The advanced glycation end product Nε-carboxymethyllysine and its precursor glyoxal increase serotonin release from Caco-2 cells

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Abstract
Advanced glycation end products (AGEs), comprising a highly diverse class of Maillard reaction compounds formed in vivo and during heating processes of foods, have been described in the progression of several degenerative conditions such as Alzheimer’s disease and diabetes mellitus. Nε-Carboxymethyllysine (CML) represents a well-characterized AGE, which is frequently encountered in a Western diet and is known to mediate its cellular effects through binding to the receptor for AGEs (RAGE). As very little is known about the impact of exogenous CML and its precursor, glyoxal, on intestinal cells, a genome-wide screening using a customized microarray was conducted in fully differentiated Caco-2 cells. After verification of gene regulation by qPCR, functional assays on fatty acid uptake, glucose uptake, and serotonin release were performed. While only treatment with glyoxal showed a slight impact on fatty acid uptake (P < 0.05), both compounds reduced glucose uptake significantly, leading to values of 81.3% ± 22.8% (500 μM CML, control set to 100%) and 68.3% ± 20.9% (0.3 μM glyoxal). Treatment with 500 μM CML or 0.3 μM glyoxal increased serotonin release (P < 0.05) to 236% ± 111% and 264% ± 66%, respectively. Co-incubation with the RAGE antagonist FPS-ZM1 reduced CML-induced serotonin release by 34%, suggesting a RAGE-mediated mechanism. Similarly, co-incubation with the SGLT-1 inhibitor phloridzin attenuated serotonin release after CML treatment by 32%, hinting at a connection between CML-stimulated serotonin release and glucose uptake. Future studies need to elucidate whether the CML/glyoxal-induced serotonin release in enterocytes might stimulate serotonin-mediated intestinal motility.

KEYWORDS
Caco-2 cells, glucose uptake, glyoxal, Nε-carboxymethyllysine, serotonin release

Abbreviations: AGE, advanced glycation end product; BSA, bovine serum albumin; CML, Nε-carboxymethyllysine; MTT, 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; RAGE, receptor for advanced glycation end products; SGLT, sodium-glucose linked transporter.

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1 | INTRODUCTION

Advanced glycation end products (AGE), formed in vivo and during thermal processing of foods have been associated with the progression of several degenerative conditions such as Alzheimer’s disease\(^1\) and diabetic retinopathy,\(^2\) in addition to potentially causing kidney damage as a result of long-term intake.\(^3\) The major epitope of AGE-modified proteins,\(^4\) N\(^\text{-carboxymethyllysine}\) (CML), is found in a number of foods, reaching concentrations of up to 85 mg per serving in meat dishes.\(^5\) The daily CML exposure of healthy adults on a standard diet has been estimated to center around 5.4 mg.\(^6\) The main contributors to CML intake were identified to be bread and dough.\(^6\) Similarly, glyoxal, a reactive carbonyl species and precursor of CML, has been detected in several beverages such as wine (0.97 \(\mu\)g \(\text{mL}^{-1}\)) or baked products such as bread (0.3 \(\mu\)g \(\text{g}^{-1}\)) or toast (0.5 \(\mu\)g \(\text{g}^{-1}\)).\(^7\) A study by Arribas-Lorenzo et al\(^8\) estimated the daily intake of glyoxal, a precursor of CML, from cookies to center around 213 \(\mu\)M in the Spanish population. Both, CML and glyoxal are detectable in human plasma, with mean CML concentrations close to 3 \(\mu\)M and mean glyoxal concentrations of 0.3 \(\mu\)M.\(^9\) In both cases, elevated concentrations are encountered in health-compromised individuals, for instance in diabetics. Han et al\(^10\) reported glyoxal concentrations to reach 1 \(\mu\)M in young type 1 diabetes patients free from complications. Likewise, Lieuw-A-Fa et al\(^11\) showed CML concentrations up to 12 \(\mu\)M in type 1 diabetes patients with reduced renal function. Several reports point to partial absorption of dietary AGEs, leading to increased plasma levels and urinary excretion. A study on the dose-dependent utilization of casein-linked AGEs showed an absorption rate of almost 30% for protein-linked CML in rats,\(^12\) whereas PET data from fluorine-18 labeled free CML injected into the tail vein of rats indicated an utilization of up to 40%.\(^13\) Similarly, increases in AGE plasma concentrations have been demonstrated in type 2 diabetics consuming a diet rich in AGEs for 2 weeks, whereas consumption of meals with only one fifth of the AGE content of the high AGE meal led to a reduction in AGE levels.\(^14\) Furthermore, a 46% increase in plasma CML levels and a 60-fold increase in CML urinary excretion have been described in infants who received sterilized infant formula only, indicating both CML uptake and rapid urinary excretion.\(^15\) CML release from proteins below 1 kDa in size comparable to native amino acids has been reported by Hellwig et al\(^16\) in experiments involving simulated gastrointestinal digestion of casein samples. However, free AGEs have been shown to not serve as substrates for intestinal lysine transporters,\(^17\) with the transepithelial flux of CML across Caco-2 cell monolayers falling below the limit of detection.\(^18\) The carboxyalkylated peptides, Ala-CML, CML-Ala, have been indicated as likely PEPT1 substrates.\(^17\) Furthermore, the glycated dipeptides were hydrolyzed intracellularly, yielding free modified amino acids with liberated CML predominately remaining inside Caco-2 cells.\(^17\) Increased AGE concentrations have also been shown in the GI tract of inherited type 2 diabetic rats and streptozotocin-induced diabetic rats.\(^19,20\) Closer examination of the epithelial cells of intestinal villi and crypts showed stronger AGE-staining in the diabetic group of rats.\(^19\)

