index (BMI). Correlation coefficient is given for this relationship. C, Weight loss by bariatric surgery in severely obese subjects is associated with increased CML plasma levels. D, CML plasma levels were associated with glucose infusion rate, which is a measurement for insulin sensitivity.
We further extended these in vitro observations to in vivo ones using a murine model of RAGE deficiency, obesity, and type 2 diabetes mellitus, RAGE+/+/Leptin–/– mice. Deletion of RAGE was protective against inflammation and was associated with greater insulin sensitivity in insulin tolerance test and improved glucose metabolism in glucose tolerance test. RAGE–/– animals had slightly higher body weights than their littermate RAGE+/+ control mice. Because dosing for glucose tolerance test and insulin tolerance test was based on total body weight, we cannot exclude this difference in body weight and hence difference in dosing of glucose and insulin influenced the outcome of the metabolic tests. However, if anything, this would lead to an underestimation of the improved glucose tolerance in RAGE–/– mice. Previous research has also demonstrated that RAGE deficiency significantly decreased the expression of pro-inflammatory mediators and lower levels of oxidative stress, whereas adiponectin levels and antioxidative defense mechanisms were higher in RAGE-deficient mice.24–28 In addition, the favorable effect of RAGE deficiency on insulin sensitivity and glucose metabolism was also demonstrated in RAGE knockout mice fed a high-fat diet.7,26 RAGE deficiency was also associated with higher glucose transporter-4 expression in adipose tissue in these mice.26 In cultured adipocytes, nondefined AGEs impair insulin signaling by increasing generation of intracellular reactive oxygen species and activation of inflammatory pathways.29,30 Ueno et al25 recently demonstrated attenuated insulin-stimulated glucose uptake in RAGE-overexpressing 3T3-L1 adipocytes. Therefore, the RAGE system represents an important risk factor for the development of obesity-associated inflammation and insulin resistance. Recent reports suggest that RAGE itself could be involved in the progression of obesity. In ApoE/RAGE double knockout mice, it was demonstrated that RAGE regulated adiposity. Monden et al28 demonstrated that an increase in body weight induced by high-fat diet is suppressed in RAGE knockout mice. In contrast to these studies, we did not observe an effect of RAGE on adiposity in the Leptin–/– background. We could speculate that the effect of RAGE on body weight does not outweigh the effect of severe obesity caused by the genetic mutation in the receptor for leptin. More research is needed to clarify these conflicting results and to reveal the underlying mechanism.

In the present study, we were intrigued by the finding that CML plasma levels were increased in obese, RAGE-deficient mice when compared with RAGE+/+Leptin–/– mice, whereas other AGEs, such as Nε-(carboxyethyl)lysine, were not affected. This finding, together with the strong colocalization of CML and RAGE in human adipocytes and the in vitro CML-RAGE-mediated adipokine dysregulation, indicates that CML is the major ligand for RAGE-mediated inflammation and insulin resistances, and that RAGE represents a mechanism for regulating CML plasma levels. CML plasma levels were unchanged in mice deficient for other AGE receptors, such as galectin-3, scavenger receptor A, and CD36 (data not shown), indicating that these scavenger receptors do not influence circulating CML levels. We demonstrated that injected fluorescently labeled CML was preferentially taken up in adipose tissue in mice expressing RAGE when compared with RAGE-deficient mice. These data, therefore, demonstrated for the first time a RAGE-mediated trapping of CML in adipose tissue and provided a mechanistic explanation for the improved grade of low-grade inflammation and insulin sensitivity observed in the obese RAGE-deficient mice.

