Capacity of $\text{H}_2\text{O}_2$ Release from Monocytes in Steroid-Sensitive Nephrotic Syndrome

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TANAKA, H., WAGA, S., SUGIMOTO, K., KAKIZAKI, Y. and YOKOYAMA, M. Capacity of $\text{H}_2\text{O}_2$ Release from Monocytes in Steroid-Sensitive Nephrotic Syndrome. Tohoku J. Exp. Med., 1996, 178 (3), 271-277 —— The pathogenesis of steroid-sensitive nephrotic syndrome (SSNS) is poorly defined. We previously demonstrated that monocytes from SSNS patients with proteinuria were activated to display exaggerated phagocytosis of opsonized particles and paradoxically reduced chemotaxis. In this study, we evaluated the capacity of hydrogen peroxide ($\text{H}_2\text{O}_2$) release from monocytes in 19 patients with SSNS and 13 healthy controls, by exposure to phorbol myristate acetate (PMA), using scopeolitin method. Of 19 patients of SSNS, 7 were proteinuric and 12 in remission. The $\text{H}_2\text{O}_2$ release was significantly higher in SSNS patients with proteinuria than those in remission or normal controls [177.49 ± 94.75 (mean ± s.d.) vs. 60.67 ± 58.89 (p < 0.02) or 85.03 ± 48.62 nmol/90 min/mg cell protein (p < 0.05)]. Follow-up measurements in two SSNS patients showed that $\text{H}_2\text{O}_2$ release was reduced when proteinuric condition was improved to be in remission. Our data suggest that monocytes in SSNS with proteinuria were activated and were prepared to receive some extracellular signaling leading to protein kinase-C activation for releasing $\text{H}_2\text{O}_2$. ——— monocyte function; steroid-sensitive nephrotic syndrome; hydrogen peroxide; proteinuria

The cellular immune alterations in steroid-sensitive nephrotic syndrome (SSNS) have been extensively studied, but these are inconsistently observed, have not been confirmed, or are a consequence of the nephrotic state (Melvin et al. 1984). Function of monocyte-macrophage series as higher hierarchical cells of lymphocyte system also has been studied in SSNS. In previous papers, we demonstrated that monocytes from SSNS patients with proteinuria displayed enhanced phagocytosis of opsonized particles and hexose-monophosphate pathway, and paradoxically suppressed chemotaxis (Nagata et al. 1981; Waga et al. 1983). Recent studies suggest that estimation of the capacity of hydrogen peroxide ($\text{H}_2\text{O}_2$) release is a useful biochemical hallmark of macrophage/monocyte activation (Nathan et al. 1979; Nakagawara et al. 1981; Tsunawaki and Nathan 1984). In the present study, we examined to see if previous observation of

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peculiar monocyte activation in SSNS is also reflecting capacity of \( \mathrm{H}_2\mathrm{O}_2 \) release, using scopoletin method with simple spectrofluorometry (Nathan 1981).

**Materials and Methods**

*Patients*

SSNS. Nineteen patients, aged from 3 to 12 years, were examined. Seven patients were in active stage of SSNS with daily urinary protein excretion ranging from 1.5 to 9.2 g/day, and 12 in remission. At the time of examination, 16 patients received prednisolone and 3 did not. Of the 3 patients, 2 were at onset of the disease and 1 in complete remission. The rest of patients received alternative day prednisolone therapy at a dose of 10–15 mg. Patients who were in active stage of SSNS at the time of examinations subsequently received full dose of prednisolone to control their disease. Follow-up measurements were undertaken in two patients before and after elimination of proteinuria. Renal biopsy was performed in 15 patients because of frequent relapses, and revealed that they had minor glomerular abnormalities.

*Normal controls.* Thirteen healthy adult volunteers aged from 28 to 32 year-old were examined as normal controls.

*Preparation of monocytes on coverslips*

Ten ml of peripheral blood was drawn from median cubital vein and heparinized. Mononuclear cells (MNC) were prepared by centrifugation of the blood on a Ficoll metrizoate gradient and washing the cells twice with 0.15 M phosphate buffered saline (PBS, pH 7.2). The sedimented cells were suspended in RPMI 1640 (GIBCO, Grand Island, NY, USA) containing 10% fetal calf serum, 100 unit/ml of penicillin and 100 \( \mu \)g/ml of streptomycin to give \( 1 \times 10^7 \) MNC/ml. \( 1 \times 10^8 \) MNC in 100 \( \mu l \) of medium were mounted on the glass coverslips (24 × 32 mm), which had been soaked in 75% ethanol for at least 5 days, dipped in 95% ethanol and flamed (Nathan 1981). After incubation for 2 hr at 37°C in 5% \( \text{CO}_2 \) incubator, the coverslips were rinsed gently with Hanks balanced solution without glucose and phenol red (HBSS) twice and with normal saline three times at room temperature. Adherent monocytes on the coverslips showed similar distribution as certified by staining with Diff-Quick (Kokusai Shiyaku, Kobe). Four sheets of MNC on coverslips were prepared from one source of MNC, of which the first sheet was for confirmation of attachment of monocytes, the second for determination of protein amount of attached cells, and the third and fourth for measurement of \( \mathrm{H}_2\mathrm{O}_2 \) release.