The intestinal brush-border membrane builds a digestive-absorptive surface,\(^21\) the fluidity of which has been shown to decrease correlating with an increase in non-enzymatic glycation in the jejunum and the duodenum.\(^22\) The human colon carcinoma cell line Caco-2, undergoes differentiation into cells exhibiting small intestine mature villus epithelial cell properties, including a brush border membrane, intracellular tight junctions, and fatty acid binding protein expression.\(^23,24\) Therefore, these cells have been described as a transport model system for the small intestinal epithelium\(^24\) and several studies report effects of AGEs on Caco-2 cells, such as p44/42 MAP kinase activation\(^25\) or influences on heat shock protein expression.\(^26\)

Most reports featuring the effects of AGEs on the gastrointestinal tract are carried out on protein-linked compounds. However, as free AGEs have also been suggested to elicit effects in some studies, we aimed at analyzing the effects of free CML and its precursor glyoxal in this study. As the majority of peripheral serotonin is located in the gastrointestinal tract, and serotonin release from Caco-2 cells has been shown previously,\(^27\) we assessed the effects of free CML and glyoxal on serotonin receptor expression and serotonin release. In addition, we measured free fatty acid and glucose uptake, as peripheral serotonin has been described in the regulation of glucose and lipid metabolism.\(^28\) In order to get some insight into the involvement of RAGE, we carried out co-incubation experiments using the RAGE-antagonist FPS-ZM1.

2 | METHODOLOGY

2.1 | Materials

Protein- and hydrochloride-free, chemically synthesized N-\(\varepsilon\)-carboxymethyl-L-lysine was ordered from Iris Biotech (Marktredwitz, Germany). The RAGE antagonist FPS-ZM1 was obtained from Merck (Millipore, Vienna, Austria). All other reagents used were obtained from (Sigma-Aldrich, Vienna, Austria) unless indicated otherwise.

2.2 | Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (ATCC), maintained at 37°C and 5% CO\(_2\) in a humidified incubator and cultivated in Dulbecco’s modified Eagle medium (DMEM, 4.5 g L\(^{-1}\) glucose), supplemented with 10% fetal bovine serum, 4 mM L-glutamine and 1%
penicillin/streptomycin (100 units penicillin and 171 μmol streptomycin/L). Cells between passage 13 and 22 were used in experiments and passed at 80-85% confluence. All experiments were carried out on enterocyte-like cells, obtained by differentiating Caco-2 cells in 6-well, 12-well, or 96-well plates for 21 days. The medium was exchanged every second to third day within this time frame. Serum-free medium or incubation buffers were used in all experiments as specified at the corresponding method.

