DOI 10.1002/mnfr.200400111 Mol. Nutr. Food Res. 2005, *49*, 710 – 715

Fate of the glucose degradation products 3-deoxyglucosone and glyoxal during peritoneal dialysis

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Conventional fluids for peritoneal dialysis (PD) contain reactive glucose degradation products (GDPs) as a result of glucose breakdown during heat-sterilization. GDPs in PD fluids (PDFs) have been associated with the progressive alteration of the peritoneal membrane during long-term PD by cytotoxic effects and formation of advanced glycation endproducts (AGEs). In this study, we investigated the possible fate of two characteristic GDPs, 3-deoxyglucosone (3-DG) and glyoxal, during PD. *In vivo*, 3-DG and glyoxal concentrations, which were analyzed by high-performance liquid chromatography (HPLC), decreased in PDFs by 78% and 88% during 4 h of dwell time. The PDFs were then incubated *in vitro* in the presence of the most important reaction partners of GDPs in the peritoneal cavity. Neither human peritoneal mesothelial cells, human peritoneal fibroblasts, soluble protein, an insoluble collagen surface, nor components of spent dialysate led to a significant reduction of 3-DG or glyoxal after 6 h. Only after long-term incubation, a noticeable decrease of 3-DG was observed (–37% after three weeks), more likely due to spontaneous degradation reaction than formation of advanced glycation endproducts. These results suggest that in the course of PD, 3-DG, and glyoxal are absorbed into the organism and thus might contribute to the systemic pool of reactive carbonyl compounds.

Keywords: Advanced glycation endproducts / Continuous ambulatory peritoneal dialysis / 3-Deoxyglucosone / Glucose degradation products / Glyoxal

Received: December 28, 2004; revised: April 8, 2005; accepted: April 8, 2005

1 Introduction

Conventional fluids for continuous ambulatory peritoneal dialysis (CAPD) contain glucose as osmotic agent. During heat-sterilization or long-term storage of these solutions, breakdown of glucose occurs, resulting in the formation of glucose degradation products (GDPs). So far, a variety of GDPs was identified in peritoneal dialysis fluid (PDF), among which 3-deoxyglucosone (3-DG), methylglyoxal, and glyoxal are present at highest concentrations [1]. GDPs in PDF are thought to cause local detrimental effects in the peritoneal cavity in at least two different ways: (i), they exert direct cytotoxic effects on peritoneal cells *in vitro* [2]. (ii) GDPs may indirectly harm peritoneal structures by promoting formation of advanced glycation endproducts (AGEs) [3]. It could be shown that GDPs are much more potent precursors of the formation of AGEs than glucose *per se* [1]. In patients on CAPD, AGEs accumulate in the peritoneum. The extent of accumulation is positively correlated with the progression of interstitial fibrosis and microvascular sclerosis, which finally might lead to a loss of ultrafiltration [4]. Thus far, it is not clear if GDPs, which originate from PDF, might also cause systemic complications in addition to the above-mentioned local effects: it has been demonstrated that GDPs in PDF disappear during a 4

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Abbreviations: AGE, advanced glycation endproduct; **CAPD**, continuous ambulatory peritoneal dialysis; **3-DG**, 3-deoxyglucosone; **GDP**, glucose degradation product; **HPFB**, human peritoneal fibroblast; **HPMC**, human peritoneal mesothelial cell; **MTT**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-*2H*-tetrazolium bromide; **PD**, peritoneal dialysis; **PDF**, peritoneal dialysis fluid

h dwell time during CAPD [5]. In another study, a rapid decrease of methylglyoxal concentration in the PDF was detected already after 2 h of dwell time. After 2 and 8 h of dwell time, spent dialysate showed also a reduced potential to form pyrraline, a nonfluorescent AGE, *in vitro* [6]. The decrease of GDPs during CAPD could be due to their local reactions in the peritoneal cavity, but also to their diffusion into the circulation. In the latter case, GDPs could directly impair systemic cellular functions [7] or lead to elevated serum AGE levels, which have been linked to increased severity of microangiopathy and the development of cardiovascular disease [8]. In order to estimate the role of absorption of GDPs *versus* their reaction with peritoneal targets, the reactivity and the possible reaction sites of two main GDPs in the peritoneal cavity, 3-DG and glyoxal, were investigated.