Because we observed that obesity is associated with CML accumulation and increased RAGE expression in human adipose tissue, this might indicate that the increased expression of RAGE may lead to increased trapping of CML in adipose tissue, thereby contributing to the accumulation of CML in adipose tissue and to lower CML plasma levels. In our obese population, we indeed demonstrated significantly lower CML plasma levels in obese subjects when compared with those in lean subjects. Previous studies by Sebeková et al31 demonstrated decreased CML plasma levels in obesity. In obese adolescents, lower levels of plasma CML were found when compared with their lean counterparts. These authors recently confirmed their data in a large cohort of obese and normal weight controls (n=437), and, in addition, they demonstrated that CML plasma levels showed a decreasing trend with rising numbers of risk factors for the metabolic syndrome.32 CML plasma levels were also inversely related to fat mass, indicating that obesity represents a main determinant for the decline of CML plasma levels.33 Nevertheless, these epidemiological studies do not encounter the underlying mechanism. Our present data provide a mechanistic explanation for these findings. In our study, we observed increased CML-RAGE accumulation in obese subjects when compared with lean subjects, and moreover, CML-RAGE accumulation was higher in VAT when compared with SAT of obese subjects. From these observations, we may deduce that in obese subjects and in VAT, there is more RAGE-mediated CML trapping present that explains the decrease of circulating CML levels.

Our human study showed, in addition to the CML accumulation in adipocytes, a strong CML staining in macrophages of obese adipose tissue. As the adipose tissue is infiltrated by inflammatory macrophages during obesity, CML accumulation in these macrophages and macrophage activation could also play an important role in adipose tissue inflammation. It has already been demonstrated that nondefined AGEs stimulate the production of tissue factor in cultured human monocytes and tumor necrosis factor-α production in rat macrophages. Therefore, the role of RAGE-CML in macrophage activation, inflammation, and insulin resistance in obesity should be addressed in future studies.

Taken together, our study provides, for the first time, evidence of a novel function of RAGE in trapping of CML in adipose tissue in obesity. This RAGE-mediated CML accumulation is an important mechanism involved in the dysregulation of adipokines in obesity, thereby contributing to the development of obesity-associated insulin resistance. Hence, RAGE-mediated CML trapping in adipose tissue is a potentially useful target for developing new therapies against obesity-associated dysregulation of adipokines and its complications.

**Acknowledgments**

We acknowledge Prof Wabitsch for providing the Simpson-Golabi-Behmel syndrome preadipocytes and GlaxoSmithKline (the Netherlands) for providing rosiglitazone. This work is dedicated to Prof Angelika Bierhaus. She passed away on April 15, 2012. She was of inestimable value for our insights into the receptor for advanced glycation endproducts. We own her a great debt of gratitude.
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Disclosures

None.

References


Significance

This study provides, for the first time, evidence that the Nepsilon-(carboxymethyl)lysine receptor for advanced glycation end product axis is a novel pathway linking obesity to inflammation and more importantly to the development of obesity-related insulin resistance. Targeting this axis may have future therapeutic potential in the management of the obesity-associated complications.
Nε-(Carboxymethyl)lysine-Receptor for Advanced Glycation End Product Axis Is a Key Modulator of Obesity-Induced Dysregulation of Adipokine Expression and Insulin Resistance


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SUPPLEMENT:

CML-RAGE Axis is a Key Modulator of Obesity-Induced Dysregulation of Adipokine Expression and Insulin Resistance

**SUPPLEMENTAL TABLE**

**Table I General Characteristics of Severely Obese Subjects.**

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</tbody>
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Data are presented as mean ± SD, or as median [interquartile range], or as percentage. Insulin resistance was estimated using the homeostasis model assessment for insulin resistance (HOMA-IR) ([http://www.dtu.ox.ac.uk](http://www.dtu.ox.ac.uk)).
Figure I. Activation of the CML-RAGE axis mediates dysregulation of adipokines in human preadipocytes.

To examine the potential biological effects of CML on the expression of adipokines, preadipocytes were incubated during 24h, 48h and 72h with 0.5 µM control albumin (white bars), minimally- (gray bars) and highly-modified (black bars) CML-albumin. Changes in expression of RAGE, PAI-1, IL-6 and adiponectin were examined by real time PCR. Incubation with preadipocytes during 24h and 48h did not change RAGE, PAI-1 and IL-6 gene expression, whereas incubation of preadipocytes during 72h significantly increased RAGE, PAI-1 and IL-6 gene expression compared with control albumin. *p<0.01 vs control-albumin.
Figure II. CML-induced dysregulation of adipokines is mediated via RAGE in preadipocytes.