2.3 Viability assay

Cell viability was assessed using the MTT (3-[4, 5-dimethylthiazolyl-2]-2, 5-diphenyltetrazolium bromide) assay. Caco-2 cells were differentiated in 96-well plates for 21 days after seeding at a density of 3 × 10^4 cells per well. The test compounds were diluted in serum-free medium and subsequently used in 90 min incubations in a humidified incubator. Thereafter, cells were treated with MTT working solution (100 μL, 1 mg mL\(^{-1}\) MTT reagent diluted in serum-free medium) for 15 min to allow for the formation of purple formazan crystals. The formed crystals were dissolved in DMSO (150 μL) and the absorbance recorded at 570 nm.

2.4 cDNA microarrays

Maskless microarray synthesis, as described by Abgavwe et al\(^{29}\) was applied for DNA microarray synthesis. In order to synthesize two independent microarrays at once, a modified reaction chamber was utilized as described by Sack et al.\(^{30}\) Differentiated Caco-2 cells in six-well plates were treated with CML (500 μM) or glyoxal (3 μM) for 90 min prior to RNA isolation using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The integrity of RNA samples with A₂₆₀/₂₃₀ and A₂₆₀/₂₈₀ ratios between 1.8 and 2.0 was assessed by agarose gel electrophoresis. A total of 10 μg RNA was reverse transcribed with SuperScript III (Thermo Fisher Scientific, Vienna, Austria) and Cy3-labeled random nonamer primers (Tebu Bio, Offenbach, Germany) as described by Ouellet et al.\(^{31}\) The generated Cy3-labeled cDNA was purified using QIAquick PCR purification columns (Qiagen) prior to microarray hybridization. For quality control purposes, three synthetic DNA sequences, one 25mer and two 60mers, were added to the hybridization solution. All microarrays were hybridized for 20 h under constant rotation at 42°C in the dark. After hybridization, the microarrays were washed and scanned with an Axon GenePix 4400A scanner (Molecular Devices, Sunnyvale, CA). Data analysis was carried out with NimbleGen 2.1 software (NimbleGen, Madison, WI), employing robust multichip analysis for normalization purposes. The extracted intensities were used for calculating fold-changes, comparing relative expression of treated samples to controls. Genes reaching a fold-change above 1.2 up- or down-regulation were further analyzed with DAVID pathway analysis tool (http://david.abcc.ncifcrf.gov). The annotation clusters created by DAVID were sorted by enrichment score. The clusters with the highest enrichment scores were further investigated, with clusters having enrichment scores above 1.3 being potential candidates.\(^{32}\)

2.5 qPCR

Caco-2 cells were seeded into six-well plates at a density of 4.2 × 10^4 cells cm\(^{-2}\) and differentiated for 21 days. Fully differentiated Caco-2 cells were incubated with CML (500 μM) or glyoxal (3 μM) for 30, 60, or 90 min prior to RNA isolation using the PeqGold Total RNA kit (Peqlab, VWR, Austria). The isolated RNA was reverse transcribed using the high capacity cDNA kit (Thermo Fisher Scientific). The generated cDNA was used in qPCR experiments with fast SYBR green master mix (Thermo Fisher Scientific) on a StepOnePlus Applied Biosystems, Thermo Fisher Scientific device. Reverse and forward primers have been used in previous studies and their sequences are shown in (Table 1).\(^{33,34}\)

| Target | Forward primer | Reverse primer | Product length (bp) |
|--------|----------------|----------------|---------------------|
| FATP2  | TGGAAACCACAGGTGCTACTC | ACCGAAGCAGTTCACCGATA | 116 |
| CD36   | TGTAACCACAGGACGTGAGG | GAAGGTCCAGAATGCGACC | 69 |
| 5HTR1A | TACCTGTGCTTTCTGCTTC | CCGGGTAAAGAGAGAGGTT | 108 |
| 5HTR1B | CTGTGCTTGGTCTTCTCCAT | AGAGGATGTTGGCGGTGTTC | 109 |
| 5HTR2A | GGTGCTTACCTGCCGATGATA | TGGCAAGATCCTACACACACAA | 144 |
| GLUT2  | CATGCTCTGGTCCGGTCTGCTATC | AACCCCTACAGGGAGCTACACT | 150 |
| SGLT1  | CCGATATCACATCTCGGTATAC | CACGATTGTTGGGAAACATGC | 78 |
| HPR1   | CCGGTCGTCGTGATTAGTGA | CGTAGAACGCGCTGCTT | 137 |
| GAPDH  | AGGTGGGCGGCTGATGATTGGT | GGAGTCATTGATGGCACAATA | 95 |
| RAGE   | ACTACCAGTGCCGTGCTTAC | GGAACACCACGGTGAGTT | 79 |
The geometric mean of the expression levels of the endogenous controls HPRT1, GAPDH, and PPIA was used for normalization purposes. Data analysis was carried out with LinReg v2013.0.35,36