2 Materials and methods

Unless otherwise stated, chemicals were purchased from the Sigma-Aldrich group (Deisenhofen, Germany). All PD solutions used were conventional single chamber bag PDFs (CAPD 2 and CAPD 3; Fresenius Medical Care, Bad Homburg, Germany). All tissue culture plastics were obtained from Falcon.

2.1 Determination of GDPs

400 μL of each sample were mixed with 150 μL phosphate buffer (0.5 M, pH 4.3), and 40 μL of an *o*-phenylenediamine solution (0.02 M in methanol) was added to obtain the quinoxaline derivatives. According to literature, the reaction is quantitative and derivatives are stable over weeks [9]. After overnight incubation at room temperature, this solution was directly used for HPLC analysis. HPLC analysis was carried out with a diode-array detector system (pump PU-1580, degaser 980-50, ternary gradient unit LG-980-02S, diode array detector MD 1510 from JASCO, Groß-Umstadt, Germany, quantification at 237/316 nm) using an RP-18 column (LC-18 DB 25 cm × 4.6 mm; Supelco). For elution, a binary gradient was used with 0 – 70% B from 0 to 18 min and 70% B from 18.1 to 25 min (solvent A: 5 mM ammoniumformate buffer, pH 7.4; solvent B: methanol). For quantification, standard curves were obtained after derivatization of pure 3-deoxyglucosone [10] and glyoxal. Average retention time of the quinoxaline derivative of 3-DG was 17.9 min, retention time of the quinoxaline derivative of glyoxal was 20.4 min.

2.2 Determination of imidazolone by ELISA

Imidazolone ELISA was performed as described in [1].

2.3 Time course of 3-DG and glyoxal concentrations during CAPD

Dialysates from six different patients were obtained after 0, 0.5, 1, 2, 4 h, and overnight dwell as part of a regular peritoneal equilibration test and assessed for 3-DG and glyoxal.

2.4 Incubation of PDF with HPMC and HPFB

Isolation of human peritoneal mesothelial cells (HPMCs) and fibroblasts (HPFBs): HPMCs and HPFBs were isolated from specimens of omentum obtained from consenting nonuremic patients undergoing elective abdominal surgery. Cells were isolated and characterized as described previously [11, 12]. HPMC were propagated in M 199 and HPFB in F 12 culture medium, supplemented with L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), hydrocortisone (0.4 μg/mL), and 10% v/v fetal calf serum (FCS; Gibco BRL, Life Technologies, Eggenstein, Germany). Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. All experiments were performed using cells only from the first three passages, since later subcultures contained an increasing number of senescent cells [11]. HPMC were plated into multiwell clusters and grown until confluence. The standard medium containing 10% FCS was then replaced with medium supplemented with 0.1% FCS in order to render the cells quiescent.

2.5 Acute exposure with PDFs

After rendering the cells quiescent for 48 h, cells were incubated with either low (1.5%) or high (4.25%) glucose-containing PDFs for up to 6 h. PDFs were mixed 1 : 1 with serum free cell culture medium and pH was adjusted to 7.4 in order to reduce acute toxicity and to keep the cell viability until the end of experiments. After the incubation period, supernatant was collected and stored at –70°C, until HPLC analysis was performed. Cells underwent a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay in order to verify the viability. These experiments were performed in quintuplicate for HPFBs and with nine different cell lines for HPMCs. As a control experiment, PDFs were also incubated without cells.

