Nε-(Carboxymethyl)lysine-Receptor for Advanced Glycation End Product Axis Is a Key Modulator of Obesity-Induced Dysregulation of Adipokine Expression and Insulin Resistance

N^{ε}-(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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Objective—Dysregulation of inflammatory adipokines by the adipose tissue plays an important role in obesity-associated insulin resistance. Pathways leading to this dysregulation remain largely unknown. We hypothesized that the receptor for advanced glycation end products (RAGE) and the ligand N^{ε}-(carboxymethyl)lysine (CML) are increased in adipose tissue and, moreover, that activation of the CML–RAGE axis plays an important role in obesity-associated inflammation and insulin resistance.

Approach and Results—In this study, we observed a strong CML accumulation and increased expression of RAGE in adipose tissue in obesity. We confirmed in cultured human preadipocytes that adipogenesis is associated with increased levels of CML and RAGE. Moreover, CML induced a dysregulation of inflammatory adipokines in adipocytes via a RAGE-dependent pathway. To test the role of RAGE in obesity-associated inflammation further, we constructed an obese mouse model that is deficient for RAGE (ie, $RAGE^{-/-}/Leptr^{Db-/-}$ mice). $RAGE^{-/-}/Leptr^{Db-/-}$ mice displayed an improved inflammatory profile and glucose homeostasis when compared with $RAGE^{+/+}/Leptr^{Db-/-}$ mice. In addition, CML was trapped in adipose tissue in $RAGE^{+/+}/Leptr^{Db-/-}$ mice but not in $RAGE^{-/-}/Leptr^{Db-/-}$. RAGE-mediated trapping in adipose tissue provides a mechanism underlying CML accumulation in adipose tissue and explaining decreased CML plasma levels in obese subjects. Decreased CML plasma levels in obese individuals were strongly associated with insulin resistance.

Conclusions—RAGE-mediated CML accumulation in adipose tissue and the activation of the CML–RAGE axis are important mechanisms involved in the dysregulation of adipokines in obesity, thereby contributing to the development of obesity-associated insulin resistance. **(*Arterioscler Thromb Vasc Biol*. 2014;34:1199-1208.)**

Key Words: advanced glycation end product ■ inflammation ■ N^{ε}-(carboxymethyl)lysine ■ obesity ■ receptor for advanced glycation end products

Obesity is closely linked to a wide array of pathophysiological conditions, including insulin resistance.[1] The adipose tissue is an important endocrine organ that produces and secretes biologically active molecules, collectively known as adipokines.[2] 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.[3–5] It has been proposed that this dysregulation is a key feature in the pathogenesis of obesity-related insulin resistance.[6–8] 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 cell-surface molecule expressed on different cell types, such as adipocytes,

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Nonstandard Abbreviations and Acronyms	
AGEs	advanced glycation end products
CML	N^{ε}-(carboxymethyl)lysine
PAI	plasminogen activator inhibitor
RAGE	receptor for AGEs
SAT	subcutaneous adipose tissue
SGBS	Simpson–Golabi–Behmel syndrome
VAT	visceral adipose tissue

endothelial cells, and macrophages.[9] RAGE is initially identified as the receptor for advanced glycation end products (AGEs),[10] but, in addition to AGEs, RAGE also interacts with multiple members of the proinflammatory S100/calgranulin family and high motility group box 1 protein.[9,11] 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.[9,12] 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. N^{ε}-(carboxymethyl)lysine (CML) is a major AGE and is an important ligand for RAGE.[10] CML is formed on proteins by nonenzymatic glycation and oxidation reactions.[13] Alternative routes for CML formation have been described, including lipid peroxidation of polyunsaturated fatty acids.[14,15] In fact, lipid peroxidation is a more important source for CML formation than glycoxidation reactions.[14] 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 and where CML can interact with RAGE. However, the role of CML-RAGE in obesity, obesity-associated inflammation, and insulin resistance has to date not been investigated.