2.6 | Fatty acid uptake

Caco-2 cells were differentiated in 96-well plates and starved with serum-free growth medium for 1 h prior to treatment with CML (0.05-500 μM) or glyoxal (0.03-30 μM) for 30 min at 37°C and 5% CO₂ in a humidified incubator. Nonivamide (100 μM) served as a positive control in this experiment.37 Subsequently, 100 μL of QBT fatty acid uptake (Molecular devices) loading buffer were added and the emission at 515 nm recorded for 60 min in kinetic cycles of 20 s each.

2.7 | Glucose uptake

Glucose uptake was assessed by 2-deoxy-2-((7-nitro-2,1,3-benozaxadiazol-4-yl)amino)-D-glucose (2-NBDG) assay as described previously.34 Briefly, fully differentiated Caco-2 cells in 96-well plates were starved with DMEM without phenol red, glucose or any additives for 1 h prior to treatment with the test compounds. After treatment with CML (5-500 μM) or glyoxal (0.03-30 μM) for 30 min, 50 μL 2-NBDG at a final concentration of 200 μM were added and incubated for another 30 min in the dark. The plate was then washed with ice-cold PBS three times. The emission of 2-NBDG taken up by the cells was recorded at 550 nm in 100 μL PBS.

2.8 | Serotonin release

Serotonin release was measured as described previously.37 Briefly, Caco-2 cells were differentiated in 12-well plates, washed with pre-warmed PBS and incubated with 150 μL test compound (CML 0.5-500 μM, glyoxal 0.03-30 μM, cinnamaldehyde 5 mM, clorgyline 50 nM, 0.1% ethanol or 0.1% DMSO as solvent control) or Krebs-Ringer-HEPES buffer (pH 7.4) for 5 min in the dark. All experiments using the RAGE antagonist FPS-ZM1 were carried out by co-incubating 10 μM of the antagonist with the corresponding test compound. In case of the co-incubation with phloridzin, 500 μM of the inhibitor were used.37 The supernatant was then collected and used in a serotonin-sensitive ELISA (DLD Diagnostika, Hamburg, Germany).

2.9 | Statistical analysis

Statistical analysis was carried out using SigmaPlot 11. Data are displayed as average with the corresponding standard deviation of at least three biological replicates. Controls were set to 1 or 100% and all treatments were calculated in relation to control samples, denominated T/C (treated over control) in the figures.

3 | RESULTS

3.1 | MTT assay

The assessment of effects of glyoxal and CML on cellular metabolic activity was carried out on the basis of the reduction of the yellow MTT tetrazole to purple formazan crystals by metabolically active cells. In this experiment, no statistical significant differences between treatment with glyoxal (0.03-30 μM) or CML (0.05-500 μM) and the untreated control were observed (P > 0.05, data not shown).

3.2 | DNA microarrays

Customized DNA microarrays, containing probes for all known human genes in addition to 3-16 probes for several genes associated with the regulation of satiety, were used in initial experiments for the identification of a subset of genes for further analysis.33 In the microarray experiments presented in this work, differentiated Caco-2 cells were treated with 3 μM glyoxal or 500 μM CML and fold-changes to untreated control calculated. Figure 1 shows scatterplots of log₂ intensities of the treatment, either CML or glyoxal, versus log₂ intensities of the corresponding controls. Probes reaching fold-changes above 1.2 up- or down-regulation in all three biological replicates were chosen and subsequently entered into the DAVID web tool for pathway analysis. A total of 1481 DAVID IDs were found to fit these criteria after CML treatment while 813 DAVID IDs fell within the cut-off range after glyoxal treatment. Clusters reaching an enrichment score above 1.3 were treated as potentially interesting for further study. In both cases, for CML and glyoxal, the first cluster encompassed genes associated with amine receptor activity (Table 2). Similar enrichment scores of 6.9 and 6.7 were observed for cases, for CML and glyoxal, the first cluster encompassed genes associated with the regulation of satiety, were used in initial experiments for the identification of a subset of genes for further analysis.33 In the microarray experiments presented in this work, differentiated Caco-2 cells were treated with 3 μM glyoxal or 500 μM CML and fold-changes to untreated control calculated. Figure 1 shows scatterplots of log₂ intensities of the treatment, either CML or glyoxal, versus log₂ intensities of the corresponding controls. Probes reaching fold-changes above 1.2 up- or down-regulation in all three biological replicates were chosen and subsequently entered into the DAVID web tool for pathway analysis. A total of 1481 DAVID IDs were found to fit these criteria after CML treatment while 813 DAVID IDs fell within the cut-off range after glyoxal treatment. Clusters reaching an enrichment score above 1.3 were treated as potentially interesting for further study. In both cases, for CML and glyoxal, the first cluster encompassed genes associated with amine receptor activity (Table 2). Similar enrichment scores of 6.9 and 6.7 were observed for CML and glyoxal, respectively. Closer examination of the cluster revealed the presence of serotonin receptors 1A and 2A.