2.6 MTT assay

Cell viability was assessed by measuring their ability to metabolize the MTT salt [13]. The assay was performed essentially as described previously [14].

2.7 Incubation of PDFs with soluble protein

PDFs containing 4.25% and 1.5% of glucose were buffered with sodium phosphate so that a final concentration of 5 mM and a physiological pH of 7.4 were reached. Human serum albumin (HSA) was then added to give a concentration of 1 mg/mL, which represents the concentrations of total soluble protein in an overnight dwell. The PDFs spiked with protein were incubated at 37°C, and samples were drawn at time points of 0, 2, 4, and 6 h. Samples were kept frozen at −20°C prior to analysis. As a control, a phosphate buffered PDF without protein was subjected to the same procedure. This experiment was performed in quadruplicate.

2.8 Incubation of PDFs with bound protein

96 well-plates from Nunc (Roskilde, Denmark) were coated with a solution of 1 mg/mL of soluble collagen over night. After washing twice with washing buffer (PBS with 0.05% Tween 20), 4.25% or 1.5% glucose PDFs (buffered to pH 7.4 as described before) were pipetted into the wells. This plate was incubated at 37°C and samples were taken after 0, 2, 4, and 6 h. Samples were kept frozen at −20°C prior to analysis. This experiment was performed in quadruplicate.

2.9 Incubation of PDFs with effluent dialysate

Unused single-chamber PDFs containing 4.25% or 1.5% glucose were incubated with effluent dialysate of six different patients. 5 mL of fresh PDF were mixed with 5 mL of the dialysate and incubated at 37°C. Sample volumes of 2 mL were drawn after 0, 2, 4, and 6 h and assessed for GDPs. As a control, phosphate-buffered saline (pH 7.4, 150 mM NaCl) was incubated with PDF using the same ratio. Controls were carried out in duplicate.

2.10 Long-term incubation of PDF with/without HSA

A PDF (4.25% glucose) containing 5 mM sodium phosphate was buffered to pH 7.5. This solution was incubated with and without 1 mg/mL HSA at 37°C. Samples were taken after 0, 1, 3, 7, 14, and 21 days and assessed for 3-DG and imidazolone formation.

2.11 Statistical analysis

For evaluation of statistically significant differences between the samples at different time points and the controls, a paired *t*-statistic was used. Differences between samples were considered as statistically significant for *p*-values <0.05. All experiments were performed with PDFs containing 4.25% and 1.5% glucose (data not shown), and similar results were obtained.

3 Results

Changes of GDP concentrations in PDFs during CAPD and *in vitro* were monitored by HPLC. Several model systems representing the possible targets in the peritoneal cavity were used. As markers for GDPs, 3-DG, which is the GDP found in highest concentration in PDFs, and glyoxal, were measured.

3.1 Time course of 3-DG and glyoxal during CAPD

Figure 1 shows a typical decrease in 3-DG and glyoxal as observed during the peritoneal dwell of PD fluids. 3-DG decreased by 78% in 4 h ($p < 0.001$), glyoxal decreased by 88% during 4 h ($p < 0.01$).

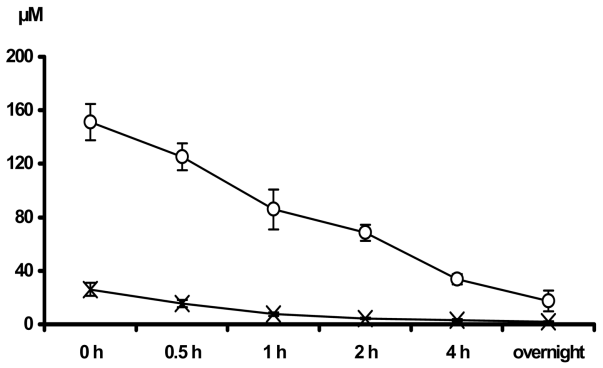

Figure 1. Time course of 3-DG and glyoxal in dialysate during PD. (o) Concentration of 3-DG; (x) concentration of glyoxal. Data are expressed as mean ± SEM.

