Polymorphonuclear leukocyte injury by methylglyoxal and hydrogen peroxide: a possible pathological role for enhanced oxidative stress in chronic kidney disease

Masaaki Nakayama¹, Keisuke Nakayama¹, Wan-Jun Zhu¹, Yuko Shirota², Hiroyuki Terawaki¹, Toshinobu Sato², Masahiro Kohno³ and Sadayoshi Ito¹,²

¹Research Division of Dialysis and Chronic Kidney Disease, Tohoku University Graduate School of Medicine, ²Department of Blood Purification, Tohoku University Hospital and ³Tohoku University, New Industry Creation Hatchery Center, Life Particle Interaction Engineering Creation, Sendai, Japan

Abstract

Background. Accelerated burst of polymorphonuclear leukocytes (PMNs) may be involved in the primary pathology of enhanced oxidative stress in patients with chronic kidney disease (CKD); however, the precise mechanism remains unknown. Methylglyoxal (MGO), an α-oxoaldehyde reportedly elevated in CKD, could induce apoptosis in several cell lines, and generates radicals by the reaction with hydrogen peroxide (H₂O₂). Thus, we tested if a high MGO of uraemic milieu could play a role in PMN injury by interaction with H₂O₂.

Method. Cellular viability of PMNs, isolated from healthy volunteers, was tested by ATP chemiluminescence levels under MGO and/or H₂O₂, or 4-β-phorbol 12-β-myristate 13-α-acetate (PMA). Superoxide anion (O₂⁻) generation and apoptosis were measured by the reduction of ferricytochrome C and fluorocytometric analysis, respectively. Plasma MGO levels were measured by mass spectrometry in 29 CKD patients.

Results. At low levels of MGO (1–10 µM) and H₂O₂ (12.5 µM), no differences were found in cellular viability as compared to controls, whereas their combination significantly decreased PMN viability. PMA stimulation enhanced cellular injury of MGO by a function of MGO levels and preincubation with 5,5-dimethyl-1-pyrroline-N-oxide (free radical trap agent) attenuated it. MGO suppressed O₂⁻ generation by PMA, while it accelerated apoptotic ratios in PMNs. Significant increases of plasma MGO and C-reactive protein levels were found by a function of CKD stage, and clinical level of MGO could induce PMN injury in combination with H₂O₂.

Conclusion. These results indicate the combinatory effect of MGO and H₂O₂ on PMN oxidative injury, and this pathology may be linked to enhanced oxidative stress in CKD.

Keywords: chronic kidney disease; hydrogen peroxide; methylglyoxal; oxidative stress; polymorphonuclear leukocyte

Introduction

Cardiovascular disease (CVD) is the leading cause of mortality in patients with chronic kidney disease (CKD) [1–5]. In addition, susceptibility to infectious disease is another clinical characteristic, and the second leading cause of death in chronic dialysis patients [6]. To date, the high prevalence of CVD in CKD has been attributed to classical as well as non-classical risk factors, such as inflammation, oxidative stress, advanced glycation end products, asymmetrical dimethyl arginine and disturbed calcium bone metabolism [7–11]. Nevertheless, the common pathological background of the high prevalence of CVD and infectious diseases in CKD has not been clearly explained.

It was reported that polymorphonuclear leukocytes (PMNs) present accelerated oxidative burst in CKD patients [12,13]. Furthermore, it has been made evident that the elevated levels of plasma myeloperoxidase released from PMNs could predict patient survival [14,15]. Thus, it is possible that PMN burst could play a crucial role in micro-inflammation, oxidative stress or immune dysfunction found in CKD [16,17]. However, the pathological mechanism for inducing PMN injury remains unclear.