* Determination of protein concentration of monocytes on coverslip*

Five hundred \( \mu l \) of 0.5 N NaOH was added to monocytes on the second coverslip, and 450 \( \mu l \) of the solution containing cell lysate was assayed for protein concentration by Lowry's method using bovine serum albumin as a standard
(Lowry et al. 1951).

**Measurement of \( \text{H}_2\text{O}_2 \) secreted from monocytes**

\( \text{H}_2\text{O}_2 \) was detected by horseradish peroxide mediated oxidation of reduced scopoletin (7-hydroxy-6-methoxycoumarin), which was determined as decreasing fluorescence intensity of reduced scopoletin measured by spectrofluorometer with excitation 350 nm and emission 460 nm (Andreae 1955). Optimal reaction time for oxidation of scopoletin by \( \text{H}_2\text{O}_2 \) was determined by spectrofluorometer equipped with recorder (RF-5000, Shimadzu, Kyoto). Fluorescence intensity produced by reduced scopoletin wanes to plateau from around 900 sec after adding \( \text{H}_2\text{O}_2 \) to working solution containing 2.0 \( \mu \text{M} \) scopoletin (Sigma, St. Louis, MO, USA), 0.44 purpurogallin \( \text{U/ml} \) of horseradish peroxidase (Type II, Sigma) and 1 mM sodium azide in HBSS (Fig. 1). Standard curve to determine the concentration of \( \text{H}_2\text{O}_2 \) in sample solution was produced with the use of 31.6\% \( \text{H}_2\text{O}_2 \) (atomic spectrum analysis grade, Wako Pure Chemical Industries, Osaka), adjusted concentration according to \( \varepsilon = 61 \text{ M}^{-1} \text{ cm}^{-1} \) at wave length 239 nm. \( \text{H}_2\text{O}_2 \) at the concentrations from 0 to 2.0 nM/ml was added to working solution with increment of 0.2 nM/ml to plot fluorescence intensity. Reduced scopoletin was detected by spectrofluorometer (650–10S, Hitachi, Tokyo). One hundred \% of fluorescence intensity was set at 0 nM/ml of \( \text{H}_2\text{O}_2 \) in working solution. Plotted intensities were apparently on the straight line \[ y = -39.42x + 97.98, \ y; \text{fluorescence intensity (\%)} , \ x; \text{H}_2\text{O}_2 \ (\text{nM/ml}), \ r = 0.9996, \ p = 0.0001, \] and an addition of 2 nM/ml of \( \text{H}_2\text{O}_2 \) showed 16.7\% of fluorescence intensity on the standard curve.

For determination of \( \text{H}_2\text{O}_2 \) concentration from monocytes preparation, two

![Fig. 1. Waning pattern of fluorescence of reduced scopoletin by the addition of \( \text{H}_2\text{O}_2 \).](image-url)
working solutions were prepared. One contains 100 ng/ml of phorbol myristate acetate (PMA) (Sigma, diluted in dimethyl sulfoxide) and the other without PMA. Addition of PMA did not affect fluorescence intensity in a separate experiment with cell-free system. Monocytes on the coverslips were reacted with 1.5 ml of working solution with or without PMA, at 37°C for 90 min in CO₂ incubator. Recovered solutions were directed to detect reduced scopoletin by the spectrofluorometer. Concentration of H₂O₂ was determined from the standard curve described above, and total amount of H₂O₂ from monocytes was calculated as nM of H₂O₂/90 min/mg cell protein.

Statistical analysis

Data were analyzed by one-way analysis of variance using personal computer (Stat View™ SE+Graphics, Version 1.04, Abacus Concepts Inc., Berkeley, CA, USA). Null hypothesis was rejected, if either Fisher's protected least significant difference test or Schéffe F-test showed significant level of 95%.

RESULTS

Protein concentration of monocytes on coverslip

Protein concentration of the preparation was 12.06 ± 3.97 (mean ± s.d.) μg in patients with proteinuria, 6.95 ± 2.50 μg in patients without proteinuria, and 12.69 ± 6.37 μg in normal controls.