The aim of this study was to investigate the role of CML–RAGE axis in obesity-associated 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 $Leptr^{Db-/-}$ 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/m^2). 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 1E). In addition, RAGE showed a similar localization as CML (Figure 1E). No differences in CML and RAGE localization were observed between SAT and VAT. However, a significant higher CML accumulation (Figure 1F) 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 1G and 1H) 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 adipose tissue, 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

	Lean Subjects	Obese Subjects
n	9	10
Age, y	59.2±7.4	59.6±9.9
Sex (men), %	100	100
BMI, kg/m^2	23.4±1.1	34.2±4.0*
Fasting glucose, mmol/L	5.3±0.3	5.9±0.5†
Fasting insulin, mmol/L	12.2±2.7	22.0±6.8†
GIR, μmol×kg body weight^{-1}×min^{-1}	37.1±7.9	14.3±7.9*

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.

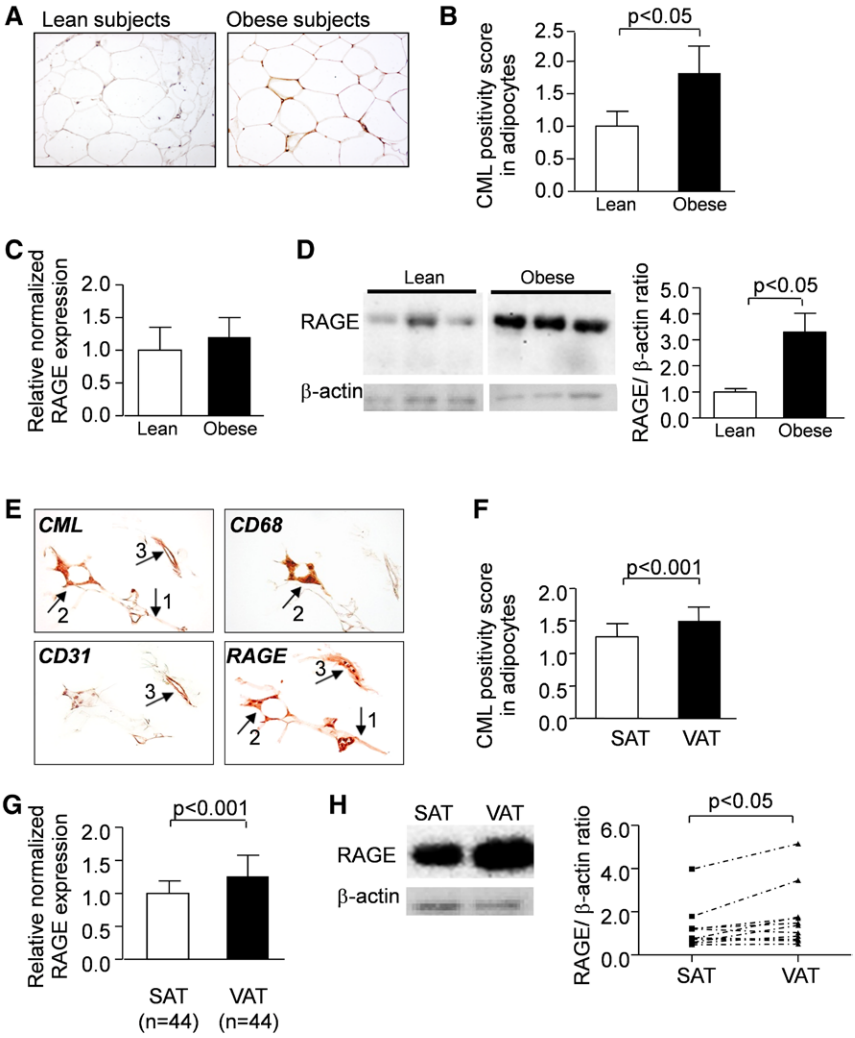

Figure 1. N$^{\varepsilon}$-(Carboxymethyl)lysine (CML) and receptor for advanced glycation end product (RAGE) in human adipose tissue in obesity. **A**, CML immunostaining of subcutaneous adipose tissue (SAT) of lean and obese subjects demonstrated that CML was only slightly detectable in SAT of lean subjects, whereas CML was abundantly present in SAT of obese subjects. **B**, Quantification of the CML immunostaining in SAT demonstrated a significant higher CML staining in obese when compared with that in lean subjects. **C**, RAGE expression was slightly higher in SAT of obese subjects when compared with that of lean subjects (P=0.189). RAGE expression levels of the obese subjects are presented relative to those of the lean subjects. **D**, Representative Western blot analyses of RAGE and β-actin protein bands of 3 lean and 3 obese subjects are given. Quantification of the RAGE protein levels demonstrated that RAGE protein levels were significantly higher in SAT of obese subjects when compared with that of lean subjects. RAGE protein levels were normalized for β-actin levels. **E**, Immunostainings of CML, CD68, CD31, and RAGE in visceral adipose tissue (VAT) of severely obese subjects demonstrated that CML and RAGE were detected in adipocytes (arrow 1), macrophages (arrow 2), and endothelial cells (arrow 3). **F**, Quantification of the CML staining demonstrated that CML staining of adipocytes of VAT was significantly higher than CML staining of SAT. **G**, RAGE expression was significantly higher in VAT when compared with SAT of severely obese subjects. RAGE expression levels in the VAT of the severely obese subjects are presented relative to those in the SAT. **H**, Representative RAGE and β-actin Western blot analyses of SAT and VAT from 1 severely obese subject are given. Quantification demonstrated that RAGE protein levels were significantly higher in VAT when compared with SAT of severely obese subjects. RAGE protein levels are normalized for β-actin levels.

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).[16] 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.