3.3 | qPCR

As a result of the DNA microarray experiments, serotonin receptors 1A, 2A, and 1B were further investigated in time-course qPCR experiments. For this purpose, differentiated Caco-2 cells were incubated with 3 μM glyoxal or 500 μM CML for 30, 60, or 90 min. After treatment with CML for 60 min, expression of 5HTR2A, 5HTR1B, and 5HTR1A was up-regulated. Likewise, effects were observed after treatment with glyoxal for 60 min. Furthermore, treatment with CML and glyoxal showed effects on CD36, FATP2, and SGLT1 expression. However, neither compound had any impact on
RAGE expression at the tested time points. Table 3 shows the fold-changes observed after treatment with CML (3A) or glyoxal (3B) for the corresponding gene. The fold-changes demonstrated in Table 3 were calculated in relation to cells receiving serum-free medium only (set to 1), for these fold-changes of 1.00 ± 0.05 were typically found.

### 3.4 | Serotonin release

Cinnamaldehyde, a compound shown to stimulate serotonin release from enterochromaffin cells,\(^\text{39}\) was used as positive control. Incubation with 5 mM cinnamaldehyde increased serotonin release to 2122 ± 1851\% \( (P < 0.05 \text{ vs control}) \). Treatment with both CML and glyoxal stimulated serotonin release in relation to control cells treated with Krebs-Ringer-HEPES buffer (0.1\% ascorbic acid, pH 7.4) only (Figure 2). Incubation with 50 \( \mu \text{M} \) and 500 \( \mu \text{M} \) CML increased serotonin release to 289 ± 189\% \( (P < 0.05 \text{ vs control}) \) and 236 ± 111\% \( (P < 0.05 \text{ vs control}) \). After incubation with 0.3, 3, and 30 \( \mu \text{M} \) glyoxal, serotonin release reached levels of 264 ± 66\% \( (P < 0.05 \text{ vs control}) \), 246 ± 96\% \( (P < 0.05 \text{ vs control}) \), and 190 ± 27\% \( (P < 0.05 \text{ vs control}) \), respectively. Co-incubation with the RAGE antagonist FPS-ZM1, decreased the CML-induced serotonin release by 34\% (CML set to 100\%, Figure 3A). The co-incubation with phloridzin reduced CML-stimulated serotonin release by 32\% on average (CML set to 100\%, \( P < 0.05 \text{ vs CML, Figure 3B} \)). The incubation with FPS-ZM1 or phloridzin alone did not have an impact on serotonin release \( (P > 0.05, \text{data not shown}) \). Incubation with the MAO-A inhibitor clorgyline for 5 min did not alter serotonin release compared to control treatment only \( (82.8 ± 27.0\%, \text{control: 100.0 ± 9.96, } t\text{-test, } n = 3) \).

