Reactive dicarbonyl compounds cause Calcitonin Gene-Related Peptide release and synergize with inflammatory conditions in mouse skin and peritoneum

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The plasmas of diabetic or uremic patients and of those receiving peritoneal dialysis treatment have increased levels of the glucose-derived dicarbonyl metabolites like methylglyoxal (MGO), glyoxal (GO), and 3-deoxyglucosone (3-DG). The elevated dicarbonyl levels can contribute to the development of painful neuropathies. Here, we used stimulated immunoreactive Calcitonin Gene–Related Peptide (iCGRP) release as a measure of nociceptor activation, and we found that each dicarbonyl metabolite induces a concentration-, TRPA1-, and Ca2+-dependent iCGRP release. MGO, GO, and 3-DG were about equally potent in the millimolar range. We hypothesized that another dicarbonyl, 3,4-dideoxyglucosone-3-ene (3,4-DGE), which is present in peritoneal dialysis (PD) solutions after heat sterilization, activates nociceptors. We also showed that at body temperatures 3,4-DGE is formed from 3-DG and that concentrations of 3,4-DGE in the micromolar range effectively induced iCGRP release from isolated murine skin. In a novel preparation of the isolated parietal peritoneum PD fluid or 3,4-DGE alone, at concentrations found in PD solutions, stimulated iCGRP release. We also tested whether inflammatory tissue conditions synergize with dicarbonyls to induce iCGRP release from isolated skin. Application of MGO together with bradykinin or prostaglandin E2 resulted in an overadditive effect on iCGRP release, whereas MGO applied at a pH of 5.2 resulted in reduced release, probably due to an MGO-mediated inhibition of transient receptor potential (TRP) V1 receptors. These results indicate that several reactive dicarbonyls activate nociceptors and potentiate inflammatory mediators. Our findings underline the roles of dicarbonyls and TRP1 receptors in causing pain during diabetes or renal disease.

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2 The abbreviations used are: MGO, methylglyoxal; GO, glyoxal; 3-DG, 3-deoxyglucosone; iCGRP, immunoreactive Calcitonin Gene-Related Peptide; PD, peritoneal dialysis; GO, glyoxal; 3-DG, 3-deoxyglucosone; iCGRP, immunoreactive Calcitonin Gene-Related Peptide; 3,4-DGE, 3,4-dideoxyglucosone-3-ene; PD, peritoneal dialysis; VGSC, voltage-gated sodium channel; AUC, area under the curve; ANOVA, analysis of variance; SIF, synthetic interstitial fluid; BK, bradykinin; PGE2, prostaglandin E2; TRP, transient receptor potential; EIA, enzyme-immunoassay; UHPLC, ultrahigh performance liquid chromatography; DAPI, 4',6-diamidino-2-phenylindole; STZ, streptozotocin; CGRP, Calcitonin Gene-Related Peptide; DRG, dorsal root ganglion; LSD, least significant difference test.

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an issue of ongoing debate (8, 13). Recently, another study identified high levels of methylglyoxal as a relevant risk factor for the development of diabetic polyneuropathy (14).

Similarly, glyoxal and 3-DG are also reactive metabolites, but there is little information whether these disease-regulated dicarbonyls contribute to activation of the nociceptive system and thereby act in concert with MGO. Up to now, only glyoxal has been investigated in TRPA1-transfected HEK cells but was ineffective in stimulating intracellular calcium increase (15).

In addition, 3,4-dideoxyglucosone-3-ene (3,4-DGE) is a highly-reactive α,β-unsaturated dicarbonyl compound found in fluids used for peritoneal dialysis (PD), which achieves blood purification in patients with renal failure. To exert hyperosmotic effects of classical inflammatory conditions. We propose that this group of dicarbonyl compounds contributes to the development of pain when they are elevated during diabetes mellitus or renal disease.

**Results**

Methylglyoxal-, glyoxal-, and 3-deoxyglucosone-induced iCGRP release

MGO, GO, and 3-DG were all found to activate peptidergic nociceptors and, by that, release iCGRP in a concentration-dependent manner. With regard to MGO, we reproduced published data (11) by showing that concentrations of 1, 3, and 5 mM lead to significant increases in iCGRP release compared with baseline levels (n = 18, 14, and 8, all p < 0.02, Wilcoxon tests, respectively; Fig. 1A). When looking at the overall release (depicted as AUC in picograms/ml), concentration dependence could be confirmed (one-way ANOVA F(2,37) = 10.9, p < 0.001; LSD post hoc p < 0.01 for both effects indicated). Likewise, GO induced a significant increase in iCGRP at 3, 5, and 10 mM (n = 8; p = 0.04, n = 7; p = 0.02 and n = 8; p = 0.02; all Wilcoxon tests, respectively, Fig. 1B), whereas at 1 mM (n = 7) no iCGRP release could be observed. The overall release induced by GO (depicted as AUC in picograms/ml) illustrates the concentration-dependent effect (one-way ANOVA F(2,20) = 19.7, p < 0.001; LSD post hoc p < 0.001 for both effects indicated). Similar to MGO, GO-induced neuropeptide release from peptidergic fibers depends on extracellular calcium and was therefore lacking at 5 and 10 mM concentrations using calcium-free solutions with EGTA (n = 4 both, Fig. 1B, right panel). In addition, GO-induced iCGRP release depended on TRPA1 receptor activation because GO at 3 and 10 mM did not induce any increase of iCGRP release in preparations from TRPA1 knockout mice (n = 6, n = 5, respectively, Fig. 1B, right panel). In skin preparations from TRPV1 knockout mice, 10 mM GO caused a significant iCGRP release that was not different from control C57BL/6J mice (n = 6, Fig. 1B, right panel) indicating that an activation of TRPV1 receptors by GO can be excluded. Interestingly, in contrast to MGO-induced release, GO-induced iCGRP release was not reversible within 5 min of washout.