It is reported that plasma levels of methylglyoxal (MGO), an α-oxoaldehyde, are elevated in patients on dialysis [18,19]. MGO is highly reactive and biologically toxic due to the formation of advanced glycation end products, protein and DNA modifications [20]. Furthermore, it has been reported that MGO induced apoptotic reactions in several cells [21–26]. In a previous study, we demonstrated...
a novel aspect of MGO to generate radical species by the non-enzymatic chemical reaction with hydrogen peroxide (H₂O₂), suggesting the interaction of MGO toxicity with H₂O₂ [27]. This led us to hypothesize the possibility that increased levels of MGO drives PMN injury especially in those with enhanced respiratory burst. To test this, we examined the combinatory effects of MGO and H₂O₂ on human PMN viability.

**Methods**

**Measurement of PMN viability**

Human PMNs were obtained from healthy volunteers. Briefly, whole blood was withdrawn from healthy volunteers, and heparinized samples were placed onto the Mono
coly Resolving Medium (Dainippon Pharmaceutical, Osaka, Japan) to collect PMNs. After centrifugation at 1800 rpm for 30 min at 18°C, the intermediate PMN-enriched layer was recovered, washed and resuspended in the RPMI 1640 culture medium supplemented with 10% foetal calf serum (FCS). Collected PMNs were adjusted to a concentration of 5–10×10⁶ cells/ml. Fifty microlitres of cell suspension was placed in a 96-well microplate, and 50µl of test solution containing MGO and/or H₂O₂ was added. Viability of PMNs was examined using a commercially available kit (Cell Titer-Glo® Luminescence Cell Viability Assay; Promega Corporation, WI, USA) as previously reported elsewhere [28]. After treatment, 100µl of the sample was placed in a microplate and 100µl of Cell Titer-Glo® Reagent, which contains beetle luciferin, and luciferase was added immediately afterward. A reaction with ATP, which is released from lysed cells to produce oxyluciferin, generated chemiluminescence as a function of these ATP levels. After incubation, luminescence was then measured using a chemiluminescence analyser (GloMax 20/20n luminometer; Promega Corporation, WI, USA). Each measurement was performed five times, and mean values for each sample were calculated for analysis after correction of cell-free levels from each sample. The coefficients of variation (CVs) of the measurements were 3.77 ± 1.12% (n = 12).

**Free radical generation from PMNs**

The rate of superoxide release from human PMNs was determined by measuring the reduction of ferricytochrome C, as reported elsewhere [12]. PMNs obtained by the same procedure mentioned above were adjusted to a concentration of 1 × 10⁶ cells/ml in a solution of 80µM cytochrome C diluted in phosphate buffered saline (PBS). Immediately after adding 10µl of control or 4-β phorbol 12-β-myristate 13-α-acetate (PMA; 100µg/ml) to 700µl of sample, 250µl of sample was placed in the microplate. Absorbance was measured for 15 min at 550 nm and expressed in nanomoles of superoxide production per 1 × 10⁶ cells/10 min.

**Analysis of apoptotic PMNs**

PMNs, obtained by the same procedure mentioned above, were adjusted to a concentration of 5–10 × 10⁶ cells/ml in RPMI 1640 with 10% FCS. When required, erythrocytes were lysed by an ACK lysing buffer (Lonza Walkersville, MD, USA). One hundred microlitres of cell suspension was placed in a 48-well microplate and a 100µl of test solution containing MGO was then added. After a 5-h incubation period, cells were rinsed with PBS twice and resuspended in a binding buffer. Next, 1µl of annexin-V (FITC-labeled) (200µg/ml) and 5µl of propidium iodide (PI) (200µg/ml) were added (Annexin V-FITC kit, Beckman Coulter, USA) to a final volume of 100µl and cells were incubated for 15 min in the dark. Cytofluorometric analysis was performed immediately after staining using a FACS can flow cytometer (BD Biosciences). Fifty thousand cells were analysed in each sample. Percentage of positive events for cell markers on gated populations was determined by software (FlowJo; Tree Star, Inc., USA). Apoptosis and secondary necrosis were defined as annexin-V positive and PI negative and annexin-V positive and PI positive, respectively.