3.2 *In vitro* incubation of human peritoneal mesothelial cells and human peritoneal fibroblasts with unused PDF

Mesothelial cells or fibroblasts were cultured in the presence of PDF. Both cell types, as the controls without cells, did not lead to significant differences in 3-DG and glyoxal levels before and after 6 h of incubation (see Table 1). MTT assay confirmed the viability of the cells after exposure to PDF (data not shown).

3.3 *In vitro* incubation of unused PDF with protein

As a model for the reaction of GDPs with soluble proteins in the peritoneum, HSA was incubated in PDF, and 3-DG

Table 1. Concentrations of the PDF-borne GDPs glyoxal and 3-DG at baseline and after 6 h of incubation of the PDF with different reaction partners for the GDPs

Reaction partner	Sample type		3-Desoxyglucosone (μM)			Glyoxal (μM)		
			0 h	6 h	Loss	0 h	6 h	Loss
HPFBs	PDF (4.25%)	$n = 5$	244.0 ± 1.9	234.6 ± 15	9.4	30.6 ± 1.0	27.4 ± 1.4	3.2
	Control	$n = 1$	254.0	247.0	7.0	33.8	31	2.8
HPMCs	PDF (4.25%)	$n = 9$	233.4 ± 9.9	239.3 ± 10.3	−5.9	33.5 ± 4.2	28.4 ± 1.9	5.1
	Control	$n = 3$	237.9 ± 9.6	228.5 ± 5.8	9.4	33.1 ± 6.0	30.1 ± 4.9	3.0
Dialysate	PDF (4.25%)	$n = 6$	240.0 ± 8.1	237.9 ± 8.6	2.1	43.5 ± 2.8	43.0 ± 1.3	0.5
	Control	$n = 2$	236.5 ± 0.4	237.6 ± 2.8	−1.1	45.3 ± 4.1	46.1 ± 3.9	−0.8
Soluble protein	PDF (4.25%)	$n = 4$	215.3 ± 1.8	211.3 ± 2.0	4.0	39.4 ± 0.3	44.5 ± 1.0	−5.1
	Control	$n = 2$	216.3 ± 0.6	212.3 ± 5.0	4.0	41.2 ± 0.9	44.3 ± 0.8	−3.1
Bound protein	PDF (4.25%)	$n = 4$	212.0 ± 1.0	219.8 ± 1.3	−7.8	39.7 ± 0.4	43.6 ± 1.5	−3.9
	Control	$n = 2$	216.3 ± 0.6	212.3 ± 5.0	4.0	41.2 ± 0.9	44.3 ± 0.8	−3.1

Controls did not contain the potential reaction partners. Data are expressed as mean ± SEM.

and glyoxal concentrations were measured. After 6 h of incubation, no significant loss of GDPs due to AGE formation was observed. In order to mimic the reaction of GDPs with insoluble matrix proteins, collagen was immobilized to a polystyrol surface and incubated in the presence of PDF. Again, AGE formation with the collagen surface did not cause significant changes in 3-DG and glyoxal concentrations after 6 h of incubation (Table 1).

3.4 *In vitro* incubation of unused PDFs with dialysate

PDFs were also incubated with spent dialysate from six different patients. No significant changes in 3-DG and glyoxal concentrations were observed after 6 h of incubation (Table 1).

3.5 Long-term incubation of PDF with/without HSA

In order to assess the stability of 3-DG, a single chamber PDF buffered to pH 7.5 was incubated with and without HSA for three weeks. 3-DG concentrations decreased by 37% during 3 weeks (Fig. 2): This decrease was irrespective of the presence or absence of HSA. Nevertheless, in the presence of HSA, imidazolone formation could be detected (Fig. 2).