3-DG significantly increased iCGRP from isolated skin preparations at 5 mM concentration (n = 12; p = 0.01; Wilcoxon test), whereas 1 and 3 mM 3-DG did not show any effect (both n = 10, Fig. 1C). The difference of the overall release (depicted as AUC in picograms/ml) between 3 and 5 mM 3-DG was significant (one-way ANOVA F(1,20) = 12.0, p < 0.01). This low reactivity motivated us to retest 3-DG in a desheathed vagus nerve preparation exhibiting high reactivity with respect to TRPA1 agonists (29). However, only 5 mM 3-DG induced a significant increase of iCGRP release (n = 6, p = 0.03; Wilcoxon test, in the figure the overall release as AUC is depicted, Fig. 1C, right panel). Using the vagus nerve preparation, we also found that 3-DG-induced iCGRP release depended on extracellular calcium (n = 4) and TRPA1, as it could not be detected when either extracellular calcium was missing or preparations from knockout mice (n = 6) were stimulated with 5 mM 3-DG (depicted as AUC in picograms/ml, Fig. 1C, right panel). In vagus preparations from TRPV1 knockout mice, we found an increased overall release (depicted as AUC in picograms/ml, Fig. 1C, right panel) compared with the control conditions (one-way ANOVA F(1,9) = 4.9, p = 0.05). Interestingly, the iCGRP release induced by 3-DG was also sustained in isolated skin preparations, similar to GO stimulation.
Comparing the acute effects of all three dicarbonyls tested so far, defined as increase over baseline level after a 5 mM stimulation, MGO and GO exhibited a comparable efficacy (2.7- and 2.8-fold increase over baseline level, respectively) in contrast to the weaker 3-DG that only resulted in a 1.9-fold increase in iCGRP over baseline. The lower reactivity of 3-DG could be due to intramolecular formation of hemiacetals or -ketals, which block the reactive carbonyl groups (30).

Temperature-dependent formation of 3,4-DGE from 3-DG in vitro

At high temperatures applied during heat sterilization of PD fluids, 3-DG is degraded, and 3,4-DGE is formed. A reversible conversion of 3-DG into 3,4-DGE has been observed (31). In human plasma, 3,4-DGE has not been detected yet, but the formation in vivo by dehydration of 3-DG is conceivable. We investigated whether 3-DG could be a source of 3,4-DGE also at a physiological temperature of 37 °C and could thus serve as a pool to release 3,4-DGE into the tissues. Thus, we measured in vitro the newly-formed 3,4-DGE in SIF solutions that contained freshly-synthesized 3-DG at a concentration of 375 μM at the beginning of the experiment. Half of these solutions were incubated at 37 °C and the other half at 39 °C to simulate normal body temperature or febrile conditions, respectively. As a control, SIF was also incubated without 3-DG. First of all, we could gain satisfying recovery rates for the quantification of 3-DG and 3,4-DGE in SIF (100.4% (n = 3) for 3-DG and 100.2% (n = 1) for 3,4-DGE, respectively), proving that the applied method was suitable for the analysis of the SIF-based samples. After 1 h at 37 °C, 0.3 ± 0.02 μM 3,4-DGE was detected, and over time a further increase of the 3,4-DGE formation occurred, although no 3,4-DGE was detectable in the control, confirming that the formed 3,4-DGE originates from 3-DG. When the same experiment was conducted at 39 °C, we detected 0.4 ± 0.04 μM 3,4-DGE after 1 h, and again a distinct increase occurred over time. These results show that a temperature difference of 2 °C has a
considerable effect on the transformation of 3-DG into 3,4-DGE (repeated measures ANOVA $F(3,12) = 8.7, p < 0.01, p < 0.05$ indicating the overall temperature effect, Fig. 2A, right diagram). After 24 h, a concentration of $-2.5 \mu M$ 3,4-DGE was measured at both temperatures, which accounts for about 0.6% of the initial 3-DG concentration. Simultaneously, we also analyzed 3-DG in the same samples and observed a continuous and linear degradation of the 3-DG content, which was also temperature-dependent (repeated measures ANOVA $F(4,16) = 26.0, p < 0.001, p < 0.02$ indicating the overall temperature effect, Fig. 2A, left panel). At 39 °C, the degradation was faster as indicated by a stronger decrease of the 3-DG content in the samples over time. These results show that 3,4-DGE is spontaneously formed from 3-DG at a physiological temperature in vitro, and its formation is accelerated at a higher temperature, e.g. during febrile states. Thus, it can be suggested that the 3-DG pool is a source for 3,4-DGE in the human body. Comparing the amount of degraded 3-DG at 24 h ($126.3 \pm 4.14 \mu M$ at 37 °C, $167.5 \pm 2.68 \mu M$ at 39 °C) with the amount of formed 3,4-DGE after 24 h ($2.4 \mu M$ at 37 and 39 °C, respectively) obviously shows that the 3-DG degradation prevails over the slight 3,4-DGE formation. We assume that some additional degradation products are formed that are not detected by the applied UHPLC method.