**Measurement of plasma MGO**

Twenty-five subjects with CKD were analysed for measurements of plasma MGO and C-reactive protein (CRP). All cases were recruited from the outpatient clinic of Tohoku University Hospital. The local ethical committee of Tohoku University approved the study, and an informed consent was obtained from patients. Patients who had any comorbidities or systemic diseases such as diabetes were not included. Blood samples were taken in the morning, or just before the regular dialysis session. CRP was measured by an auto-analyser. MGO was measured by electrospray ionization–liquid chromatography–mass spectrometry (ESI/LC/MS), as described elsewhere [29]. Briefly, 2,3-dimethylquinoxaline as internal standard (IS) and o-phenylenediamine for the derivatization of MGO were added to 0.5 ml of the sample. The samples were then applied down a prepared C18 SPE column, rinsed with formic acid, and the retentate was eluted in methanol. The derivatized dicarbonyl compound was analysed by high-performance liquid chromatography and ESI/MS, using a time-of-flight mass spectrometer (AccuTOF JMS-T100LC, JEOL Ltd, Tokyo, Japan). The derivatives were resolved by reverse-phase chromatography on a C18 column. Quantification of MGO was done by calculating the peak area ratio for dicarbonyl-derived protonated molecular ion peak intensity (MGO; m/z 145) to a protonated molecular ion peak intensity (2,3-dimethylquinoxaline; m/z 159) in the selected ion monitoring mode. H₂O₂, luminol, MGO, PBS, cytochrome C and PMA were purchased from Wako Pure Chemical Industries (Osaka, Japan).

All values are expressed as mean ± SD. Repeated measure ANOVA, one-way factorial ANOVA or the unpaired t-test were used for statistical analysis using StatMateIII (ATMS Digitals, Tokyo, Japan) and values of P < 0.05 were considered statistically significant.

**Results**

Time-course changes of human PMN viability by MGO and H₂O₂ are shown in Figure 1a. There are significant
reductions in ATP-CL by a function of MGO and H$_2$O$_2$ levels at both measurements ($^*P < 0.001$, in respective groups). Significant differences were found between MGO 1000 μM and MGO 0 μM, MGO 10 μM, MGO 100 μM ($P < 0.01$, respectively) and H$_2$O$_2$ 25.0 μM and H$_2$O$_2$ 0 μM, H$_2$O$_2$ 12.5 μM ($P < 0.01$, respectively). Post hoc analysis revealed a significant decrease at 1000 μM of MGO as compared to 10 or 100 μM MGO at 180 min ($P < 0.01$, respectively), whereas significant reductions were found in all MGO groups compared to the controls at 360 min ($P < 0.001$, respectively). Likewise, a significant decrease was found at 25 μM of H$_2$O$_2$ compared to the controls or 12.5 μM of H$_2$O$_2$ at 180 min ($P < 0.001$, respectively), whereas significant reductions were found in H$_2$O$_2$ groups compared to controls at 360 min ($P < 0.001$, respectively). Thus, we examined the combination cytotoxicity of MGO and H$_2$O$_2$ at 180-min incubation.