### 3.5 | Fatty acid uptake

The ability of CML (0.05-500 \( \mu \text{M} \)) and glyoxal (0.03-30 \( \mu \text{M} \)) to alter long-chain fatty acid uptake by differentiated Caco-2 was assessed using fluorescently labeled BODIPY-C12. Nonivamide, which was used as positive control, decreased BODIPY-C12 uptake to 58.5 ± 5.4\% \( (P < 0.05 \text{ vs control}) \) compared to control cells. Treatment with 3 \( \mu \text{M} \) glyoxal also resulted in reduced fatty acid uptake \( (87.6 ± 7.6\%; P < 0.05 \text{ vs control}) \). None of the other glyoxal concentrations tested showed statistically significant results (data not shown). Similarly,

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**TABLE 2** Cluster with the highest enrichment score after DAVID pathway analysis, probes reaching fold-changes below 0.8 or above 1.2 in all three biological replicates were used in the analysis

| A: Cluster 1, enrichment score: 6.91 | \( P \)-value | Benjamini |
|--------------------------------------|--------------|-----------|
| Amine receptor activity              | 3.6 \( \times \) \( 10^{-11} \) | 4.0 \( \times \) \( 10^{-8} \) |
| Serotonin receptor activity           | 4.2 \( \times \) \( 10^{-11} \) | 2.3 \( \times \) \( 10^{-8} \) |
| Serotonin binding                    | 1.1 \( \times \) \( 10^{-8} \) | 4.2 \( \times \) \( 10^{-6} \) |
| Serotonin receptor                    | 4.9 \( \times \) \( 10^{-7} \) | 8.9 \( \times \) \( 10^{-4} \) |
| Amine binding                        | 2.2 \( \times \) \( 10^{-4} \) | 1.3 \( \times \) \( 10^{-1} \) |

| B: Cluster 1, enrichment score: 6.7  | \( P \)-value | Benjamini |
|--------------------------------------|--------------|-----------|
| Neuroactive ligand-receptor interaction | 1.3 \( \times \) \( 10^{-18} \) | 1.8 \( \times \) \( 10^{-16} \) |
| Amine receptor activity              | 8.0 \( \times \) \( 10^{-15} \) | 6.4 \( \times \) \( 10^{-12} \) |
| Second-messenger-mediated signaling  | 3.7 \( \times \) \( 10^{-14} \) | 4.9 \( \times \) \( 10^{-11} \) |
| Regulation of cAMP metabolic process | 1.0 \( \times \) \( 10^{-10} \) | 3.8 \( \times \) \( 10^{-8} \) |
| cAMP-mediated signaling              | 9.5 \( \times \) \( 10^{-10} \) | 1.8 \( \times \) \( 10^{-7} \) |

A, output after treatment with 500 \( \mu \text{M} \) CML for 90 min in relation to untreated control; B, output after treatment with 3 \( \mu \text{M} \) glyoxal for 90 min in relation to untreated control.
none of the CML concentrations (data not shown) used had significant impact on long-chain fatty acid uptake.

3.6 | Glucose uptake

The results of the glucose uptake experiments with the fluorescently labeled glucose analogue 2-NBDG are shown in Figure 4. Treatment with 0.12 and 1.2 μM serotonin significantly decreased glucose uptake, leading to values of 83.4 ± 9.0% (P < 0.05 vs control) or 85.3 ± 10.0% (P < 0.05 vs control), respectively. Both CML and glyoxal significantly influenced the uptake of glucose by differentiated Caco-2 cells. After treatment with 500, 50, or 5 μM CML, glucose uptake decreased to 81.3 ± 22.8% (P < 0.05 vs control), 80.7 ± 27.6% (P < 0.05 vs control), or 68.1 ± 12.7% (P < 0.05 vs control), respectively. Treatment with 0.03 μM or 0.3 μM glyoxal decreased glucose uptake to 77.3 ± 14.4% (P < 0.05 vs control) and 68.3 ± 20.9% (P < 0.05 vs control).