To determine whether the effects of CML on the expression of adipokines in preadipocytes involve the activation of RAGE, CML-RAGE interaction was blocked using a RAGE antibody (5 µg/mL) or sRAGE (11.5 µg/mL). CML-induced gene expression of RAGE, IL-6 and adiponectin were significantly inhibited by RAGE antibody and sRAGE. *p<0.05 and **p<0.01 CML-albumin vs control-albumin, ##p<0.05 and ***p<0.01 anti-RAGE antibody/soluble RAGE vs CML-albumin.
Figure III. Effect of S100 protein on expression levels of adipokines in preadipocytes.

Incubation of SGBS preadipocytes with 6 and 24 μg/mL of S100 protein during 1h showed upregulation of the expression of RAGE, PAI-1 and IL-6, and downregulation of adiponectin gene expression. *p<0.01 vs control-albumin.
Figure IV

(A) CML plasma levels were measured by UPLC-Tandem MS in mice deficient for RAGE (RAGE^-/- mice) and wild-type (WT) mice (n=10 per group, 12 weeks old). Plasma CML was significantly higher in RAGE^-/- mice compared with WT mice. (B) Circulating levels of the pro-inflammatory markers IL-1β, IL-12, IL-6, mKC (IL-8), IFN-γ and TNF-α were decreased in RAGE^-/- versus WT mice, whereas the anti-inflammatory markers IL-10 and adiponectin were increased. *p-value<0.05.

Figure IV. RAGE mediates CML plasma concentrations and inflammatory markers.
Figure V. Plasma CML levels are decreased in obesity.

To confirm in a larger study population of obese and control subjects the association between CML plasma levels and obesity, we selected lean control subjects (n=77, BMI ≤ 25 kg/m²) and severely obese subjects (n=44, BMI > 40 kg/m²). The severely obese subjects were admitted to the Surgical Department of Maastricht University Hospital to undergo bariatric surgery. The control subjects were randomly selected from a population of subjects with normal glucose tolerance of the Cohort Study of Diabetes and Atherosclerosis Maastricht (CODAM), and from the population of spouses of familial combined hyperlipidemia patients. Details of these control subjects are described by Brouwers et al. and Jacobs et al. Plasma CML levels were measured with UPLC-Tandem MS as described in the material and methods section. In addition
to CML, plasma levels of N\textsuperscript{\textdegree}-(Carboxyethyl)lysine (CEL) were also measured in the same plasma sample and simultaneously with the CML analysis. For the CEL analysis, D4-CEL was used as internal standard. In addition to these analyses of CML and CEL in total plasma proteins, free levels of CML and CEL were also measured in these subjects. Free CML and CEL levels were measured in deproteinized plasma by UPLC-Tandem MS as described in the material and methods section. (A) CML plasma levels were significantly decreased in the severely obese subjects compared with the lean control subjects. Free CML plasma levels were also significantly decreased in the severely obese subjects compared with the lean control subjects. (B) CEL plasma levels were significantly increased in the severely obese subjects vs the lean control subjects; whereas free CEL plasma levels were not different between the groups. (C) and (D) CML plasma levels were inversely correlated with waist-to-hip ratio (C) and BMI (D), respectively. Correlation coefficient for each relationship is given.
REFERENCES


SUPPLEMENT: MATERIAL AND METHODS:

CML-RAGE Axis is a Key Modulator of Obesity-Induced Dysregulation of Adipokine Expression and Insulin Resistance


Human Study

We used 9 male lean (BMI \( \leq 25 \text{ kg/m}^2 \)) and 10 male obese (BMI > 25 kg/m\(^2\)) subjects, which were admitted to the Maastricht University Medical Center to study in detail subcutaneous adipose tissue (SAT) function and insulin sensitivity by a hyperglycemic-euglycemic clamp. The mean glucose infusion rate during steady state (last 30 min) was used to assess insulin sensitivity. In all subjects, following plasma variables were determined after overnight fasting using standard laboratory techniques: glucose, insulin, total cholesterol, HDL- and LDL cholesterol, triglycerides and free fatty acids. Details of the study protocol are described by Goossens et al.\(^1\) In these subjects, plasma N\(^\circ\)-Carboxymethyl)lysine (CML) levels were measured with ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-Tandem MS) and SAT biopsies were available for immunodetection and RNA isolation.