### 3,4-DGE-induced iCGRP release from skin preparations

Subsequently, we investigated whether the $\alpha,\beta$-unsaturated dicarbonyl compound 3,4-DGE activates nociceptors. It has already been shown that 3,4-DGE has a high-glycating activity and modifies proteins that result, for example, in the inactivation of enzymes (17). 3,4-DGE reacts specifically with cysteine residues so that it is an interesting candidate to modify TRPA1 receptors and, by that, activate nociceptors (32). We stimulated skin preparations with concentrations of 30 and 100 $\mu M$ 3,4-DGE and found significant increases in iCGRP release (both $n = 6, p = 0.03$ Wilcoxon tests, respectively, Fig. 2B). The overall release (depicted as AUC in picograms/ml) was concentration-dependent (one-way ANOVA $F(1,10) = 45.9, p < 0.001$). The high reactivity of 3,4-DGE is evident when compared with the stimulatory effects of 5 mM MGO, GO, and 3-DG resulting in a 2.7-, 2.8-, and 1.9-fold increase over baseline. In contrast, an about 14-fold increase of iCGRP release over baseline was observed for 100 $\mu M$ 3,4-DGE, which is a 50-fold lower concentration. The effect was also not reversible within a 5-min washout.

### CGRP-immunoreactive nociceptive nerve fibers in the parietal peritoneum

In peritoneal dialysis fluids, 3,4-DGE is found after heat sterilization, and it is known to be an important reactive component that causes a variety of local and probably also systemic effects (5, 19). During dialysis, the peritoneum is in direct contact with the dialysis fluids, and therefore, we investigated the impact of 3,4-DGE and a commercial PD fluid on nociceptors in a preparation of the parietal peritoneum.

The parietal peritoneum receives sensory innervation from phrenic and spinal viscero-somatic nerves that form a dense network in the submesothelial tissue. We observed CGRP immunoreactive fibers (Fig. 2A and B, red) in the parietal peritoneum of the mouse. Frequently, we found thick fiber bundles (Fig. 3A, arrowhead) meandering in parallel with the muscle fibers (asterisk). At a higher resolution, a homogeneous staining of thin filaments could be observed (Fig. 3B, arrowhead). Additional nuclear DAPI-staining (in blue) detected CGRP-positive cells (Fig. 3B, asterisk) within the peritoneum. We did not further identify these cells but propose that they are immune cells that appear CGRP-positive (33).

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**Figure 2.** Temperature-dependent dehydration of 3-deoxyglucosone to 3,4-DGE that effectively releases iCGRP from isolated skin. A, time courses of the formation of 3,4-DGE from 3-DG (defined start concentration) at 37 or 39 °C. Samples were taken at the depicted time points (0–24 h). On the left, the time course of degradation of 3-DG in the same solution stored at 37 and 39 °C is depicted. The experiment was performed in triplicate, mean value ± S.E. Structural formulas depict the proposed temperature-dependent conversion between 3-DG and 3,4-DGE. Abbreviation used: cis-3,4-DGE, cis-isomer of 3,4-dideoxyglucosone-3-ene (31). B, time course of 3,4-DGE-induced iCGRP release from isolated hairy skin of C57BL/6J WT mice. On the right, overall release induced by 3,4-DGE is illustrated (AUC in picograms/ml, means ± S.E.). Utilized concentrations and number of individual experiments are as indicated below the columns on right. *p < 0.05, significant effect of 3,4-DGE.
3,4-DGE induced iCGRP release from the peritoneum

Subsequently, the parietal peritoneum was functionally characterized by measuring iCGRP release following stimulation of the abdominal surface of the isolated peritoneum with nociceptor-specific TRPV1 and TRPA1 agonists (for the experimental setting, see Fig. 3C). Capsaicin (Cap) and mustard oil (MO) stimulation was first used to functionally test for the expression of TRPV1 and TRPA1 receptors. We also tested commercially-available dialysis fluid to stimulate the parietal surface of the peritoneum similar to the situation during dialysis. D, capsaicin stimulation elicits more iCGRP than MO stimulation, as illustrated by the overall release (AUC in picograms/ml, means ± S.E.) in columns at right. Likewise, 3,4-DGE, which is found in peritoneal dialysis fluids, activates peritoneal nociceptors and releases iCGRP into the elution chamber (E, time course of release). The release was again calcium- and TRPA1-dependent (right panel shows the overall release as AUC in picograms/ml, means ± S.E., for the color code of the columns, please see Fig. 1 legend). *, p < 0.05, significant stimulatory effect of capsaicin, MO, or 3,4-DGE.

Table 1

|                   | MGO  | GO   | 3-DG | 3,4-DGE |
|-------------------|------|------|------|---------|
| Concentrations of the main dicarbonyls in a commercially available heat-sterilized peritoneal dialysis fluid expressed in micromoles/liter, mean ± S.E. (n = 3) |      |      |       |         |
|                   | 9.8 ± 0.09 | 19.3 ± 0.13 | 251.5 ± 0.31 | 11.2 ± 0.03 |

The stimulation of the peritoneum with this solution resulted in a significant increase in iCGRP release into the eluate (n = 6, p = 0.03, Wilcoxon test, in the figure the overall release as AUC is depicted, Fig. 3D, right panel).

Also, when 3,4-DGE was applied onto the peritoneal surface in concentrations comparable with values measured in the peritoneal dialysis fluid, a significant and reversible release of iCGRP was observed (n = 6, p = 0.04 and n = 6, p = 0.03, Wilcoxon tests, respectively, Fig. 3D). Additionally, we tested the effect of a commercially available heat-sterilized PD solution that contained concentrations of the glucose degradation products as listed in Table 1.
from Table 1), we found a significant and, in this case, reversible iCGRP release. 10 and 100 μM (n = 5, p = 0.05, n = 9, p = 0.008, Wilcoxon tests, respectively, Fig. 3E) were about equally effective to release iCGRP. 3,4-DGE at 1 μM tended to induce only a small insignificant increase over baseline level (n = 5). Moreover, the 3,4-DGE–induced iCGRP release was found to be calcium- and TRPA1 receptor-dependent at 100 μM (data depicted as overall release AUC in picograms/ml, Fig. 3E). Using preparations from TRPV1 knockout mice, 3,4-DGE at 100 μM was even more effective in releasing iCGRP, but the difference to control preparations was not significant, so we excluded an activation of TRPV1 receptors by 3,4-DGE.

