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Inhibition of IgM Secretion in Murine B Cell Lymphoma by Hydrogen Peroxide

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ABSTRACT *: Reactive oxygen species (ROS) contribute to several cellular functions and are involved in the regulation of signal transduction, gene expression, and proliferation. In the present study, we investigated the effect of H_2O_2 treatment on IgM secretion in LPS-stimulated murine B lymphoma, CH12.LX. Cells were treated directly with H_2O_2 and stimulated with LPS. H_2O_2 treatment during 72 h time period inhibited IgM secretion in LPS-stimulated CH12.LX cells in a dose- and time-dependent manners. After treatment with 50 μM H_2O_2 during 72 h time period, the level of IgM in LPS-stimulated CH12.LX cells was markedly decreased, whereas cell viability was not significantly changed. Addition of H_2O_2 concomitantly with LPS, or 12 h post-LPS stimulation, produced a significant inhibition of IgM secretion, whereas inhibitory effect of H_2O_2 on IgM secretion was not observed when added 24 h after LPS stimulation. These findings suggest that H_2O_2 can inhibit the secretion of IgM in LPS-stimulated CH12.LX cells, and may alter the events necessary for terminal B cell differentiation.*

Key Words *: H_2O_2, IgM, B cell lymphoma*

I. INTRODUCTION

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide, and hydroxyl radical, are known to play multiple roles in physiological and pathological states and are constantly produced in living organisms (Yaar and Gilchrest, 1990; Halliwell and Gutteridge, 1990). Although excessive generation of ROS is associated with cell injury in a variety pathological conditions, it has been known that ROS also have normal roles as second messengers in several signal transduction pathways (Halliwell and Gutteridge, 1990; Nakamura *et al.*, 1997; Sundaresan *et al.*, 1995). At low concentrations, hydrogen peroxide is known to induce Ca^{2+} mobilization and activation of protein tyrosine kinases, mitogen-activated protein kinases and transcription factors (Nakamura *et al.*, 1997; Qin *et al.*, 1997; Suzuki *et al.*, 1996). Protein tyrosine kinases play crucial roles in a variety of cellular responses, such as cellular activation, proliferation, differentiation, and apoptosis (Ullrich and Schlessinger, 1990). At high concentrations of hydrogen peroxide, cells appeared to die through necrosis while lower concentrations induced apoptosis (Brenneisen *et al.*, 1997).

It has been reported that B cells are relatively sensitive to H_2O_2 treatment, and high doses of H_2O_2 induce cell necrosis, whereas low doses of H_2O_2 (10~100 μM) induce cellular apoptosis in B cells (Gardner *et al.*, 1997; Lee and Shater, 1997; Lee and Shater, 1999). It has been reported that Syk protein-tyrosine kinase is rapidly activated in B cells after treatment with low doses of H_2O_2 (Qin *et al.*, 1997). Syk plays a crucial role in B cell receptor (BCR)-mediated signaling (Yamada *et al.*, 1993). There are growing evidences that upon BCR-activation, Syk mediates the activation of phospholipase Cγ, resulting in the subsequent production of inositol 1,4,5-triphosphate, thereby inducing an elevation in intracellular Ca^{2+} (Takata *et al.*, 1994). These findings suggest that H_2O_2 can modify B cell differentiation following LPS stimulation.

Here, we have examined whether H_2O_2 is able to modify the differentiation process triggered by LPS

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The list of abbreviations : LPS, lipopolysaccaride; ROS, Reactive oxygen species; H_2O_2, hydrogen peroxide

stimulation in murine B cell lymphoma. It was found that H_2O_2 strongly inhibits IgM production in LPS-stimulated CH12.LX cells. Also, IgM secretion exhibited the greatest inhibition to H_2O_2 during first 24 h post-LPS stimulation.

II. MATERIALS AND METHODS

1. Chemicals

Hydrogen peroxide (30%) was purchased from Sigma-Aldrich (St. Louis, MO) and a stock solution was prepared in serum-free RPMI medium. Bovine calf serum was purchased from HyClone (Logan, UT).