We also included severely obese subjects (n=44, BMI > 40 kg/m\(^2\)) which were admitted to the Surgical Department of Maastricht University Hospital to undergo bariatric surgery.\(^2\) In all subjects, following plasma variables were determined after overnight fasting using standard laboratory techniques: glucose, insulin, total cholesterol, HDL- and LDL cholesterol, triglycerides and free fatty acids (main characteristics are presented in supplemental table S1). During bariatric surgery, SAT and visceral adipose tissue (VAT) were sampled for immunodetection and for RNA isolation.

All participants gave written informed consent for participation in the study, which was undertaken with approval from the Ethics Committee of the Maastricht University Medical Center.

Immunodetection of CML in Human Adipose Tissue

SAT of lean (n=9) and obese (n=10) male subjects were sampled during the study, fixed in 4% formalin and immediately embedded in paraffin. In addition, SAT and VAT of severely obese subjects (n=44) were sampled during bariatric surgery and embedded in paraffin after fixation in formalin. Immunohistochemistry was performed using Envision+ System-HRP (Dako, Glostrup, Denmark) to demonstrate the presence and localization of CML-modified proteins in adipose tissue. Primary antibodies that were used include: monoclonal antibody against CML-modified proteins,\(^3\) anti-CD31 antibody specific for endothelial cells (Dako), anti-CD68 antibody specific for macrophages (Dako) and antibody directed against the receptor for advanced glycation endproducts (RAGE) (R&D Systems, MN, USA).

Scoring of the CML staining of adipose tissues was performed by an investigator (HWMN) masked to the patient’s characteristics. CML-positive adipocytes were scored in three randomly selected fields and each adipocyte was given a score according to the intensity of the staining
(score 0 = no CML staining; 1 = weak CML staining; 2 = moderate CML staining; and 3 = strong CML staining). Each score was then multiplied by the number of adipocytes positive for that score, and the total CML score of an individual SAT/VAT was calculated by summing the multiplication score and dividing this sum by the total number of adipocytes.

RAGE Expression in Human Adipose Tissue

RAGE gene expression levels were measured in SAT of lean and obese subjects, and in SAT and VAT of severely obese subjects. After isolation using Tri Reagent (Sigma, MO, USA), total cellular RNA was reverse transcribed with iScript cDNA synthesis kit (Biorad). Expression of RAGE was measured quantitatively by real time PCR using SYBR Green mix (Quantace). The threshold cycle (Ct) value for every sample was measured, and mRNA expression levels of RAGE were normalized to the reference genes β-actin, cyclophilin A and β2-microglobulin. Data were analyzed by the ∆Ct method.

RAGE Protein Levels in Human Adipose Tissue

Homogenates of SAT of lean controls and obese subjects, and VAT and SAT of severely obese subjects were available to analyze RAGE protein levels by Western blotting. Total proteins of VAT and SAT were randomly distributed and separated on a 12% SDS-PAGE. Proteins were blotted onto a polyvinylidene fluoride membrane (Amersham, Buckinghamshire, UK) and blocked with 3% non-fat milk/PBS. The membrane was incubated with RAGE antibody (R&D Systems), followed by incubation with goat-anti-mouse horseradish peroxidase antibody (Dako). Proteins were detected using the chemiluminescence kit (Amersham) and visualized by Chemidoc XRS (Bio-Rad). Quantification of the RAGE band was done by Quantity One (Bio-Rad) and was normalized for β-actin.

