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Effects of intracellular reactive oxygen species generated by 6-formylpterin on T cell functions

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Abstract

The intracellular generation of reactive oxygen species (ROS) by 6-formylpterin and its effects on the human T cell functions were examined *in vitro*. When T cells isolated from fresh blood were incubated with 6-formylpterin for 1 hr, the oxygen consumption and concomitant ROS generation were observed. The incubation of T cells with 50–500 μM 6-formylpterin for 24 hr brought about the elevation of intracellular ROS without inducing cell death. In contrast, the incubation of T cells with exogenously administered hydrogen peroxide (H_2O_2) or other pterin derivatives (6-hydroxymethylpterin, pterin-6-carboxylic acid, pterin, neopterin, biopterin and folic acid) for 24 hr did not cause the intracellular ROS elevation. In the T cells stimulated with mitogenic lectin phytohemagglutinin (PHA) in conjunction with phorbol myristate acetate (PMA), 6-formylpterin suppressed the NF-κB-dependent transcription, the production of cytokines (IFN-γ and IL-2) and the cell proliferation. These suppressive effects of 6-formylpterin were all reversed by *N*-acetyl-L-cystein (NAC). However, 6-formylpterin did not inhibit the NF-κB-DNA binding of the nuclear extracts obtained from the PHA/PMA-stimulated T cells. Since the NF-κB-DNA binding assay performed *in vitro* merely shows the presence or absence of NF-κB subunit in the nuclear extracts but not guarantees the actual binding of NF-κB with DNA in the nucleus, these findings suggest that intracellular ROS generated by 6-formylpterin does not affect the translocation of NF-κB to the nucleus but that it inhibits the NF-κB-dependent transcription in the nucleus, resulting in the suppression of cytokine production and cell proliferation in the activated T cells.

Keywords: 6-Formylpterin; Reactive oxygen species; T cells; NF-κB; Cytokines; Cell proliferation

1. Introduction

6-Formylpterin is known as a potent xanthine oxidase inhibitor with an inhibition constant (K_i) of approximately 0.6 nM [1,2]. Although 6-formylpterin does not usually occur *in vivo*, it is produced from folic acid *in vivo* in some pathological conditions, such as carcinoma [3]. Recently, we showed that 6-formylpterin had the property to transfer electron from NAD(P)H to oxygen and that this property brought about the intracellular generation of reactive oxygen species (ROS) which induced apoptosis in HL-60 cells, suppressed cell proliferation in PanC-1 cells and inhibited Fas-mediated apoptosis in Jurkat cells [4]. The intracellular ROS generation by 6-formylpterin was also observed in human peripheral leukocytes: neutrophils, lymphocytes, and monocytes, and the generated ROS was involved in their cell death [5]. ROS is not only involved in cell death, but also modulates a variety of cell functions [6]. Therefore, the ROS generated by 6-formylpterin was expected to modulate the functions of leukocytes, such as bactericidal activity in neutrophils [7], inflammatory responses in

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Abbreviations: Carboxy-DCFH-DA, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; DCF, dichlorofluorescein; NAC, *N*-acetyl-L-cystein; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PI, propidium iodide; PMA, phorbol myristate acetate; ROS, reactive oxygen species; RPMI, Roswell Park Memorial Institute.

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monocytes [8] and immune responses in lymphocytes [9]. In fact, in neutrophils, the generated ROS restored the impaired bactericidal activity of diphenyleneiodonium (DPI)-treated neutrophils [10]. However, in lymphocytes, it remains still unclear how the generated ROS affects the cell functions.

In the present study, to elucidate the effects of intracellular ROS generated by 6-formylpterin on cell functions of lymphocytes, we applied 6-formylpterin to freshly isolated human peripheral blood T cells. First, in resting T cells, the effects of 6-formylpterin and exogenously administered H_2O_2 on the intracellular ROS content and cell death were compared. Then, in activated T cells stimulated with mitogenic lectin phytohemagglutinin (PHA) in conjunction with phorbol myristate acetate (PMA), the effects of 6-formylpterin on the T cell functions, NF-κB activation, cytokine production and cell proliferation, were examined.