2. Cell culture and treatment

The CH12.LX B cells, derived from the murine CH12 B cell lymphoma, were grown in RPMI 1640 (GIBCO Lab., Grand Island, NY) supplemented with heat-inactivated 10% bovine calf serum, 13.5 mM HEPES, 23.8 mM sodium bicarbonate, 100 U/m*l* penicillin, 100 μg/m*l* streptomycin, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 50 μM β-mercaptoethanol. CH12.LX cells were cultured in 24 well plates (Costar, Cambridge, MA) at a density of 2.5×10^4 cells/well in 1 m*l* treatment media (growth media as stated above but with 5% bovine calf serum) overnight at 37°C in an atmosphere of 5% CO_2. H_2O_2 was directly added to culture media the day after plating into plate.

3. Cell viability

Cell viability was measured by hemocytometry using trypan blue exclusion. One volume of trypan blue (0.4%, Sigma) was added to two volumes of cell suspension harvested from cultures. The cells were examined by inverted light microscopy. Cell viability was expressed as fold change from naive.

4. ELISA

Culture supernatants were harvested at indicated times from naive or LPS (5 μg/m*l*)-stimulated CH12.LX cells that were treated with H_2O_2 or vehicle (serum-free RPMI medium). Supernatants were analysed for IgM by sandwich ELISA as previously described (Sulentic *et al.*, 1998). Briefly, 100 μ*l* of supernatant or standard (mouse IgM κ) were added to wells of a 96 well plate (Immulon 4, Dynex Technologies Inc., Chantilly, VA) previously coated with anti-mouse Ig capture antibody (Roche Molecular Biochemicals, Indianapolis, IN), and then incubated at 37°C for 1.5 h. After the incubation period, the plate was washed with 0.05% Tween-20 PBS and H_2O. A horseradish peroxidase-linked anti-mouse IgM detection antibody was added to the plate and incubated for 1.5 h at 37°C. After washing the plate with 0.05% Tween-20 PBS and H_2O, ABTS substrate (Roche Molecular Biochemicals) was added and colorimetric detection was performed over a 1 h period using an EL808 automated microplate reader with a 405 nm filter (Bio-Tek, Winooski, VT). The concentration of total IgM in the supernatants was calculated using a standard curve generated from the absorbance readings of known IgM concentrations.

5. Statistical analysis of data

The mean±standard deviation was generated for each treatment group. The statistical difference between treatment groups and the appropriate controls was determined using a Dunnett's two-tailed *t* test.

III. RESULTS AND DISCUSSION

Bacterial lipopolysaccharide (LPS), a potent stimulant of B cells, induces B cell proliferation and differentiation into antibody secreting cells (Venkataraman *et al.*, 1999). In a first set of experiments, we studied IgM secretion pattern in LPS-stimulated CH12.LX cells. CH12.LX cells were stimulated with LPS (5 μg/m*l*). The level of IgM in supernatant was measured at indicated time periods. Figure 1 shows a time course of IgM secretion in CH12.LX cells stimulated with LPS. The level of IgM in supernatant was slightly increased 24 h after LPS stimulation, but markedly increased 48 h and 72 h post LPS-stimulation.

To investigate the effect of H_2O_2 treatment on IgM secretion in LPS-stimulated CH12.LX cells, cells were treated directly with various concentrations of H_2O_2 (10~100 μM) and stimulated with LPS (5 μg/m*l*). Cul-

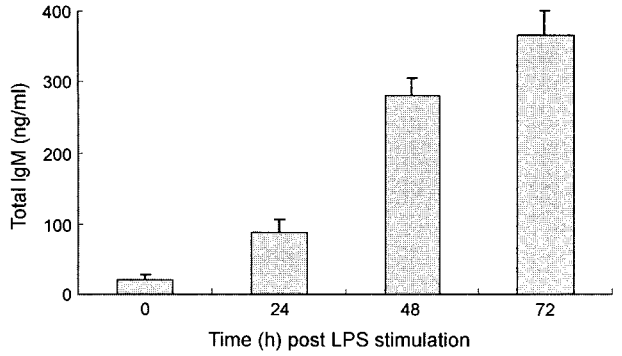

Fig. 1. IgM production of LPS-stimulated CH12.LX cells. CH12.LX cells were stimulated with LPS (5 μg/ml). Supernatants were harvested at 0, 24, 48 or 72 h after LPS stimulation and analyzed for IgM by a sandwich ELISA. IgM is represented on the *y*-axis as ng/ml. Results represent the combined mean±S.D. from three separate experiments, each with an n = 4.