Changes of human PMN viability by co-incubation with MGO and H$_2$O$_2$ are shown in Figure 1b. Data are expressed by the percent changes in the ATL-CL levels of each sample after 180-min incubation as compared to the control (MGO: 0 μM; H$_2$O$_2$: 0 μM). There were significant reductions in ATP-CL in a MGO and H$_2$O$_2$ level-dependent manner ($P < 0.01$, respectively). In combination of MGO (at 1 and 10 μM) and H$_2$O$_2$, there were significant reductions in ATP-CL ($^*P < 0.01$). MGO 1 μM + H$_2$O$_2$ 12.5 μM versus MGO 1 μM alone; $P < 0.01$. MGO 10 μM + H$_2$O$_2$ 12.5 μM versus MGO 10 μM alone; $P < 0.05$ ($n = 5$, mean ± SD). (c) Morphological changes of PMNs by MGO and H$_2$O$_2$. Representative findings of a control (left) and a case with MGO 10 μM and H$_2$O$_2$ 12.5 μM (right) are shown. After 180-min incubation, cells were mounted on a slide following cytopsinning and stained with Hoechst 33342. Apoptotic changes such as chromatin condensation or fragmented nuclei were observed in cases incubated with MGO, H$_2$O$_2$, or MGO + H$_2$O$_2$ at various degrees.
Fig. 2. (a) Changes of human PMN viability after respiratory burst and effect of co-incubation with methylglyoxal (MGO). Human PMNs isolated from blood were suspended in phosphate buffered saline (PBS) (2 × 10⁵ cells/ml) containing MGO (0–1000 µM), following which 4-β phorbol 12-β-myristate 13-α-acetate (PMA) was added (20 µg) to induce a respiratory burst. ATP chemiluminescence (CL) was measured 30 min after treatment. The percent changes in the ATL-CL levels of each sample were expressed as compared to the control (MGO: 0 µM, PMA negative). There were significant reductions in ATP-CL in a MGO level-dependent manner, both under the condition without PMA (P < 0.001) and with PMA (P < 0.01). In the presence of MGO, reductions in ATP-CL by PMA were further exaggerated (P < 0.05) (n = 8, mean ± SD). (b) Effect of MGO on O₂⁻ radical generation in human PMNs by PMA. Human PMNs isolated from blood were suspended in PBS (1 × 10⁶ cells/ml) with or without containing 100 µM MGO after which PMA was added to induce respiratory burst. The levels of cases with MGO were significantly less than those without MGO (P < 0.05) (n = 6, mean ± SD). (c) Effect of a radical trap agent on human PMN viability in the presence of MGO and PMA. Human PMNs isolated from blood were suspended (2 × 10⁵ cells/ml) in PBS with both MGO and 0.1% 5,5-dimethyl-1-pyrroline-N-oxide, a specific radical trap agent. Immediately afterwards, PMA was added. ATP-CL was measured 30 min following treatment. The percent changes in the ATL-CL levels of each sample were expressed as compared to the control (MGO: 0 µM, PMA negative). There were significant decreases in ATP-CLs in a MGO dose-dependent manner in both groups, the PMA-treated (P < 0.05) and the non-treated (P < 0.001) groups, but no significant differences were found between them at respective MGO levels (n = 5, mean ± SD).

Changes of human PMN viability after respiratory burst and effect of co-incubation with methylglyoxal (MGO) were shown in Figure 2a. The percent changes in the ATL-CL levels of each sample were expressed as compared to the control (MGO: 0 µM, PMA negative). There were significant reductions in ATP-CL in a MGO level-dependent manner, both in the condition without PMA (P < 0.001) and with PMA (P < 0.01). In the presence of MGO, reductions in ATP-CL by PMA were further exaggerated (*P < 0.05).
The effect of MGO on O$_2^-$ radical generation in human PMNs by PMA is shown in Figure 2b. The levels of cases with MGO were significantly less than those without MGO ($P < 0.05$).

The effect of the radical trap agent on human PMN viability in the presence of MGO and PMA is shown in Figure 2c. The percent changes in the ATL-CL levels of each sample were expressed as compared to the control (MGO: 0 µM, PMA negative). There were significant decreases in ATP-CLs in a MGO dose-dependent manner in both groups, the PMA-treated ($P < 0.05$) and the non-treated ($P < 0.001$) groups, but no significant differences were found between them at respective MGO levels.

Changes of apoptosis and secondary necrosis ratios of human PMNs incubated with methylglyoxal (MGO) are shown in Figure 3. In the PMA non-treated group (Figure 3a), increased ratios of apoptosis and secondary necrosis were shown by a function of MGO levels ($P < 0.05$). In the PMA-treated groups (Figure 3b), increased ratios of apoptosis and secondary necrosis were also shown, but no significant differences were found as a function of MGO.