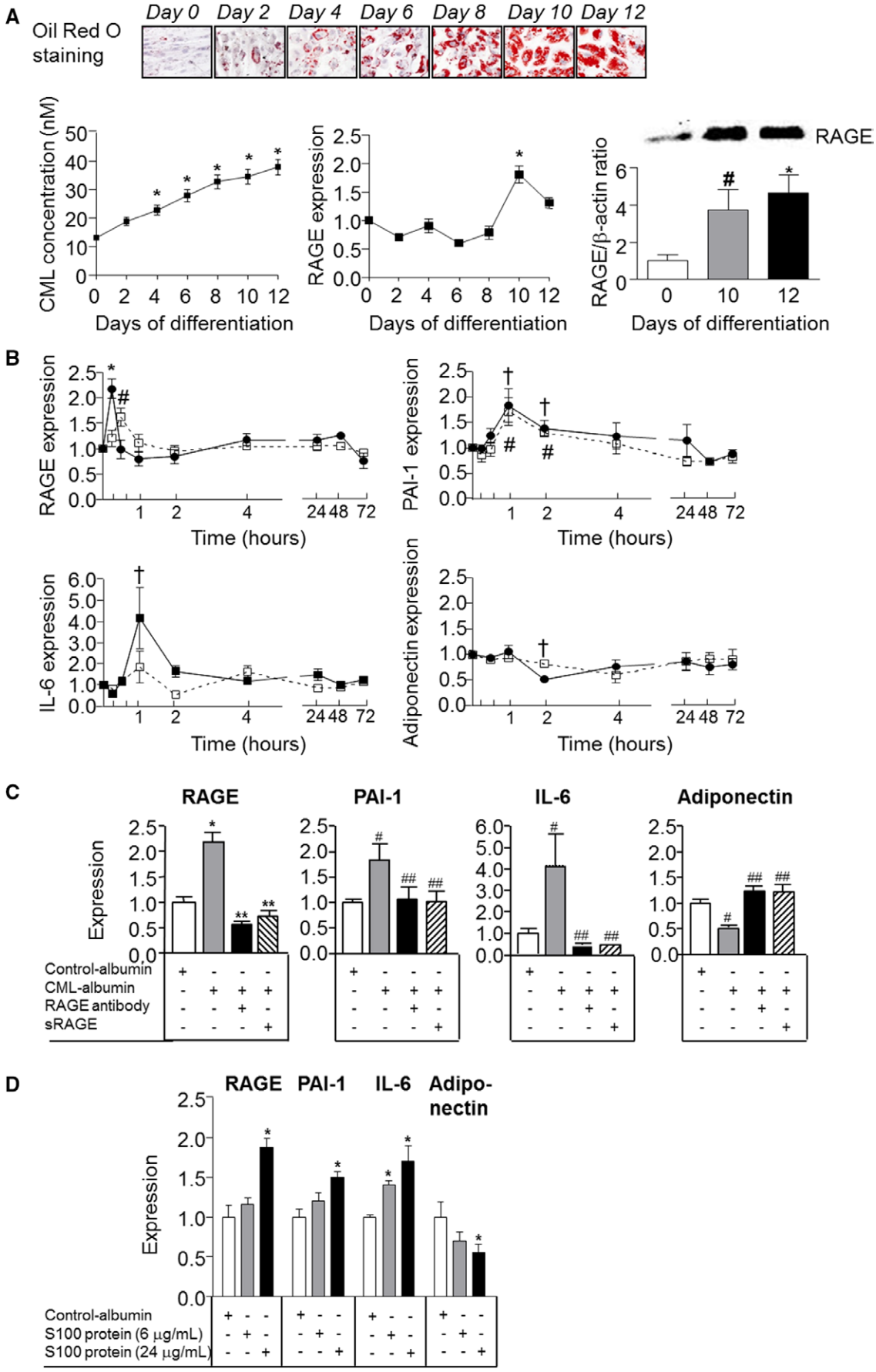

Figure 2. (Continued)

Figure 2. N^{ε}-(Carboxymethyl)lysine (CML)–receptor for advanced glycation end product (RAGE) axis is increased during adipogenesis, and activation of this pathway mediates adipokine dysregulation in human adipocytes. **A**, Differentiation of Simpson–Golabi–Behmel syndrome (SGBS) preadipocytes to adipocytes was evaluated by an Oil Red O staining and showed a significant lipid accumulation during adipogenesis. CML levels (determined by UPLC-Tandem MS [ultra performance liquid chromatography-tandem mass spectrometry]), RAGE gene expression (determined by quantitative RT-PCR [real-time polymerase chain reaction]), and RAGE protein levels (determined by Western blotting) were significantly increased during the differentiation process. #P<0.05, *P<0.01 vs day 0. **B**, Incubation of SGBS adipocytes with minimally (dotted line) and highly modified CML-albumin (black line) increased the expression of RAGE and downstream genes plasminogen activator inhibitor (PAI)-1 and interleukin (IL)-6, and decreased adiponectin expression in a time- and concentration-dependent manner. Minimally and highly modified CML-albumin were prepared and characterized as described before.[16] The expression levels of each gene are presented relative to those of the control-albumin–treated cells for each time point. #P<0.05 minimally modified CML vs control-albumin, †P<0.05 and *P<0.01 highly modified CML-albumin vs control-albumin. **C**, Preincubation of SGBS adipocytes with anti-RAGE antibody or soluble RAGE (sRAGE) inhibited the CML-increased expression of RAGE, PAI-1, and IL-6 and the CML-decreased expression of adiponectin. #P<0.05 and *P<0.01 CML-albumin vs control-albumin, ##P<0.05 and **P<0.01 anti-RAGE antibody/sRAGE vs CML-albumin. **D**, Incubation with S100 protein increased the RAGE, PAI-1, and IL-6 gene expression in SGBS adipocytes, whereas adiponectin gene expression was dowregulated on S100 incubation. *P<0.01 vs control-albumin.