**Bradykinin and prostaglandin E₂ augment and low pH reduces MGO-induced iCGRP release**

We studied a possible interaction of inflammatory mediators with MGO at a threshold concentration of 1 mM, which by itself induced a small but significant increase of iCGRP (data taken from Fig. 1 and depicted in Fig. 4, A–C, black column in right panel, for comparison, respectively). As expected, 10 μM bradykinin alone induced a significant iCGRP release (n = 8; p = 0.01, Wilcoxon test, Fig. 4A). The combination of 10 μM bradykinin and 1 mM MGO provoked a clearly-augmented release compared with BK alone (n = 6; p = 0.03, Wilcoxon test). 100 μM prostaglandin E₂ (PGE₂) alone induced only a small but significant increase of iCGRP release (n = 8, p = 0.03, Fig. 4B). When PGE₂ and MGO were combined, iCGRP release was clearly increased compared with PGE₂ alone (100 μM PGE₂ and 1 mM MGO, n = 10, p < 0.01; Wilcoxon test, Fig. 4B). The overall iCGRP release (depicted as AUC in picograms/ml) induced by the combined stimuli, as compared with the respective single ones, showed a supra-additive effect for both inflammatory mediators: BK (one-way ANOVA F(2,29) = 24.4, p < 0.001; LSD post hoc p < 0.001, p < 0.001 for both comparisons depicted, Fig. 4A, right panel) and PGE₂ (one-way ANOVA F(2,33) = 8.4, p < 0.01; LSD post hoc p < 0.001, p < 0.01 for both comparisons depicted, Fig. 4B, right panel).
Different reactive dicarbonyls activate nociceptors

Low pH values occur in certain tissues under diabetic conditions (34). Therefore, we tested the combination of low pH with a dicarbonyl stimulus. The acidic SIF solutions at pH 6.1 (data not shown) and pH 5.2 alone resulted in significant pH-dependent iCGRP release over baseline (n = 6, p = 0.03; n = 7, p = 0.02, Wilcoxon tests, respectively, Fig. 4C). In a separate set of experiments, we tested stimulation at pH 5.2 in skin preparations, harvested from TRPV1 knockout mice, and confirmed that low pH-induced release depended on TRPV1 activation (data not shown). When MGO was dissolved at the threshold concentrations of 1 mM in acidic SIF solutions, MGO-induced release was not augmented either at pH 6.1 or pH 5.2. In contrast and most obviously at pH 5.2, the combined stimulus resulted in a strong and significant reduction of iCGRP release compared with the pH stimulation alone (one-way ANOVA F(2,29) = 37.7, p < 0.001; LSD post hoc p < 0.001 for the effect depicted, Fig. 4C, right panel). We assume that MGO may inhibit low pH-induced release similar to lactate (35).

Discussion

(MGO, GO, and 3-DG are the main reactive glucose metabolites, which cause dicarbonyl stress within the context of diabetes or chronic kidney disease (1, 3). It has already been shown that MGO activates the nociceptive system through TRPA1 receptor-channels (9, 11, 36). Here, we show that besides MGO, GO, 3-DG, and the α,β-unsaturated dicarbonyl compound 3,4-dideoxyglucosone-3-ene (3,4-DGE) can also activate nociceptors and therefore are all relevant candidates to contribute to acute pain or the development of painful neuropathy. We demonstrate that in vitro at a physiological temperature of 37 °C, 3-DG is partially dehydrated to 3,4-DGE (see Fig. 2A, Equilibrium). This process was accelerated at an elevated temperature of 39 °C that appears during fever or locally in inflamed tissue. From that, a transient and local formation of 3,4-DGE within the tissue could take place. We also investigated a synergistic effect of MGO with classical inflammatory mediators like BK or PGE2 and found a supra-additive stimulatory effect when both stimuli were combined. When solutions of low pH were combined with MGO, an inhibition of pH-induced iCGRP release was found, possibly due to a simultaneous inhibition of TRPV1 receptors. Together, these findings expand our knowledge on the effects of reactive dicarbonyl compounds alone and in combination with inflammatory tissue conditions on primary sensory neurons.

In this study, we employed stimulated CGRP release as a measure of nociceptor excitation; hence, we only studied effects on peptidergic sensory neurons. In the adult mouse, TRPA1 receptors are expressed in peptidergic as well as in nonpeptidergic sensory neurons (37), and there is no functional difference known between TRPA1 receptors in these different neuronal populations. Thus, the limitation of our study applies to effects related to nonpeptidergic nociceptors. However, the contribution of CGRP-positive nociceptors to the development of heat hyperalgesia in a model of neuropathic pain underlines the relevance of peptidergic nociceptors studied here (38).