Measurements of CML levels

Protein-bound CML levels in plasma or cell homogenates were measured by UPLC-Tandem MS (Waters, UPLC/Xevo TQ MS, Etten-Leur, the Netherlands).4 Total proteins were reduced with 100 mM sodium borohydride in 0.2 M sodium borate buffer at pH 9.2 for 2 hours at room temperature. The proteins were then precipitated with trichloroacetic acid and hydrolyzed with 6 M HCl overnight at 110°C. HCL was evaporated at 80°C under nitrogen, and residues were resolved in 0.5 mM tridecafluoroheptanoic acid. Analysis was performed using C18 reversed phase column with a linear gradient of acetonitril. CML levels were measured using a positive ionization mode with D4-CML as internal standard.

Cell Culture of Human SGBS Adipocytes

Human SGBS (Simpson-Golabi-Behmel-Syndrome) preadipocytes were maintained in DMEM/HAM’s F12 culture medium containing 10% FCS, 1 mg/mL biotine, 1 mg/mL pantothenate and 1% penicillin/streptozotocin, and were grown to confluence.5 To obtain serum-free-medium (OF medium), supplementation with FCS was omitted. After reaching confluence, adipogenic differentiation was induced by incubating the preadipocytes with OF medium.
supplemented with 0.01 mg/mL human transferrin, 20 nM insulin, 100 nM cortical and 0.2 nM triiodothyronin for 12 days. For the first 6 days, this adipogenic medium was additionally supplemented with 25 nM dexamethasone, 500 µM 3-isobutyl-1-methylxanthine and 2 µM rosiglitazone (GlaxoSmithKline, London, UK). Oil Red O staining (Sigma) of SGBS cells during differentiation was performed following the manufacturer’s instructions to determine adipogenesis. In addition, homogenates of differentiating SGBS adipocytes were harvested every other day (day 0, 2, 4, 6, 8, 10 and 12) during adipogenesis to measure protein-bound CML levels by UPLC-Tandem MS (as described above), and to analyze expression levels of RAGE by real time PCR (as described above) and RAGE protein levels by Western blotting (as described above).

**Effect of CML incubation of Human SGBS Pre- and Adipocytes on Inflammation**

SGBS preadipocytes and mature, differentiated SGBS adipocytes were incubated for 15 min, 30 min, 1 hr, 2 hr, 4 hr (short incubation), 24 hr, 48 hr and 72 hr (long incubation) with 0.5 µM control albumin, minimally- and highly-modified CML albumin. After incubation, total cellular RNA was extracted using Tri reagent (Sigma). Total RNA was reverse-transcribed with iScript cDNA synthesis kit (Biorad) and expression of RAGE, plasminogen activator inhibitor (PAI)-1, interleukin (IL)-6 and adiponectin were measured quantitatively by real time PCR using SYBR Green mix (Quantace). The threshold cycle (Ct) value for every sample was measured, and mRNA expression levels of each gene were determined by the ΔCt method using β-actin, cyclophilin A and β2-microglobulin as reference genes.

To determine the mechanism of CML-mediated dysregulation of inflammatory markers, SGBS preadipocytes and differentiated SGBS adipocytes were pre-incubated with anti-RAGE antibody (5 µg/mL) (R&D Systems) or soluble form of RAGE (11.5 µg/mL) (provided by Dr. M. Gebbink, Utrecht University, the Netherlands) for 1 hr to block the endogenous RAGE pathway or to block CML binding to RAGE, respectively. Cells were then incubated with 0.5 µM control-albumin or CML-modified albumin. After incubation, RNA was extracted and cDNA was synthesized as described above. Differences in gene expression of RAGE, PAI-1, IL-6 and adiponectin were determined by real time PCR.

As positive control, we incubated SGBS preadipocytes and adipocytes with S100 protein (6 and 24 µg/mL) (Merck, NJ, USA) during 1h. S100 protein is a pro-inflammatory ligand for RAGE and the effect of S100 protein incubation on RAGE, IL-6, PAI-1 and adiponectin was examined by real time PCR.

**Animal experiments**

Obese, RAGE deficient mice (RAGE+/LeprDb−) were generated by crossing obese, non-insulin-dependent diabetic C57BLKS-LeprDb (LeprDb−/−) (Charles River, Maastricht, the Netherlands) with RAGE−/− mice (obtained from Heidelberg University) for several generations. All experiments were approved by the local ethics committee on the use of laboratory animals.