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,[17] 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 ($RAGE^{-/-}$/$Leptr^{Db-/-}$) and obese, wild-type ($RAGE^{+/+}$/$Leptr^{Db-/-}$) mice. In the $Leptr^{Db}$ background, no effect of RAGE on body weight was observed (39±5 and 35±5 g, respectively) at 9 weeks. $RAGE^{-/-}$/$Leptr^{Db-/-}$ mice exhibited an improved inflammatory profile when compared with $RAGE^{+/+}$/$Leptr^{Db-/-}$ 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 analoge of human interleukin-8]), levels were also lower in $RAGE^{-/-}$/$Leptr^{Db-/-}$ 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 $RAGE^{-/-}$/$Leptr^{Db-/-}$ mice when compared with those in $RAGE^{+/+}$/$Leptr^{Db-/-}$ 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 $RAGE^{-/-}$/$Leptr^{Db-/-}$ mice when compared with that in $RAGE^{+/+}$/$Leptr^{Db-/-}$ 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 $RAGE^{+/+}$/$Leptr^{Db+/+}$ 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, $RAGE^{-/-}$/$Leptr^{Db-/-}$ mice were also associated with altered metabolic phenotype. Glucose tolerance test demonstrated significant differences in response to glucose load between $RAGE^{-/-}$/$Leptr^{Db-/-}$ and $RAGE^{+/+}$/$Leptr^{Db-/-}$ mice (Figure 3B). $RAGE^{-/-}$/$Leptr^{Db-/-}$ mice had an improved glucose metabolism when compared with $RAGE^{+/+}$/$Leptr^{Db-/-}$ mice (Figure 3B). After insulin injection, glucose did not significantly decrease in $RAGE^{+/+}$/$Leptr^{Db-/-}$ 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 $RAGE^{-/-}$/$Leptr^{Db-/-}$ 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 $RAGE/Leptr^{Db}$ Mice