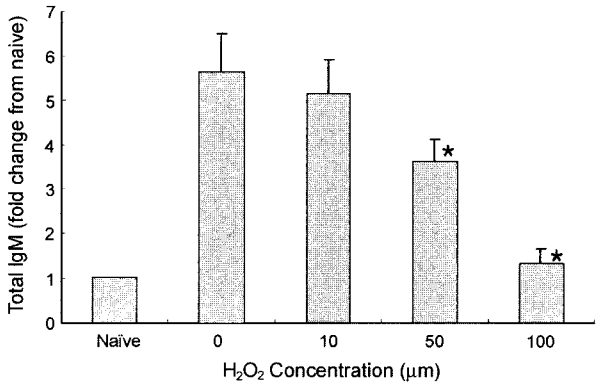

Fig. 2. The level of IgM in LPS-stimulated CH12.LX cells after 72 h post-H_2O_2 treatment. CH12.LX cells were stimulated with LPS (5 μg/ml) and treated with H_2O_2 or vehicle (serum-free medium). Supernatants were harvested at 72 h post-LPS stimulation and analyzed for IgM by a sandwich ELISA. The level of IgM was expressed as fold change from naive. Results represent the combined mean±S.D. from three separate experiments, each with an n = 4. *, donates a value significantly different from vehicle at $p < 0.05$.

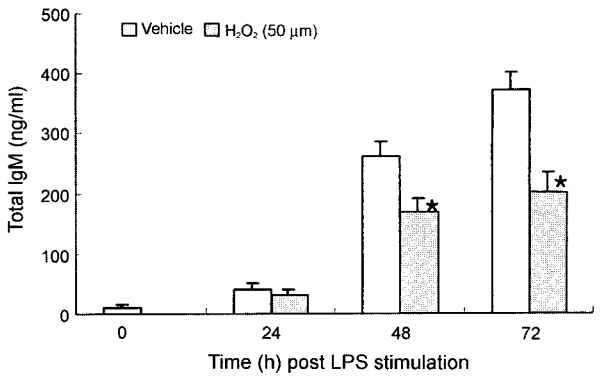

Fig. 3. Effect of H_2O_2 on IgM production in LPS-stimulated CH12.LX cells. CH12.LX cells were stimulated with LPS (5 μg/ml) and treated with H_2O_2 (50 μM) or vehicle (serum-free medium). Supernatants were harvested at 0, 24, 48 or 72 h after LPS stimulation. The level of IgM in supernatant was analyzed by a sandwich ELISA. Results represent the combined mean±S.D. from three separate experiments, each with an n = 4. *, donates a value significantly different from vehicle at $p < 0.05$.

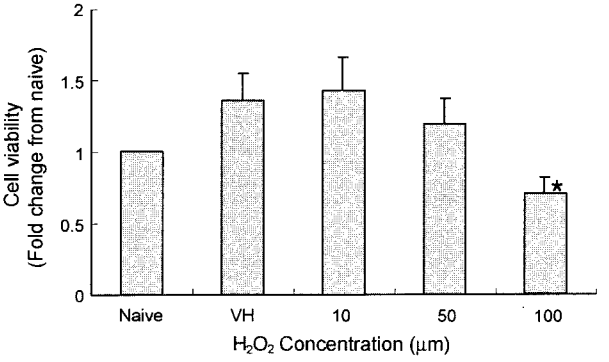

Fig. 4. Cell viability of LPS-stimulated CH12.LX cells after 72 h post-H_2O_2 treatment. CH12.LX cells were stimulated with LPS (5 μg/ml) and treated with H_2O_2 at various concentrations. Cells were harvested at 72 h post-H_2O_2 treatment. Cell viability was measured by trypan blue exclusion method. Results represent the combined mean±S.D. from three separate experiments, each with an n = 4. *, donates a value significantly different from vehicle at $p < 0.05$.

ture supernatants were harvested at indicated time periods after LPS stimulation. The level of IgM in culture supernatant was measured by a sandwich ELISA. Figure 2 and Fig. 3 show concentration- and time-dependent response curve, respectively. As shown in Fig. 2, the level of IgM was significantly decreased after H_2O_2 treatment at doses of 50 and 100 μM in LPS-stimulated CH12.LX cells.