**Markers of Inflammation in Mice:** At 9 weeks, plasma of RAGE+/LeprDb− mice (n=10) and their littermate RAGE+/LeprDb− controls (n=10) were collected to measure circulating inflammatory markers. Mouse inflammatory panels for IL-1β, IL-12p70, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, mKC (IL-8), IL-10 and adiponectin assay were purchased from Meso Scale
Discovery (MSD, MD, USA). All reagents were provided with the MSD kit, and measurements were performed according to the manufacturer’s instructions. Interassay CV were 14.9% for IL-1β, 9.0% for IL-12p70, 9.3% for IFN-γ, 12.1% for IL-6, 14.0% for TNF-α, 7.7% for mKC (IL-8), 5.8% for IL-10 and 4.0% for adiponectin. To better understand the effect of RAGE deficiency on inflammation in obesity, an overall inflammation score was calculated in RAGE−/−/Leptin Db−/− and RAGE+/+/Leptin Db−/− mice by averaging the z-scores of each of the individual inflammatory markers [z-score of an individual markers = (individual’s observed value – mean) / SD]. This score enables a more robust characterization of the overall mechanism of inflammation than that as characterized on the basis of each marker separately. In addition, this score has the advantage of reducing the influence of measurement error/biological variability.

CML Plasma Levels in Mice: Protein-bound CML plasma levels of RAGE−/−/Leptin Db−/− (n=10, 9 weeks) and littermate RAGE+/+/Leptin Db−/− mice (n=10, 9 weeks) were measured with UPLC-Tandem MS as described above.

Glucose and Insulin Tolerance Tests in Mice: Glucose tolerance tests (GTT) were performed in 12 weeks old RAGE−/−/Leptin Db−/− (n=8) and littermate RAGE+/+/Leptin Db−/− (n=10) mice. Following a 16 hr overnight fasting period, a small tail cut was made and whole blood glucose was measured by a glucometer (Contour) (Bayer, Mijdrecht, the Netherlands) from the second drop of blood expressed at time 0 and at time 30, 60, 90 and 120 min following intraperitoneal injection of glucose (2 g/kg bodyweight in filtered saline solution) (Sigma). Insulin tolerance tests (ITT) were performed in the same RAGE−/−/Leptin Db−/− and RAGE+/+/Leptin Db−/− at 13 weeks of age. Food was removed 6 hr prior to insulin treatment (0.75 U/kg bodyweight, intraperitoneal injection) (Actrapid Penfill, Novo Nordisk, Bagsvaerd, Denmark), which was administered at time 0, following measurements of bodyweight and baseline glucose. A small tail cut was made for glucose measuring at 0, 30, 60, 90 and 120 min.