Of interest, CML plasma levels were significantly higher in $RAGE^{-/-}$/$Leptr^{Db-/-}$ mice when compared with $RAGE^{+/+}$/$Leptr^{Db-/-}$ mice (923±114 nmol/L versus 684±78nM, P<0.050), 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 $Leptr^{Db-/-}$ mice. We injected $Leptr^{Db-/-}$ 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 $Leptr^{Db-/-}$ mice. After injection, plasma clearance of fluorescently labeled CML-albumin was significantly faster than that of control-albumin ($t_{1/2}$=59±5 minutes

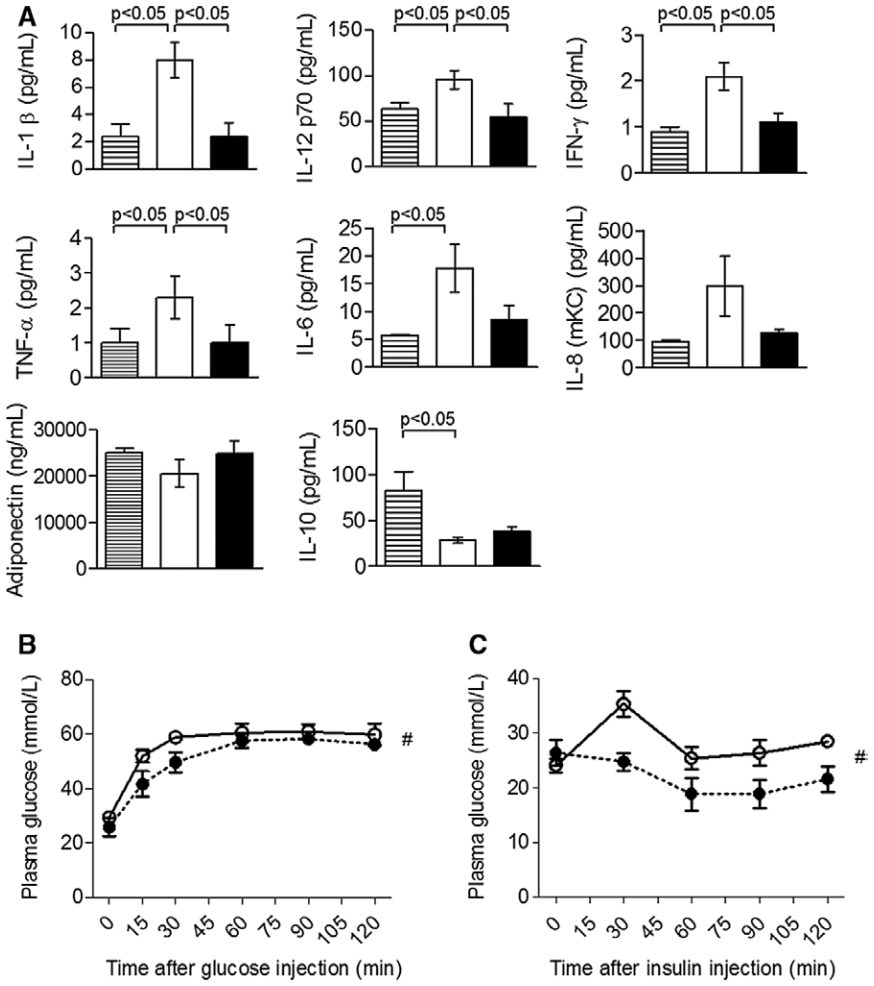

Figure 3. Improved regulation of adipokines and glucose homeostasis in obese, RAGE-deficient mice. **A**, Circulating levels of proinflammatory markers—interleukin (IL)-1β, IL-12p70, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, IL-6, and mKC (IL-8)—and anti-inflammatory cytokines—adiponectin and IL-10—were measured in RAGE$^{+/+}$/Leptr$^{Db+/+}$ mice (shaded bars), RAGE$^{+/+}$/Leptr$^{Db-/-}$ mice (white bars), and RAGE$^{-/-}$/Leptr$^{Db-/-}$ mice (black bars) and compared between groups. **B**, After injection of glucose, glucose levels were lower at all time points in RAGE$^{-/-}$/Leptr$^{Db-/-}$ (●, dotted line) vs RAGE$^{+/+}$/Leptr$^{Db-/-}$ (○, black line) mice. #Overall P<0.01. **C**, After injection of insulin, maximum glucose decline was 30% in RAGE$^{-/-}$/Leptr$^{Db-/-}$ (●, dotted line) mice, whereas glucose concentrations did not change in the RAGE$^{+/+}$/Leptr$^{Db-/-}$ mice (○, black line). #Overall P<0.01.

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 Leptr$^{Db-/-}$ 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 Leptr$^{Db-/-}$ 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 mice model (ie, RAGE$^{-/-}$/Leptr$^{Db-/-}$ and RAGE$^{+/+}$/Leptr$^{Db-/-}$ mice). RAGE$^{-/-}$/Leptr$^{Db-/-}$ and RAGE$^{+/+}$/Leptr$^{Db-/-}$ mice were injected with fluorescently labeled CML-albumin (red fluorescently signal). A significant accumulation of CML-albumin was observed in adipose tissue of RAGE$^{+/+}$/Leptr$^{Db-/-}$ mice, whereas the fluorescently labeled CML-albumin was completely absent in adipose tissue of RAGE$^{-/-}$/Leptr$^{Db-/-}$ 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$^{+/+}$/Leptr$^{Db-/-}$ 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