Comparison of H₂O₂-release among patients

Secretion of H₂O₂ was not observed in any patients when PMA-avoided working solution was exposed. H₂O₂ was released only by exposure of the solution containing PMA. Amount of H₂O₂ release was 177.49 ± 94.75 nM/90 min/mg cell protein in SSNS with proteinuria, 60.67 ± 58.67 nM/90 min/mg cell protein in SSNS without proteinuria and 85.02 ± 48.60 nM/90 min/mg cell protein in normal controls. Statistical significance between SSNS patients and other groups were observed (Fig. 2). There was no significance between SSNS patients without proteinuria and normal controls. Follow-up measurements of H₂O₂ were undertaken in two SSNS patients with proteinuria who subsequently improved to the stage of remission. One patient with onset of SSNS showed declining H₂O₂ from 108.00 to 5.82 nM/90 min/mg cell protein, and the other patient with frequent relapses of SSNS from 361.53 to 33.65 nM/90 min/mg cell protein (arrows in Fig. 2).

H₂O₂ release in correlation with laboratory data

When the level of H₂O₂ release was compared with serum total protein and total cholesterol in the group of SSNS with proteinuria, high correlation coefficient value was observed, although p-value was not significant possibly because of insufficient number of patients studied (r = 0.52; p = 0.29 and r = 0.67; p = 0.14,
Monocyte $\text{H}_2\text{O}_2$ Release in Nephrotic Syndrome

![Graph showing $\text{H}_2\text{O}_2$ release in Nephrotic syndrome](image)

Fig. 2. $\text{H}_2\text{O}_2$ release of monocyte from the patients with SSNS.

- ● SSNS patients with proteinuria; ○ SSNS patients without proteinuria;
- △, normal controls.

Arrows indicate follow-up examinations. Column and bar reflect mean and s.d., respectively.

* $p < 0.05$; n.s., not significant.

respectively). The level of urinary protein excretion did not correlate with the amount of $\text{H}_2\text{O}_2$ secretion ($r = 0.06; \ p = 0.9$).

### Discussion

The present study demonstrates that PMA-triggered monocytes from SSNS patients with proteinuria released significant amount of $\text{H}_2\text{O}_2$, compared with patients in remission and normal controls. In two patients, $\text{H}_2\text{O}_2$ release was decreased as their proteinuria subsided after therapy. These findings suggest that monocytes from proteinuric SSNS patients were activated to be prepared for $\text{H}_2\text{O}_2$ release by exposure of PMA, a protein kinase-C activator (Berridge et al. 1994).

The enhanced capacity to release $\text{H}_2\text{O}_2$ triggered by PMA, detected with scopoletin method, has been established as a useful biochemical correlate of macrophage/monocyte activation (Nathan et al. 1979; Nakagawara et al. 1981; Tsunawaki et al. 1984). Induction of $\text{H}_2\text{O}_2$ release required both activated macrophages and exposure of a triggering agent such as PMA to the cells, where omission of either of these made $\text{H}_2\text{O}_2$ undetectable (Nathan and Root 1977). Capacity of $\text{H}_2\text{O}_2$ release from monocytes depends on the maturation of the cells induced by prolonged incubation of monocytes (Nakagawara et al. 1981), or depends on the exposed activating factor such as Bacille Calmette-Guérin (Nathan and Root 1977). For monocyte activation, our previous study demonstrated that
phagocytosis of opsonized particles as well as the activity of hexosemonophosphate pathway estimated with nitroblue tetrazolium dye test were exaggerated, but chemotaxis was not in the proteinuric stage of SSNS (Nagata et al. 1981; Waga et al. 1983). Activating factors preparing monocyte to elicite enhanced capacity of H$_2$O$_2$ release by PMA was not clarified in this study.

The significance of H$_2$O$_2$ and other reactive oxygen species has been demonstrated in experimental and human mesangial proliferative glomerulonephritis, focusing on the histological participation of neutrophilic polymorphonuclear cells and its capacity of H$_2$O$_2$ release (Rehan et al. 1985; Chen et al. 1992; Diamond 1992; Johnson et al. 1994). However, these may not explain proteinuria in SSNS, in which any of infiltrating cells have not been demonstrated. In contrast, reactive oxygen species was proved to develope proteinuria solely (Yoshioka et al. 1991) or in association with macrophages (Dileepan et al. 1993), and the effect did not require infiltrating cells to the glomeruli.

Thus, it must be interesting to consider that the activated monocytes leading to H$_2$O$_2$ release may have a role in the development of proteinuria in SSNS, although elucidation of interaction between the monocytes and glomeruli of the patients is crucial.

References