Plasma levels of MGO and CRP are shown in Table 1. There were significant differences in MGO as well as CRP levels.

We examined the cytotoxic effect of 1.0 µM MGO level which was revealed to be equivalent to CKD 5D cases as shown in Table 1. Human PMNs isolated from blood were incubated in RPMI for 210 min ($0.5 \times 10^6$ cells/well) containing MGO (1.0 µM), H$_2$O$_2$ (10.0 µM) and combination of both. ATP-CL of each well was measured at 210 min. The ATP-CLs in MGO 1.0 µM alone tended to be lower than the control, although it was not statistical significant. However, there were significant differences in ATP-CL among the groups examined (Table 2: $P < 0.001$), the lowest in the group with combination of MGO and H$_2$O$_2$.

**Discussion**

We employed an ATP-CL method to estimate the number of live cells. Regarding the ATP reduction rate (%) at 180 min (Figure 1b), no significant changes were found at lower levels of single MGO (1 and 10 µM) and H$_2$O$_2$ (12.5 µM), whereas a combination of them resulted in significant reductions of ATP-CL levels. Morphologically, chromatin condensation and nuclei aggregation were found in cases with decreased levels of ATL-CL (Figure 1c), indicating that the acceleration of apoptotic reaction could involve a mechanism of cellular injury. Thus, it is suggested that nontoxic levels of MGO and H$_2$O$_2$ could enhance PMNs injury by their combination.

PMA induces respiratory bursts of PMNs, while it is reported that the PMN oxidative burst is exaggerated in uraemic patients [12,13]. We thus examined the effect of MGO on the oxidative burst phenomenon by PMA. In a preliminary study, we confirmed that PMA increased superoxide anion generations under an MGO-free condition, and the dose of PMA used in this study significantly decreased ATP-CL levels of PMNs (Figure 2a), suggesting the progression of cellular damage after oxidative burst by PMA. In fact, reduction of ATP-CL by PMA load was further facilitated in a dose-dependent manner of MGO, indicating that MGO may potentiate cellular injury by PMA load. Furthermore, cellular function to generate superoxide anion was shown to be significantly reduced in PMNs in the presence of MGO (Figure 2b).

As MGO chemically reacts with H$_2$O$_2$ to generate hydroxyl and carbon radicals, we examined whether radicals generated from MGO plays some role in injury. To test this, we employed DMPO, a specific radical trap agent, and examined its effect on PMN oxidative burst by MGO. As a result, exaggeration of cellular injury of MGO was abolished by DMPO (Figure 2c), suggesting a crucial role of radicals for the mechanism of MGO toxicity during the course of the respiratory burst phenomenon.

Flow cytometrical analysis revealed an increased ratio of apoptotic cells by MGO load (Figure 3a). On the other hand, in the presence of MGO, apoptotic ratios by PMA were not decreased as compared to the control (MGO: 0 µM) (Figure 3b). Thus, it is indicated that MGO enhances oxidative injury of PMNs by PMA, a mechanism independent of enhanced physiological respiratory burst.

To date, studies on the mechanisms of enhanced PMN injury in uraemic patients have been limited. Increased production of H$_2$O$_2$ from PMNs was supposed as one candidate, but it was not supported by the recent study [30].

The present study could demonstrate the significance of combinatory effects of MGO and H$_2$O$_2$ on PMN injury in vitro. And it was shown that plasma MGO levels are increased by a function of the CKD stage, indicating a possible pathological involvement of MGO for PMN injury of CKD. Thus, we further examined the cytotoxic effect of the 1.0 µM MGO level which was revealed to be equivalent to CKD 5D cases as shown in Table 1. And it was found that the combination of MGO and H$_2$O$_2$ significantly decreased PMN viability, although no statistical changes were found in the group with MGO alone (Table 2). Thus, in terms of clinical relevance of the results, we speculate the following pathological process in CKD patients; generation of H$_2$O$_2$ from primed PMNs could facilitate PMN injury by the combinatory effect of high MGO in plasma. In fact, it is reported that renal function has close relationships with oxidative stress markers [31,32], and PMNs injury [12]. Taken together those findings and the present study, MGO may be one of uraemic toxins for the pathology of immune dysfunction in CKD. We think that further clinical examinations are crucially needed to conclude this important issue.