Methylglyoxal, glyoxal, and 3-deoxyglucosone induce neuropeptide release at high concentrations

We applied MGO, GO, and 3-DG as short (5 min) incubation stimuli to preparations from healthy animals. Here high concentrations in the millimolar range were effective to stimulate iCGRP release. These millimolar concentrations are above plasma levels measured in patients suffering from diabetes or renal disease and therefore might be considered unphysiologically. In the plasma of diabetic or uremic patients, low micromolar concentrations are detected (8, 39–41). Altered sugar metabolism, loss of renal clearance, or an impaired glyoxalase system elevate reactive dicarbonyl concentrations, and the harmful effects can accumulate over time (1). Here, we used only a short-term incubation of the intact tissue preparations from healthy animals that caused a local and transient chemical stimulation. In such preparations, high (e.g. millimolar) dicarbonyl concentrations are needed to overcome the intact glyoxalase detoxification system (4). It has been shown that 75-fold of the physiologically measured MGO has to be injected i.p. into a healthy mouse to achieve an only transient increase of plasma MGO reaching levels found in diabetic mice (8). Also, passage through the cell membrane could be an important factor. Assuming that dicarbonyl compounds can pass cell membranes quite quickly (42, 43), we have measured MGO in the cytosol of cultured DRG neurons after a 3 mM MGO treatment lasting for only 90 s. Under these conditions, about 0.1% of the applied concentration could be detected in the cytosol (11). Similarly, myoblasts treated with 2.5 mM methylglyoxal incorporated only about 3% after 10 min of incubation (44). Transferring these observations to our experimental setting, we suppose that the amount of reactive dicarbonyl reaching the intracellular targets, e.g. the TRPA1-binding sites, after a 5-min–long high-millimolar application, could be in the concentration range of dicarbonyl compounds found under diabetic or uremic conditions, which we intended to mimic at least for a short period of time. Accordingly, similar studies investigating the effects of MGO in expression systems, cultured sensory neurons or other cell types, apply MGO in very high concentrations and report EC20 values in the high micromolar or even millimolar range (9, 45, 46).

C6-dicarbonyl compounds 3-DG and 3,4-DGE activate sensory neurons

We show that also C6-dicarbonyl compounds like 3-deoxyglucosone and the α,β-unsaturated 3,4-dideoxyglucosone-3-ene stimulate nociceptors and induce iCGRP release. To our knowledge, this is the first direct evidence that these C6-dicarbonyl compounds may also contribute to the development of painful neuropathy.

3-DG is formed by glucose degradation or the polyol pathway and can additionally pass from PD solutions into the circulation (47, 48). Consequently, its concentration is high in the plasma of diabetic and uremic patients, where it has been claimed to be responsible for various complications due to dicarbonyl stress (49). During heat sterilization of PD fluids, 3-DG is formed by thermal glucose degradation and is then partially converted to 3,4-DGE (5). Accordingly, a transformation of 3-DG into 3,4-
DGE can also be expected in vivo. Our data support this hypothesis and clearly show that 3-DG is converted into 3,4-DGE also at a physiological temperature. 3,4-DGE exhibits a high reactivity against different cellular targets resulting, for example, in the depletion of GSH, reduction of enzyme activity, or cytotoxic and immunosuppressive effects (17, 19, 50). Because of its additional double bond in αβ-position of a carbonyl group in the C6-carbon backbone, 3,4-DGE is an especially reactive dicarbonyl compound (32). In heat-sterilized peritoneal dialysis fluids, 3,4-DGE is found at a concentration of about 15 μM (5, 16), and during peritoneal dialysis, it penetrates the peritoneum and causes local inflammation or fibrosis (19). 3,4-DGE may reach peritoneal nociceptors and, by that, could contribute to acute abdominal pain, from which peritoneal dialysis patients suffer (51). If it is absorbed, it likely contributes to the development of painful uremic neuropathy (52). To further elucidate the effects of 3,4-DGE on peritoneal nociceptors, we developed a new preparation that allows us to directly apply stimulating solutions to the parietal surface of the isolated peritoneum (see Fig. 3C). Nociceptive termini and fibers in the peritoneum exhibit TRPA1 and TRPV1 receptors (53), and in our preparation, capsaicin and mustard oil stimulation consistently released iCGRP into the elution chamber (Fig. 3A). Notably, our data point to a lower sensitivity of peritoneal nociceptors to TRPA1-specific stimulation compared with TRPV1-specific stimulation. This result is consistent with the observation that capsaicin was about 25 times more potent than mustard oil in inducing abdominal nociception, when injected intraperitoneally (53). In the skin, 100 μM 3,4-DGE was more effective than in the peritoneum, probably due to a lower expression of TRPA1 in the latter. In the peritoneum, 10 μM 3,4-DGE augmented iCGRP release significantly, and this concentration is exactly in the same range as the levels analyzed in the dialysis fluid applied in present experiments. Our data functionally confirm the higher reactivity of the αβ-unsaturated 3,4-DGE (32), which is effective in the micromolar range compared with the other dicarbonyls that activate nociceptors and release iCGRP only in the millimolar range, when applied as short stimuli in vitro.

**MGO, GO, 3-DG, and 3,4-DGE activate TRPA1 receptors to release CGRP**

We and others have shown that MGO activates nociceptors by a covalent modification of cysteine and lysine residues located in the intracellular terminus of the TRPA1 receptor (9, 11, 36). This reaction pattern follows the well-established activation mechanism of TRPA1 receptors that has been identified for endogenous mediators, herbs, and chemical irritants (37). By using preparations of TRPA1 knockout animals, our data clearly indicate that GO, 3-DG, and 3,4-DGE also stimulate nociceptors in the skin, along the vagus nerve, and in the peritoneum by activation of TRPA1 receptors. Like MGO, GO modifies arginine, lysine, and cysteine residues (54). 3-DG directly reacts with lysine and arginine, whereas cysteines are only modified via the 3-DG–lysine glycation product, pyrraline (55). 3,4-DGE reacts with arginine (30) but prevalently with cysteine residues (17). Using a model peptide, a stable cysteine modification has recently been identified. It was also shown that 3,4-DGE exhibits a much higher glycation activity than 3-DG and 3-deoxygalactosone (32). Our experiments substantiate a higher efficacy of 3,4-DGE to release CGRP compared with MGO, GO, and 3-DG, probably due to a stronger or faster TRPA1 receptor activation by cysteine modification.