2. Materials and methods

2.1. Reagents

6-Formylpterin was obtained from Sankyo Kasei Kogyo. 6-Hydroxymethylpterin was made in our laboratory from 6-formylpterin by reducing it with sodium borohydride. Pterin-6-carboxylic acid, pterin and folic acid were purchased from Sigma Chemicals. Neopterin and biopterin were supplied by Asahi Breweries Ltd. and Kaneka Co., respectively. Roswell Park Memorial Institute (RPMI) medium, fetal calf serum (FCS), and penicillin-streptomycin liquid were purchased from GIBCO; fluorescein isothiocyanate (FITC)-conjugated Annexin V was from Boehringer Mannheim; propidium iodide (PI) and Hoechst 33342 were from Nacalai Tesque. 6-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-DCFH-DA) was obtained from Molecular Probes. Other chemicals, such as PMA, PHA, and *N*-acetyl-L-cystein (NAC), were purchased from Sigma Chemicals.

2.2. Cell isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated from blood of healthy adult volunteers by sedimentation through two-step Percoll (Pharmacia) gradients, as previously described [11]. Resting $CD3^+$ T cells were further isolated from the PBMCs using a column-based purification technique (Stem Cell Technologies) according to the manufacturer's recommendation [12]. The purity of cells after column depletion was always greater than 97% as determined by flow cytometry with FITC-conjugated anti-CD3 antibody (Becton Dickinson). Freshly purified cells were cultured in RPMI medium containing 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37° in a 5% CO_2 incubator.

2.3. Measurement of oxygen consumption and detection of intracellular ROS generation

O_2 consumption in the T cells incubated with 6-formylpterin was measured polarographically with a Clarke oxygen electrode (model 5300, Yellow Springs Instruments) at 37°. Purified T cells (2×10^7) were transferred to a 4-mL chamber and incubated with 5 μM carboxy-DCFH-DA, a ROS-sensitive dye [13]. After baseline measurement (5 min), 6-formylpterin (final 2 mM), or an equivalent volume of phosphate-buffered saline (PBS) was added to the chamber and the oxygen consumption was continuously recorded for 60 min. Immediately after the measurement of oxygen consumption, the cells in the chamber were collected and the intracellular ROS was detected by flow cytometry.

2.4. Microscopic study

Intracellular ROS generation in T cells was also confirmed using a Nikon E800M fluorescence microscope (Nikon) equipped with a LEICA DC500 digital CCD camera (Leica) and a PC computer. Purified T cells were incubated with medium alone, 500 μM 6-formylpterin or 10 μM hydrogen peroxide (H_2O_2) for 24 hr. The cells (1×10^6 cells/mL) were then incubated with 2 μM carboxy-DCFH-DA for an additional 30 min at 37° in an incubator, washed, and plated onto glass coverslips for visualization and image acquisition. Hoechst 33342 was also used to depict the total cell distribution.

2.5. Detection of intracellular ROS and cell death

The intracellular ROS in resting and activated T cells was also detected using carboxy-DCFH-DA and flow cytometry. In the resting T cells, the cells were incubated for 24 hr with 50–500 μM 6-formylpterin or 5–50 μM H_2O_2. The effects of other pterin derivatives (6-hydroxymethylpterin, pterin-6-carboxylic acid, pterin, neopterin, biopterin, and folic acid) on the intracellular ROS were also examined. Further, to determine the fate of 6-formylpterin and the other pterin derivatives in the cell culture, the supernatant was collected before and after the 24-hr incubation, and the production-analysis was carried out using reversed-phase high-performance liquid chromatography (RP-HPLC).

In the activated T cells, the cells were stimulated with 1 μg/mL PHA in conjunction with 5 ng/mL PMA, and then incubated with 50–500 μM 6-formylpterin or 500 μM 6-formylpterin with 10 mM NAC for 24 hr. In both cases, after the 24-hr incubation, the cells (1×10^6 cells/mL) were incubated with 2 μM carboxy-DCFH-DA for an additional 30 min at 37° in an incubator, washed, and resuspended in PBS. The intracellular ROS was detected by the FACSCalibur flow cytometer (Becton Dickinson) and analyzed using the CELLQuest software (Becton Dickinson).

The measurement of cell death in the resting and the activated T cells was performed simultaneously with the detection of intracellular ROS. The cells (1×10^6 cells/mL) incubated for 24 hr under the above-mentioned conditions were stained with FITC-conjugated Annexin V and PI according to a previously described protocol [14], and analyzed on the FACSCalibur flow cytometer using the CELLQuest software.