In conclusion, the combination of MGO and H$_2$O$_2$ accelerates oxidative injury of human PMNs, and this may link to the primary pathology of enhanced oxidative stress in patients with CKD. The suppression of such an axis could prove to be a new therapeutic target to CKD patients.

**Conflict of interest statement.** None declared. A part of this study was presented at the annual meeting of American Society of Nephrology (San Francisco 2007).
Fig. 3. (a and b) Changes of apoptosis and secondary necrosis ratios of human PMNs incubated with methylglyoxal (MGO), and the effect of respiratory burst. Human PMNs isolated from blood were incubated in RPMI with FCS 5% final) for 300 min (5 × 10⁶ cells/well) containing MGO (0–1000 µM). Cells were stimulated either by 4-β phorbol 12-β-myristate 13-α-acetate (PMA) or vehicle at 240 min, followed by another 60-min incubation. Cytofluorometric analysis was performed after staining with Annexin-V (FITC-labelled) and propidium iodide (PI). More than 3000 events were counted by flow cytometry. Apoptosis was defined by Annexin-V positive with PI negative, whereas secondary necrosis was defined by double positive staining. In the PMA non-treated group (a), increased ratios of apoptosis and secondary necrosis were shown by a function of MGO levels (P < 0.05). In the PMA-treated groups (b), increased ratios of apoptosis and secondary necrosis were also shown, but no significant differences were found as a function of MGO (n = 6, mean ± SD). (c) Representative data of cytofluorometric analysis by MGO and PMA load. Representative results are shown: (A) control at 0 min (ratios of apoptosis and secondary necrosis: 7.92%), after the 300 min incubation, (B) without PMA load under MGO 0 µM (19.67%), (C) with PMA load under MGO 0 µM (32.61%), (D) without PMA load under MGO 1000 µM (32.60%) and (E) with PMA load under MGO 1000 µM (40.39%).
Table 1. CRP (mg/dL) ± Post hoc seven cases.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Age (years)</th>
<th>CRP (mg/dL)</th>
<th>MGO (µM/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CKD1-2</td>
<td>8</td>
<td>51.8 ± 16.1</td>
<td>0.15 ± 0.09</td>
<td>263.5 ± 29.1</td>
</tr>
<tr>
<td>CKD3-5</td>
<td>11</td>
<td>52.6 ± 10.0</td>
<td>0.19 ± 0.19</td>
<td>380.3 ± 130.2</td>
</tr>
<tr>
<td>CKD5D</td>
<td>10</td>
<td>54.0 ± 9.8</td>
<td>0.45 ± 0.35</td>
<td>1002.2 ± 460.9</td>
</tr>
</tbody>
</table>

*Post hoc* analysis by the Tukey test.

Table 2. Mean ATP-chemiluminescence levels in the respective group

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: control</td>
<td>91 179</td>
<td>(89 262–93 095)</td>
</tr>
<tr>
<td>B: MGO 1 µM</td>
<td>89 719</td>
<td>(87 802–91 636)</td>
</tr>
<tr>
<td>C: H2O2 10 µM</td>
<td>87 442</td>
<td>(85 525–89 359)</td>
</tr>
<tr>
<td>D: MGO 1 µM + H2O2 10 µM</td>
<td>83 326</td>
<td>(81 410–85 243)</td>
</tr>
</tbody>
</table>

A versus C; P < 0.05.
A versus D: P < 0.001.
B versus D: P < 0.001.
C versus D: P < 0.05.

Data are expressed as mean values of five independent measurements from seven cases.

References