4 | DISCUSSION

The advanced glycation end product CML and its precursor glyoxal, both ingested as part of a Western diet, have been detected in plasma samples of healthy and compromised individuals, such as diabetics. In addition to higher plasma concentrations, increased AGE concentrations have been reported in the GI tract of streptozotocin-induced and inherited type 2 diabetic rats. More specifically, the epithelial cells of intestinal villi and crypts of diabetic rats have been shown to yield a stronger AGE-staining compared to healthy controls.
While glyoxal concentrations in foods are typically in the μg range, CML concentrations are in the mg range, reaching up to 85 mg per average portion size in some meat dishes. Although several studies indicate at least partial uptake of CML and its liberation from proteins below 1 kDa in size has been shown, transepithelial flux of CML across Caco-2 monolayers could not be demonstrated. However, free CML has been shown to remain predominately within Caco-2 cells after uptake and consequent release from glycated dipeptides. Free CML has been detected in both plasma and urine samples, revealing plasma levels of free CML of up to 100 nM. Furthermore, a recent study by Li et al suggested conversion of free exogenous CML to protein-bound CML in vivo, concluding that long-term exposure of rats on a high fat diet to high levels of free CML may put them at risk for degenerative diseases, for example, diabetes mellitus or Alzheimer's disease. As most studies focus on AGEs bound to proteins, but some reports indicate effects of free AGEs as well, we aimed at investigating the effect of free, exogenous CML, and its precursor glyoxal on fully differentiated Caco-2 cells. First, we conducted a genome-wide screening using a customized microarray as there is no reference on the genome-wide effects of CML and glyoxal in intestinal cells. For both compounds, the cluster reaching the highest enrichment score after pathway analysis with DAVID, consisted of genes related to second-messenger-mediated signaling, specifically serotonin receptors. Consequently, we analyzed the expression of 5HTR1A, 5HTR2A, and 5HTR1B in a time-course qPCR experiment, showing effects of both CML and glyoxal on the expression of these receptors, predominately after incubation for 60 min. As Caco-2 cells have been shown to synthesize and degrade serotonin and have been reported capable of releasing serotonin, we next conducted serotonin release experiments. In order to exclude serotonin degradation to influence our serotonin release experiments, we incubated Caco-2 cells with 50 nM of the MAO-A inhibitor clorgyline. However, no significant difference between control and MAO-A inhibitor treatment

**FIGURE 3** Serotonin release after co-incubation with RAGE antagonist FPS-ZM1 (A) or SGLT-1 inhibitor phloridzin (B) compared to serotonin release after incubation with CML only. Data shown as average ± standard deviation in relation to CML treatment (500 μM) only, at least three independent biological replicates. Statistics: Student's t-test, * = P-value < 0.05 (A) or Mann-Whitney U t-test, * = P-value < 0.05 (B)

**FIGURE 4** Glucose uptake by differentiated Caco-2 cells after incubation with 0.12-1200 μM serotonin (A), 5-500 μM CML (B), or 0.03-3 μM glyoxal (C). Data are shown as average ± standard deviation in relation to untreated control (T/C) with at least three independent biological replicates. Statistics: Kruskal-Wallis one-way ANOVA on ranks, multiple comparisons versus control group (Dunn's method) * = P-value ≤ 0.05
was observed, indicating serotonin degradation to be negligible in the brief incubation window of 5 min. Incubation with CML or glyoxal for 5 min, led to significant increases in serotonin release. We have previously reported treatment with 500 μM CML to increase serotonin release from SH-SY5Y cells, used as a model system for central serotonin release. Circulating serotonin has been reported to have effects on lipid metabolism and peripheral glucose levels. A study by Watanabe et al in which serotonin was administered to seven-week-old male C57BL/6 mice, revealed lower plasma triglyceride and cholesterol concentrations 30 min after administration. In addition, increased glucose plasma levels between 60 and 90 min after administration and inhibition of glucose uptake from blood to tissues by serotonin were reported. Furthermore, a study by Hajduch et al reported incubation of L6 myotubes with 1 nM-100 μM serotonin to increase 2-(1,2-3H)-deoxy-D-glucose uptake. In addition, the serotonin degradation product 5-hydroxyindole acetic acid has been indicated to decrease glucose uptake in isolated rat soleus muscle. While the studies mentioned above described effects of serotonin on glucose uptake, the impact of glucose on serotonin release has also been reported: A study by Kim et al using enterochromaffin BON cells, demonstrated that incubation with glucose stimulates serotonin release from intracellular stores. The stimulation of serotonin release by incubation with glucose has very recently also been shown in Caco-2 cells, a study in which homoeriodictyol induced serotonin release and glucose uptake in a SGLT-1 mediated manner.