CML Trapping in Mice: Trapping of CML in obesity was first investigated in a murine model of obesity and type 2 diabetes, i.e. Leptin Db−/− mice (Charles River). Leptin Db−/− mice (n=6, 6-8 weeks) were intravenously injected with fluorescently-labeled CML-albumin and fluorescently-labeled control-albumin to visualize distribution/accumulation of circulating CML among different organs. To produce fluorescently-labeled CML-albumin and fluorescently-labeled control-albumin, Alexa-labeled albumin 488 (green fluorescent signal) and 555 (red fluorescent signal) (Invitrogen, CA, USA) were incubated overnight with or without 100 mM glyoxal, respectively, to induce CML-modification in albumin. Glycation and grade of modification of the fluorescently-labeled CML-albumin and control-albumin was confirmed by UPLC-Tandem MS and Western blotting (data not demonstrated). Fluorescently-labeled CML-albumin and control-albumin (3.5 mg/kg bodyweight) were simultaneously injected intravenously into the tail vein of these Leptin Db−/− mice. Blood samples were collected before and at different time points after injection from the tip of the tail using a heparin-coated microcapillary (Microvette CB 300 LH, Sarstedt, Nümbrecht, Germany) and centrifuged. Plasma fluorescence was measured at 515 and 565nm with the NanoDrop 3300 Fluorospectrometer (Thermo Fisher Scientific, Wilmington, Delaware, USA) to study the clearance of fluorescently labeled CML-albumin and unmodified-albumin from the circulation. Four hrs after injection, mice were sacrificed and adipose tissue, liver and kidney were sampled, washed in 0.9% NaCl and then embedded in 1% agarose (Biorad). These intact tissues were immediately, and without further processing, analyzed by two-photon microscopy to detect trapping of the injected fluorescently-labeled CML-albumin. Two-photon laser scanning microscopy (TPLSM) images were recorded with a Leica DM6000 CFS upright microscope incorporated in a Multiphoton optical imaging system (Leica TCSPC SP5, Leica Microsystems, Mannheim, Germany). Fluorophores and autofluorescence of the tissues were excited by a mode-locked Coherent Chameleon Ti:Sapphire laser (Coherent, Santa Clara, CA, USA) with an
880 nm central wavelength and a 120 fs pulse width at the focal plane. Datasets were acquired using a 20× water-dipping objective lens (Leica, WD 3 mm) with a 1.00 numerical aperture and subsequent optical zoom. Fluorescence in Lepr<sup(Db-)</sup>/mice was detected in the direct detector system with emission filters IF525/50 and IF585/40 and color-coded in green (CML-albumin) and red (unmodified albumin), respectively. Autofluorescence of adipocytes was also detected with the green filter replaced by a BP460/50 filter, color-coded in blue. The chosen combination of emission filters minimized leak-through, while simultaneously optimizing sensitivity. All images shown have a pixel field of 512×512 pixels and are imaged with a line frequency of 400 Hz, resulting in a pixel dwell time of 4.9<sup>3</sup>s and an image frame time of 1.28 s.

Next, we repeated this CML trapping experiment in RAGE<sup(-/-)</sup>/Lept<sup(Db-)</sup>/ and littermate RAGE<sup(+/+)</sup>/Lept<sup(Db-)</sup>/ mice (n=10 in both groups, 9 weeks) to investigate the role of RAGE in the distribution and accumulation of circulating CML among different organs. Because of the presence of an EGFP/RAGE construct in RAGE<sup(-/-)</sup>/Lept<sup(Db-)</sup>/ mice, mice were injected with red fluorescently-labeled CML-albumin (Alexa-labeled albumin 555). Production of fluorescently-labeled CML-albumin and trapping experiments was performed as described above. Processing and analysis of the tissues by two-photon microscopy was performed as described above. Fluorescence in RAGE<sup(-/-)</sup>/Lept<sup(Db-)</sup>/ and RAGE<sup(+/+)</sup>/Lept<sup(Db-)</sup>/ mice was detected in the direct detector system with emission filters 397-451 nm, 500-450 nm and 550-661 nm and color-coded in blue (collagen), green (EGFP/RAGE construct) and red (CML-albumin), respectively.

**Statistics**

All analyses were performed with the Statistical Package for Social Sciences (SPSS) version 15.0 for Windows (SPSS Inc, Chicago, Illinois, USA). Variables with a skewed distribution were log transformed prior to further analyses. Differences in CML immunostaining, RAGE gene expression levels and RAGE protein levels between SAT of lean and obese subjects, and between SAT and VAT of severely obese subjects were analyzed by unpaired or paired Student's t-test, respectively. Differences in gene expression levels of RAGE, PAI-1, IL-6 and Adiponectin after incubation of SGBS adipocytes with CML were investigated using a Student’s t-test. In addition, changes of CML concentration, RAGE gene expression levels and RAGE protein levels during differentiation of SGBS preadipocytes to adipocytes were also investigated using a Student’s t-test. In mice, differences in circulating levels of inflammatory markers and CML plasma levels were also investigated using a Student’s t-test. A Two Way ANOVA with Bonferroni correction was used to analyze the results of GTT and ITT. Comparisons of characteristics between lean and obese subjects were performed with a Student’s t-test or Chi-square test. Associations between CML plasma levels, BMI and glucose infusion rate were determined by partial correlation and were adjusted for age and sex. P-values less than 0.05 were considered statistically significant. All data are presented as mean ± SD.
REFERENCES