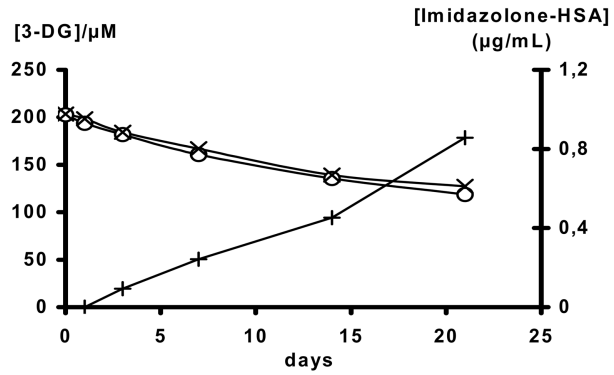

Figure 2. Decrease in 3-DG during incubation of a single chamber PDF (x) with or (o) without 1 mg/mL HSA at pH 7.5. The secondary axis shows the concomitant increase in the AGE imidazolone, when PDF was incubated with HSA (+).

4 Discussion

In contrast to totally reactive carbonyl species, which also include carbonyl compounds descending from the circulation [15], 3-DG and glyoxal, originating from PDF, decrease during the peritoneal dwell time (Fig. 1, [5]). Partly, the decrease in these GDPs might be explained by a dilutional component, since water joins the intraabdominal fluid through ultrafiltration. However, this effect must be considered as small in comparison to the rapid decline observed: decreases in GDPs by about 80% that are observed after 4 h of PD would require an increase in volume by 400%, if only the dilutional effect was accountable. In literature, GDPs are often described as highly reactive compounds and, therefore, 3-DG and glyoxal might be depleted during PD as a result of reactions with peritoneal targets. To examine this hypothesis, we set up different model systems that were designed to comprise the main reaction partners for GDPs during the peritoneal dwell as completely as possible. Since the full spectrum of GDPs is not known yet, 3-deoxyglucosone and glyoxal were exam-

ined vicariously. 3-DG and glyoxal belong to the three GDPs usually present at highest concentrations in PDF [1], 3-DG itself being a potent accelerator of AGE formation [16].

First, cellular components of the peritoneum are potential reaction partners of 3-DG and glyoxal. Cells could lead to a decrease in GDP concentrations by three different pathways: uptake of 3-DG and glyoxal by the cells, direct glycation reaction of 3-DG and glyoxal with the cell surface, or by detoxification [17]. HPMCs should be the main target of GDPs in an intact peritoneum. However, during long-term PD, the peritoneum is often denuded of its mesothelial cell monolayer, so that the interstitium and its cellular component, HPFBs, are exposed directly to the PDF. Therefore, interaction of GDPs with HPFBs might be more relevant than that with HPMCs. Consequently, the interaction of HPMCs and, for the first time, of HPFBs with 3-DG and glyoxal was investigated: neither HPMCs, nor HPFB significantly altered the concentrations of 3-DG or glyoxal in the PDF. A recent study by Linden *et al.* [18] showed that 3-DG did not show significant reactivity with HPMC, whereas methylglyoxal and formaldehyde concentrations were markedly reduced after the incubation with human peritoneal mesothelial cells.