We further tested whether dicarbonyl compounds activate TRPV1 receptors. We have already shown that MGO does not excite nociceptors by a direct activation of TRPV1 receptor channels (11). The same mechanism was now found for GO, 3-DG, and 3,4-DGE in skin, vagus nerve, and peritoneum preparations using TRPV1 knockout animals. Interestingly, the C6-dicarbonyl compounds 3-DG and 3,4-DGE induced an even higher iCGRP release compared with C57BL/6J control animals when TRPV1 receptors were missing. We speculate that both C6-dicarbonyl compounds may modulate the TRPV1 receptor function and, by that, the interaction between TRPV1 and TRPA1 receptors. Mutual interactions causing an activation or inhibition of the respective other receptor have been described (56–58). If C6-dicarbonyl compounds modulate TRPV1 receptor function, a post-translational modification of cysteine residues is conceivable. Cysteine modification of TRPV1 receptors by different endogenous stimuli has already been shown. Oxidative stress induced a robust and long-lasting sensitizing effect via formation of disulfide bonds in the intracellular terminus (59). Likewise, reactive metabolites of acetaminophen activate and sensitize TRPV1 receptors by cysteine modification (60).

In addition, a functional interaction of the co-expressed TRPV1 and TRPA1 receptors has been observed (37). In isolated skin preparations, a mutual heterologous desensitization of capsaicin- and mustard oil-induced CGRP release has been described, which occurs via a calcium–calcineurin-dependent mechanism (61). C6-dicarbonyl compounds may also induce a similar cross-desensitization of TRPA1 receptors under control conditions that only becomes evident when the causative TRPV1 receptors are missing.

We also investigated the interactions of MGO with low pH, because acidosis occurs in inflamed tissue. In contrast to the results above, MGO inhibited TRPV1 under these conditions, indicating that dicarbonyl compounds may have additional effects on the TRPV1 receptor function (see below).

**Time course of neuropeptide release**

In skin preparations, MGO-induced CGRP release was reversible, while a sustained release occurred when GO, 3-DG or 3,4-DGE was applied. Here, CGRP levels remained elevated after the 5-min–long stimulation with the dicarbonyl compounds. This result may indicate a continuing activation of nociceptors or different permeation behavior through the membrane. Because of its additional methyl group, MGO is more lipophilic than GO and therefore could pass faster through the membrane. 3-DG and 3,4-DGE are both even more hydrophilic compared with GO due to their additional hydroxyl groups.

**Bradykinin and prostaglandin E2 over-additively augment MGO-induced release**

We have already shown that 4-HNE and PGJ2, both products of oxidative stress and effective TRPA1 agonists, exhibit a coop-
Different reactive dicarbonyls activate nociceptors

Operative effect with MGO on nociceptor activation (11). Besides the occurrence of oxidative stress, it is also known that diabetes produces inflammatory stress and releases classical inflammatory mediators by activation of the kallikrein system and phospholipase A_2/cyclooxygenase system within the cells (8, 62). We therefore mimicked the coincidence of inflammatory and diabetic conditions. When MGO at the threshold concentration to induce iCGRP release was co-applied with bradykinin or prostaglandin E_2, MGO responses were clearly augmented in a supra-additive manner. In healthy skin, bradykinin activates nociceptors directly by binding to constitutively expressed G protein- coupled B2 receptors (63) and releases neuropeptides like CGRP (64). In addition, bradykinin activates phospholipase A_2, probably through G protein- coupled receptors and, by that, augments prostaglandin production (63). We have shown that in healthy skin a short bradykinin stimulus causes an immediate increase of prostaglandin E_2 release (65). Our results clearly show that under physiological conditions the application of PGE2 sensitizes TRPA1-dependent MGO responses. PGE2 binds to G protein- coupled EP receptors (e.g. EP4) leading to PKA activation, and the sensitization of TRPA1 receptors by PKA-dependent phosphorylation is generally accepted (37). In addition, bradykinin can augment TRPA1-mediated responses also by an activation of bradykinin receptors that are coupled to phospholipase C (63).

Interaction with low pH

Tissue acidosis is common under diabetic and inflammatory conditions. In inflamed or ischemic tissues, increased proton concentration activates and sensitizes nociceptors and potentially causes pain by activation of human TRPA1 and TRPV1 channels (66). In preliminary stages of diabetic coma, accumulation of ketone bodies is accompanied by extracellular acidosis (67). In addition, experimental hyperglycemic hypoxia leads to extracellular acidification in sensory nerves (68). Schneider et al. (69) showed that under hyperglycemic hypoxia an increase of neuronal excitability of myelinated axons occurs by inhibition of potassium channels. Likewise, nociceptive C-fibers recorded from skin of STZ-diabetic rats are activated when their receptive fields are superfused with hyperglycemic and hypoxic solutions at neutral as well as acidic pH 5.7 (70).