As a result of these reports, we analyzed the expression of GLUT-2 and SGLT-1 in qPCR experiments, carried out glucose uptake measurements and analyzed serotonin release after co-incubation with the SGLT-1 inhibitor phloridzin. First, we assessed the impact of serotonin on glucose uptake. This experiment revealed, contrary to the effects reported on L6 myotubes, serotonin to reduce glucose uptake in differentiated Caco-2 cells. Treatment with CML starting at concentrations of 5 μM as well as treatment of glyoxal at 0.03 and 0.3 μM led to a decrease in glucose uptake. In both cases, these concentrations are in a plasma relevant range, as plasma concentrations with glyoxal center around 0.3 μM and CML plasma concentrations of up to 12 μM have been observed in individuals suffering from type 1 diabetes with impaired renal function. Furthermore, CML influenced SGLT-1 expression after 30 and 60 min incubation. AGEs have been associated with alternations in glucose uptake in previous reports. By using AGE-BSA, Unoki et al demonstrated reduced basal and insulin-stimulated glucose uptake in 3T3-L1 cells. Similarly, we observed decreased basal glucose uptake after treatment of 3T3-L1 cells with 50 μM free, exogenous CML in a previous study. The here presented experiment using the SGLT-1 inhibitor phloridzin showed reduced serotonin release after co-incubation with the inhibitor and CML as compared to CML treatment only. This is in agreement with the effects observed after treatment with homoeriodictyol and philoridzin, further pointing to a link between serotonin release and glucose.

In terms of assessing the effects of CML and glyoxal on fatty acid uptake, we noticed a slight decrease of fatty acid uptake after treatment with 3 μM glyoxal. However, none of the other concentrations tested produced any significant results, nor did the treatment with any of the CML concentrations applied. However, in both cases, we observed significant changes in the expression of CD36 and FATP2 after 60 min of treatment. Unlike our previous study on SH-SY5Y cells, we did not observe any changes in RAGE gene expression at any of the tested time points. AGE-modified proteins, the major epitope of which has been identified to be CML, have been demonstrated to induce RAGE activation, thereby modulating gene expression. Although failure of free CML to engage RAGE has been reported, from our experiments, an activation of RAGE after incubation with free CML cannot be excluded. However, the cellular fate of the administered CML should be considered carefully, as Caco-2 cells have been reported to not take up CML and conversion of free CML to protein-linked CML has been suggested. Regardless of free CML's lack of stable RAGE binding, several reports indicate a link between protein-free CML and RAGE activation. A study by Zill et al on HEK-293 cells expressing full length RAGE revealed both casein-CML and protein-free CML to lead to an increase in p38 MAPK activation. Similarly, in HEK-293 cells expressing only a C-terminally truncated version of the receptor, thus lacking the cytosolic domain required in intracellular signaling pathways, a RAGE-related pathway not only for protein-bound but also for free CML was indicated. Also using HEK-293 cells, a reduction of MAP kinase activation in cells only expressing the truncated version of the receptor for both casein-linked and free CML was shown by Somoza et al. Schmid et al conducted experiments on Caco-2 cells, utilizing both free and casein-bound CML. This study on the expression of heat shock proteins also suggests a RAGE-mediated pathway in both cases. To gain some mechanistic insight, we performed experiments using the RAGE antagonist FPS-ZM1. This tertiary amide is a highly affine blocker of RAGE V-domain-mediated ligand binding. The experiment using FPS-ZM1 showed CML treatment attenuated serotonin release, suggesting RAGE involvement. As it has been reported that free CML is not actively taken up by Caco-2 cells, CML may interact with transmembrane receptors like RAGE to produce intracellular effects. Our current study suggests RAGE-involvement in the signaling cascade leading to CML-induced serotonin release. However, further studies are required to elucidate this pathway. A study by Collison et al suggests a link between Ca²⁺ mobilization, which has been
shown to mediate serotonin release, and RAGE activation. In our previous study on SH-SY5Y cells, we were able to show increases in Ca\(^{2+}\) mobilization and an up-regulation in RAGE gene expression which was blocked after pre-incubation with neomycin, a compound known to inhibit Ca\(^{2+}\) release from ryanodine-sensitive and IP3 intracellular calcium stores. Consequently, further experiments may be carried out on Ca\(^{2+}\) mobilization and other second messengers like cAMP, in addition to experiments with either RAGE knock-down cells or cells lacking the cytosolic domain of RAGE. Additionally, further studies on the link between glucose uptake, serotonin release, and the involvement of RAGE should be carried out.

In conclusion, our findings show decreased glucose uptake and increased serotonin release from Caco-2 cells after treatment with both CML and glyoxal. The reduction in CML-induced serotonin release by the RAGE antagonist FPS-ZM1 suggests a RAGE-mediated pathway. Whether the CML-/glyoxal-induced serotonin release plays a role in serotonin-mediated gastric motility has to be elucidated in future studies.

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CONFLICTS OF INTEREST

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