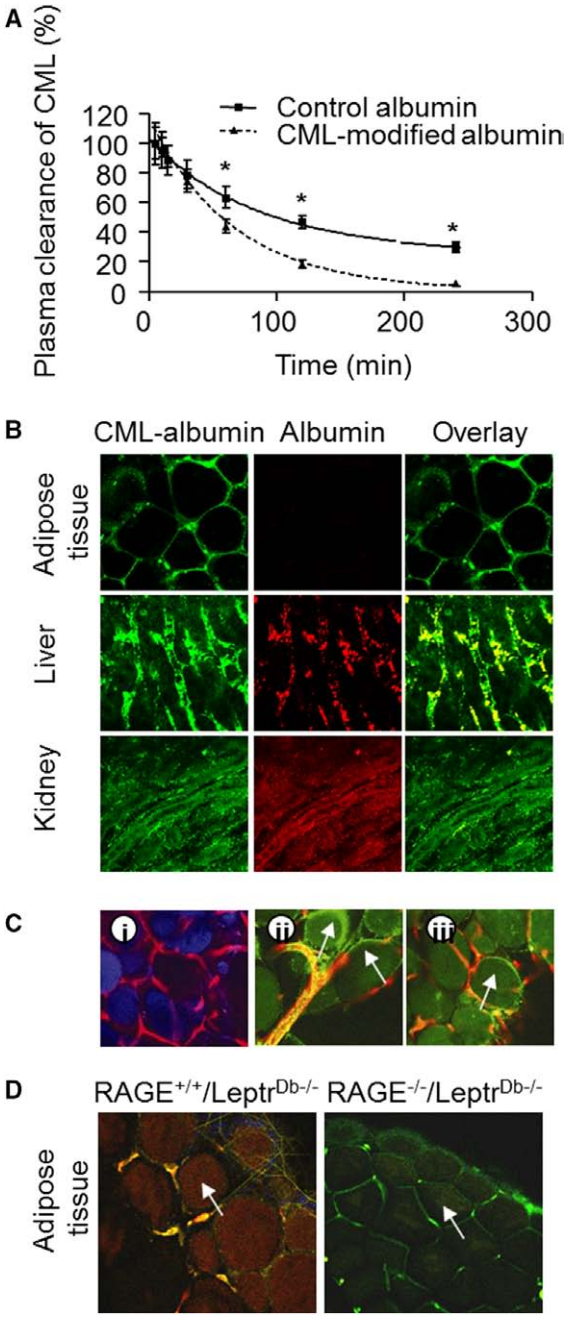

Figure 4. Trapping of N^{ε}-(carboxymethyl)lysine (CML) in adipose tissue is mediated by receptor for advanced glycation end product (RAGE). **A**, Clearance of CML-albumin from circulation was significantly faster than the clearance from control-albumin. **P*<0.001. **B**, Trapping of CML-albumin (green fluorescent signal) and control-albumin (red fluorescent signal) in adipose tissue, liver, and kidney was studied in $Leptr^{Db-/-}$ 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 $RAGE^{+/+}/Leptr^{Db-/-}$ mice, whereas no CML-albumin was trapped in $RAGE^{-/-}/Leptr^{Db-/-}$ mice (see arrows).

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/m^2 before surgery and 36.3±4.4 kg/m^2 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 $RAGE^{-/-}/Leptr^{Db-/-}$ 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 $RAGE^{-/-}/Leptr^{Db-/-}$ mice. In line with this concept, we also found decreased CML plasma level 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

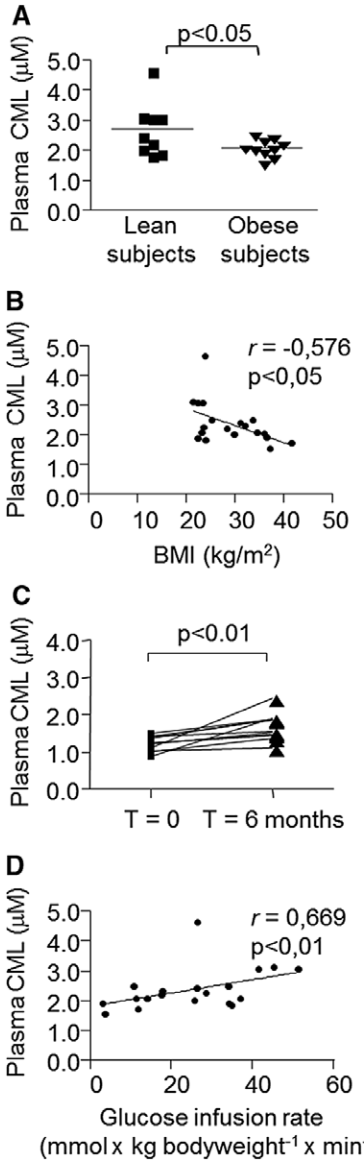

Figure 5. Nᵋ-(Carboxymethyl)lysine (CML) plasma levels are decreased in obesity and associated with insulin sensitivity. **A**, CML plasma levels were significantly lower in obese subjects when compared with those in lean controls. **B**, CML plasma levels were inversely related with body mass index (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.

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 in other tissues that the obese state is associated with local CML accumulation. Our previous study demonstrated that development of fatty livers was associated with increased formation of CML in lipid-laden hepatocytes.[19] In addition, CML accumulation was observed in the muscle tissue of obese subjects, which was correlated with weight gain.[20] 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.[21]

CML exerts biological effects via altered gene expression mediated by RAGE.[9] 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.[9] 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.[12] 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[22] 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.[23] 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.

We further extended these in vitro observations to in vivo ones using a murine model of RAGE deficiency, obesity, and type 2 diabetes mellitus, $RAGE^{-/-}/Leptr^{Db-/-}$ 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 then 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 proinflammatory 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.[27,28] RAGE deficiency was also associated with higher glucose transporter-4 expression in adipose tissue in these mice.[28] 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 al[25] 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 al[28] 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 $Leptr^{Db-/-}$ 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 $RAGE^{+/+}/Leptr^{Db-/-}$ mice, whereas other AGEs, such as N^{ε}-(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 al[31] 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.

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Disclosures

None.

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Significance

This study provides, for the first time, evidence that the N^{ε}-(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.