We explored a possible interaction of low pH and MGO and therefore stimulated the skin with MGO solutions at low pH. Surprisingly, we found that pH 5.2-induced iCGRP release was significantly reduced by co-application of MGO compared with pH 5.2 alone, possibly by an interaction of MGO and protons at TRPV1 receptors. A direct activation of TRPV1 receptors by MGO can be excluded by the results of our experiments performed in TRPA1 knockout animals, when MGO-induced iCGRP release was abolished. However, the results of combined stimuli suggest an inhibition of TRPV1 receptors by MGO. We assume that this inhibition becomes evident when TRPV1 is simultaneously activated, e.g. by protons.

In mice, TRPV1 receptors expressed in cutaneous nociceptors are responsible for proton-induced activation (71). A separate set of experiments supports this mechanism by showing that low pH-induced iCGRP release is also reduced in skin from TRPV1 knockout mice (data not shown). Recently it has been shown that L-lactate is a potent inhibitor of TRPV1 receptors (35). Lactate inhibits TRPV1 receptors from the extracellular side and independently of the intracellular pH. It accumulates during ischemia in the working muscle reaching millimolar concentrations (72). Very similar to our experiments, L-lactate inhibits pH-induced iCGRP release from isolated sciatic nerves at pH 6.2 and 5.1. The mechanism showing how L-lactate inhibits TRPV1 receptors is not fully understood, but a conformational change of the lower gate is proposed (35). The possible mechanism of MGO-induced TRPV1 inhibition also requires further investigation.

Impact on the development of painful diabetic neuropathy

We showed that different reactive dicarbonyl compounds like MGO, GO, 3-DG, and 3,4-DGE activate the peripheral nociceptive system in preparations from healthy animals via TRPA1 receptor channels. Up to now, it is not clear how these effects are manifested under diabetic conditions, when tissue levels of diabetic or inflammatory metabolites are elevated and meet an altered excitability of sensory neurons. In diabetic patients and patients with uremia, levels of reactive dicarbonyl compounds are higher or fluctuate over time, while tissue pH is lowered.
Different reactive dicarbonyls activate nociceptors

The expression level and grade of sensitization of TRPA1 receptors under diabetic conditions will also be decisive for effects of dicarbonyl compounds. However, in this respect conflicting evidence is reported. TRPA1 expression in DRG neurons of STZ-diabetic rats was increased (74), although no change of TRPA1 mRNA in DRG neurons of STZ-treated rats and diabetic db/db mice was observed (75, 76). Behavioral tests for cold or heat hyperalgesia in diabetic animals conclusively suggest an important role of TRPA1 receptors (77–79).

In conclusion, we have shown that besides MGO, GO, 3-DG, and 3,4-DGE also activate nociceptors and have to be considered when the contribution of reactive dicarbonyl compounds to painful neuropathy is discussed. Under diabetic or uremic conditions, a possible link of dicarbonyl compounds and inflammatory stress may cause or perpetuate a hyperexcitable nociceptive system. Future experiments will investigate the effects of dicarbonyl compounds on nociceptors under experimental diabetic conditions.

Experimental procedures

Animals and preparations

Ethical approvals for breeding, euthanasia, animal handling, and experimental procedures were given by the Ethics Committee of the regional government (Würzburg, Germany). C57BL/6j control mice and all knockout mice were born and raised in the institute’s animal house. TRPA1^+/− mice were a kind gift of Drs. Kelvin Kwan and David Corey (81), and TRPV1^+/− mice were received from Dr. John Davis (82). The knockout strains were backcrossed to C57BL/6j every 3rd or 4th generation to maintain congenicity.

The hairy skin from both hind paws, both vagus nerves, or the peritoneum was harvested from adult mice after sacrificing them in a rising CO2 atmosphere as described previously (83, 29). For preparation of the mouse parietal peritoneum, the hairy belly skin of the mouse was removed to exhibit the outer muscular layers (musculus obliquus externus and musculus rectus abdominis) of the abdominal wall. Along the midline, the muscular layer was cut longitudinally. Both parts of the muscular layers with the parietal peritoneum attached were then excised with a semicircular cut on both flanks of the abdomen. This procedure resulted in two tissue pieces of about 4 cm2 area each. Each tissue piece was tied to the bottom of a hollow glass cylinder using a surgical thread (see Fig. 3C). The glass cylinder with the attached tissue section formed a small watertight chamber with the parietal peritoneum exposed at the inner side (circular area 95 mm2).

Sampling and stimulation

The preparations were equilibrated for 30 min in carbogen-gassed (O2 95%, CO2 5%) SIF (84) containing (in mM) NaCl 107.8, KCl 3.5, MgSO4 0.69, NaHCO3 26, NaH2PO4 1.7, CaCl2 1.5, sodium gluconate 9.6, glucose 5.6, and saccharose 7.6.

One release experiment consisted of four incubation steps (S1–S4) each lasting 5 min. After the initial washout, the preparations were first incubated twice for 5 min in test tubes containing SIF to determine basal iCGRP release (S1 and S2). This procedure was followed by a 5-min stimulatory incubation (S3) in tubes containing solutions of MGO, GO, 3-DG, or 3,4-DGE or the inflammatory mediators BK or PGE2 dissolved in SIF to the final concentration, or SIF at a pH of 5.2 and 6.1. The final 5-min incubation period (S4) in SIF was performed to assess reversibility of stimulated iCGRP release.

Enzyme-immunoassay (EIA) for immunoreactive Calcitonin Gene-Related Peptide

As described previously, the incubation fluids were processed immediately after the release experiment using a commercial iCGRP-EIA kit (Bertin Pharma, France) with a detection limit of 5 pg/ml (83). The antibodies used are directed against human α/β-CGRP but are 100% cross-reactive against mouse CGRP. The EIA plates were determined photometrically using a microplate reader (Dynatech, Channel Islands, UK).