Secondly, apart from cells, proteins might act as reaction partners for 3-DG and glyoxal. An extracellular collagen matrix is the main component of the peritoneal interstitium, which is exposed in long-term PD patients. Furthermore, soluble proteins are present in the dialysate. In overnight dwells, total soluble protein concentrations of 1–1.5 mg/mL were measured [19]. Since it is known that GDPs in PDFs cause an extensive formation of AGEs in the presence of soluble [1] and matrix-bound proteins [20], GDPs should consequently decrease. Especially 3-DG has been reported to react quickly with proteins leading to imidazolone formation [17]. However, in our experiments, the presence of proteins did not induce a measurable decrease in 3-DG and glyoxal concentrations during 6 h. Therefore, the stability of 3-DG in the presence and absence of proteins was further investigated. The result, that concentrations of 3-DG and glyoxal do not decrease detectably upon the incubation with proteins, could be confirmed for 3-DG in long-term incubations. In addition, imidazolone formation was measured (Fig. 2). Imidazolone is an AGE, which is formed by the condensation of 3-DG with arginine residues of proteins [21]. 3-DG in a single-chamber PDF buffered to pH 7.5 decreased by 37% during 3 weeks. This decrease was not influenced by the presence or absence of protein (1 mg/mL HSA). Nevertheless, in the presence of HSA, considerable imidazolone formation could be detected by ELISA (Fig. 2). As the formation of AGEs cannot occur in the absence of proteins, the decrease in 3-DG observed must be ascribed to processes different from AGE formation. Such reactions might be the further degradation of 3-DG, resulting in the formation of 5-hydroxymethylfurfural or metasaccharinic acid [22]. Therefore, to compensate for this loss of 3-DG, long-term incubation models of the peritoneum should include daily changes of PDF [1]. In summary, it can be concluded that, though imidazolone formation in coincubates of 3-DG and proteins can be readily detected with sensitive methods, only a negligible part of available 3-DG participates in the formation of AGEs. In our long-term incubation experiments, after 20 days of incubation, we measured an imidazolone formation corresponding to less than 1 μg/mL imidazolone-HSA standard. Assuming a modification rate of the standard of 33% (every third arginine residue is modified as imidazolone), the measured imidazolone concentration after 20 days would be caused by less than 0.1 μmol/L 3-DG. This amount is negligible compared to a concentration of about 200 μM 3-DG in the PDF. The limited reactivity of 3-DG could be attributed to its bicyclic structure (at least 11 isomers [22]), that is formed by intramolecular hemiacetales. In contrast, glyoxal is not able to form intramolecular acetales and its reactivity should be higher compared to 3-DG. However, after 6 h, glyoxal, which reacted with proteins, was also very low compared to the overall glyoxal concentration.

Thirdly, substances present in the dialysate that descended from the circulation, *e.g.*, urea and creatinine, might react with GDPs. Similar to other experiments [18], 3-DG and glyoxal concentrations do not decrease significantly by the reaction with components of spent dialysate.

Taken together, these findings suggest that, as in the case of glucose, diffusion of 3-DG and glyoxal into the circulation and lymphatic system might be the reason for their decrease during PD. This would not be surprising, since both GDPs have similar polarity and lower molecular weight than glucose itself, which are two main determinants of the diffusion rate. We assume that diffusion of 3-DG and glyoxal takes place through the small pores in the peritoneal membrane, which are permeable for water and small molecules [23] as well as through the lymphatic lacunae. It must be investigated in detail whether other GDPs, which are present in lower concentrations in PDF, act in a similar way or do react with peritoneal targets. As a consequence, absorption of 3-DG and glyoxal into the organism could elevate their serum levels. Thus, it can be expected that 3-DG and glyoxal originating from heat-sterilized PDF could not only impair the function of the peritoneum locally, but that they might also lead to detrimental systemic effects. These could result from cytotoxic activity of both GDPs or from enhanced systemic AGE formation. This hypothesis is supported by a recent study by Zhang *et al.* [24, 25], who showed that the concentration of the AGE imidazolone in blood serum is higher in patients treated with GDP-rich single-chamber PDF in comparison to patients who are dia-

lyzed against double-chamber bag PDF low in 3-DG and glyoxal. A comparable result was obtained by Zeier *et al.* [6] for the AGE markers plasma fluorescence and plasma carboxymethyllysine. In summary, a large proportion of 3-DG and glyoxal originating from PD fluids could be absorbed into the organism. These absorbed GDPs might supply a reactive pool for systemic AGE formation.

This study was supported by the "Fonds der Chemischen Industrie".

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