To assess whether H_2O_2-induced inhibition of IgM secretion in LPS-stimulated CH12.LX cells was due to cytotoxicity of H_2O_2 to CH12.LX cells, cytotoxic effects of different concentrations of H_2O_2 were measured. CH12.LX cells were incubated with increasing concentrations of H_2O_2 during 72 h, and cytotoxic effects were assayed by trypan blue exclusion method. As shown in Fig. 4, cell viability (fold change from naive) in a vehicle group, stimulated with LPS but not treated with H_2O_2, was 1.31 0.21, which might be due to increased proliferation in LPS-stimulated CH12.LX cells. Concentrations of H_2O_2 below 10 μM had no effect on cell viability over a 72 h time period (Fig. 4). However, as shown in Fig. 4, cell viability (fold change from naive) of CH12.LX cells exposed to 100 μM H_2O_2 was significantly decreased to 0.67 0.17, whereas cell

viability at 50 μM H_2O_2 was slightly decreased as compared to vehicle group, but not significantly different.

As shown in Fig. 2 and Fig. 4, after treatment with 50 μM H_2O_2 during 72 h time period, the level of IgM in LPS-stimulated CH12.LX cells was markedly decreased, whereas cell viability was not significantly changed. These findings suggest that H_2O_2-induced inhibition of IgM secretion in LPS-stimulated CH12.LX cells is not due to cell death, but partly due to interference of H_2O_2 in activation processes of LPS-stimulated B lymphoma cells. It is well known that ROS contribute to several cellular functions and are involved in the regulation of signal transduction, gene expression, and proliferation (Burdon, 1995).

LPS dramatically increases IgM secretion in CH12.LX cells at 48 and 72 h post-LPS stimulation (Fig. 1), while the levels of IgM in the presence of H_2O_2 (50~100 μM) were significantly decreased (Fig. 2). In time of addition studies, TCDD had to be added to mouse splenocyte culture within the first 24 h after antigen treatment to produce an inhibition of the antibody forming cell response (Tucker *et al.*, 1986). This critical time period of sensitivity for H_2O_2-induced inhibition of IgM secretion in LPS-stimulated CH12.LX cells was also observed in our experiments (Fig. 5). Addition of H_2O_2 concomitantly with LPS, or 12 h post-LPS stimulation, produced a significant inhibition of IgM secretion. However, the inhibitory effect of H_2O_2 on IgM secretion was not observed when added 24 h after LPS stimulation. These findings suggest that H_2O_2 may alter the events necessary for terminal B cell differentiation.

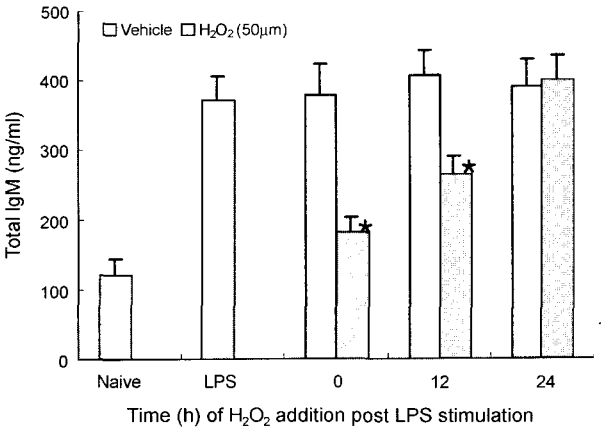

Fig. 5. Effects of time of addition on inhibition of IgM secretion in LPS-stimulated CH12.LX cells. CH12.LX cells were treated with H_2O_2 (50 μM) or vehicle (serum-free medium) at 0, 12, or 24 h after LPS stimulation. Supernatants were harvested at 72 h post-LPS stimulation and analyzed for IgM by a sandwich ELISA. Results represent the combined mean±S.D. from three separate experiments, each with an n = 4. *, donates a value significantly different from vehicle at $p < 0.05$.

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