Degradation of 3-DG and formation of 3,4-DGE

We investigated the degradation of 3-DG and the formation of 3,4-DGE as follows: 375 μM 3-DG solutions were prepared in SIF using freshly-synthesized 3-DG and then incubated at 37 and 39 °C, respectively. As a control, 3-DG–free SIF solutions were incubated as well. Samples were then collected after 1, 5, 9, and 24 h, subsequently degassed in an ice-cold ultrasonic bath, and analyzed according to Mittelmaier et al. (85). This incubation experiment was performed in triplicate, and to make sure that the used quantification method was suited for the applied SIF matrix, 3-DG and 3,4-DGE were added to SIF, and the recovery rates were analyzed.

Chemicals

GO and 3-DG were purchased from Sigma-Aldrich (Taufkirchen, Germany); MGO was synthesized as described previously (86). 3,4-DGE was synthesized as described previously by Mittelmaier et al. (85), with the exception that the second SPE, methanol (10% in water), was used instead of ethanol (10% in water) for the elution of 3,4-DGE (85). 3-DG, applied for the elucidation of its hypothesized degradation to 3,4-DGE in SIF, was synthesized according to Gensberger-Reigl et al. (32) and Gensberger et al. (87) using glucose instead of galactose as the starting material.

A commercially-available heat-sterilized PD solution, containing glucose as the osmotic agent, was also investigated. Screening and quantification of the main dicarbonyls in the
Different reactive dicarboxyls activate nociceptors

applied PD fluid were carried out by UHPLC/DAD, as shown previously (85).

Bradykinin and prostaglandin (BK and PGE2 (both from Sigma-Aldrich) were dissolved in water or ethanol as \(10^{-3} \text{ m}\) stock solution) were freshly diluted in SIF to obtain final concentrations. SIF solutions with low pH were produced by replacing \(\text{NaHCO}_3\) (26.2 mM) with different proportions of phosphate buffer. To investigate \(\text{Ca}^{2+}\) dependence of stimulated neuropeptide release, we used stimulation solutions that were made with \(\text{Ca}^{2+}\)-free SIF (SIF + EGTA 10 mM; Merck, Darmstadt, Germany).

Histochemistry

The peritoneum samples were fixed in PBS (pH 7.4) with a final formalin concentration of 4%. For indirect immunofluorescence, whole mounts of the peritoneum were rinsed in PBS, preincubated for 2 h at 4 °C in a solution of 5% goat normal serum (Dianova, Hamburg, Germany) with 0.5% Triton X-100 and 1% BSA rinsed in PBS, and incubated overnight with rabbit polyclonal antiserum raised against rat CGRP (working dilution 1:100, Dianova, Hamburg, Germany). Thereafter, the preparations were rinsed in PBS three times for 10 min and incubated with the secondary antibody goat anti-rabbit IgG conjugated with indocarbocyanine (working dilution 1:100, Cy3, Dianova, Hamburg, Germany) for 2 h at room temperature.

Samples were mounted on glass slides, air-dried, and cover-slipped with Roti®-Mount Fluor Care (Roti, Karlsruhe, Germany) containing DAPI for nuclear DNA staining. Fluorescence was analyzed using an LSM 780 light and confocal microscope (Carl Zeiss Mikromalaging GmbH Jena, Germany) mounted on an inverted Axio Observer Z1. Two dry objective lenses (\(10\) and \(20\) with numerical apertures of 0.3 and 0.8) were used. Structures were observed in the light path mode using red and green filters. Confocal images were taken using filter settings for Cy3 (excitation 514 nm and emission 530–566 nm). The number of image pixels was 1024 × 1024 or 512 × 512. Pictures were converted to a 12-bit RGB TIFF file using confocal assistant software ZEN 2010.

Statistics

Data are displayed as measured by EIA in picograms/ml (mean ± S.E.). To reduce the inter-individual and baseline variability, all data were corrected by subtracting the second individual baseline value from all four data points resulting in iCGRP release over baseline (in picograms/ml in time-course figures). The column diagrams show the overall stimulated release. For that purpose, the values of the stimulated and the successive sample (S3 + S4) were added up, and the sum of the baseline values (S1 + S2) was subtracted to gain a quasi-area under the curve (AUC in picograms/ml). AUC data from the peritoneum were calculated using only the second incubation step as baseline because of high iCGRP levels in the first incubation step, possibly due to the muscular tension of the preparation (see Fig. 3, D and E, right panel, respectively). Within one experimental group, the time-lapse data of iCGRP release were analyzed by Wilcoxon matched pairs test. Multiple groups were compared by one-way ANOVA followed by Fisher’s LSD post hoc test. The experiment regarding the 3-DG degradation to 3,4-DGE was performed in triplicate, and probes were taken at five consecutive time points and at 37 and 39 °C, respectively. The measured 3-DG and 3,4-DGE concentrations at two temperatures were compared using an ANOVA with repeated measurements. All statistical tests were performed with Statis tica 7 software (StatSoft, Tulsa, OK); differences were considered significant at \(p < 0.05\) as marked with an asterisk.

Data availability

All data are contained within the article.

Author contributions—A. K. B., A. A., and S. K. S. data curation; A. K. B. formal analysis; A. K. B., A. A., and K. M. investigation; A. K. B. visualization; A. K. B. and S. K. S. writing-original draft; A. A., M. P., K. M., T. F., and P. W. R. writing-review and editing; T. F. methodology; S. K. S. conceptualization; S. K. S. funding acquisition; S. K. S. project administration.

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