2.6. NF-κB-DNA binding assay

Activation of the p65 subunits of NF-κB in the activated T cells was determined using NF-κB p65 ELISA-based transcription factor assay kits (Active Motif). The detecting antibodies recognize epitopes on p65 that are accessible only when NF-κB is activated and bound to its target DNA (containing the NF-κB consensus binding site, 5′-GGGACTTTCC-3′) attached to 96-well plates [15]. Purified T cells (1×10^7) were stimulated with 1 μg/mL PHA in conjunction with 5 ng/mL PMA, and then incubated with 50–500 μM 6-formylpterin or 500 μM 6-formylpterin with 10 mM NAC for 2 hr. After the 2-hr incubation, the cells were harvested and the nuclear extract preparation and NF-κB ELISA were carried out according to protocols supplied by the manufacturer.

2.7. NF-κB reporter gene assay

A phagemid vector pNF-κB-Luc, which contains five copies of NF-κB-binding motif of the murine immunoglobulin κ light chain enhancer controlling luciferase expression, was purchased from Stratagene. Transient transfection of the pNF-κB-Luc into T cells was performed as previously described [16] with some modification. Briefly, purified T cells (2×10^7) were cultured with 5 μg/mL PHA overnight. Between 19.5 and 20 hr after PHA stimulation, 5×10^6 cells were electroporated in a cuvette with 10 μg of pNF-κB-Lu at 250 V and 690 μF, using a Bio-Rad Gene Pulsar II electroporater (Bio-Rad). The cells were transferred to fresh culture medium at 5×10^5 cells/mL and rested for 2 hr with periodic agitation. Cell viability was ascertained by Trypan blue exclusion, and 1×10^6/mL live cells were cultured in 96-well U-bottom microtiter plates (Falcon, Becton Dickinson) with fresh culture medium. The transfected cells were stimulated with 1 μg/mL PHA in conjunction with 5 ng/mL PMA, and then incubated with 50–500 μM 6-formylpterin or 500 μM 6-formylpterin with 10 mM NAC for 6 hr. After the 6-hr incubation, the cells were harvested and the luciferase activity of the lysates was determined using a commercial assay system (Promega) with a luminometer, Lumat LB9507 (Berthold, GmbH & Co., KG, Bad Wildbad). The relative luciferase activity was calculated by assigning the luciferase activity obtained from the unstimulated cells to the value 1.

2.8. Measurement of cytokine production

The production of cytokines (IFN-γ and IL-2) in the PHA/PMA-stimulated T cells, incubated with 50–500 μM 6-formylpterin or 500 μM 6-formylpterin with 10 mM NAC for 24 hr, was measured by sandwich ELISA using Ab pairs (PharMingen), according to the manufacturer's recommended procedures. The lower limits of sensitivity in the ELISA were 4.7 pg/mL (IFN-γ) and 7.8 pg/mL (IL-2), using human IFN-γ and IL-2 as standards.

2.9. Cell proliferation

The cell proliferation for 48 hr in the PHA/PMA-stimulated T cells was measured using the CyQUANT kit (Molecular Probes) as previously described [17]. Briefly, purified T cells were seeded at 1×10^4 per well in 96-well plates (Falcon, Becton Dickinson) in culture medium. The cells were stimulated with 1 μg/mL PHA in conjunction with 5 ng/mL PMA, and then incubated for 48 hr with 50–500 μM 6-formylpterin or 500 μM 6-formylpterin with 10 mM NAC. After centrifugation (500 *g* for 15 min), the supernatant was removed and the plates were frozen at -80° at least overnight. Proliferation was calculated from the change in total nucleic acid content in each well using the CyQUANT kit that measures the signal generated by binding of a fluorescent dye to nucleic acids, a signal that increases in proportion to the number of cells [18]. Fluorescence signals were read from a 96-well plate using a fluorometric microplate reader (Fluoroskan Ascent; Labsystems) with excitation and emission wavelengths of 485 and 538 nm, respectively.

2.10. Statistical analysis

Values are shown as means ± SD. Statistical comparisons were made using the repeated-measures analysis of variance, followed by Student's paired *t*-test with a Bonferroni correction for multiple comparison of the group means. A P-value < 0.05 was regarded as being statistically significant.

3. Results

3.1. Oxygen consumption and intracellular ROS generation

The oxygen concentration of the medium containing T cells and 6-formylpterin decreased more than that of the medium containing T cells alone (Fig. 1A). Further, the DCF fluorescence obtained from the cells with 6-formylpterin was greater than that obtained from the cells without 6-formylpterin (Fig. 1B). These results showed that the ROS was generated in the T cells with 6-formylpterin from the dissolved dioxygen in the medium.

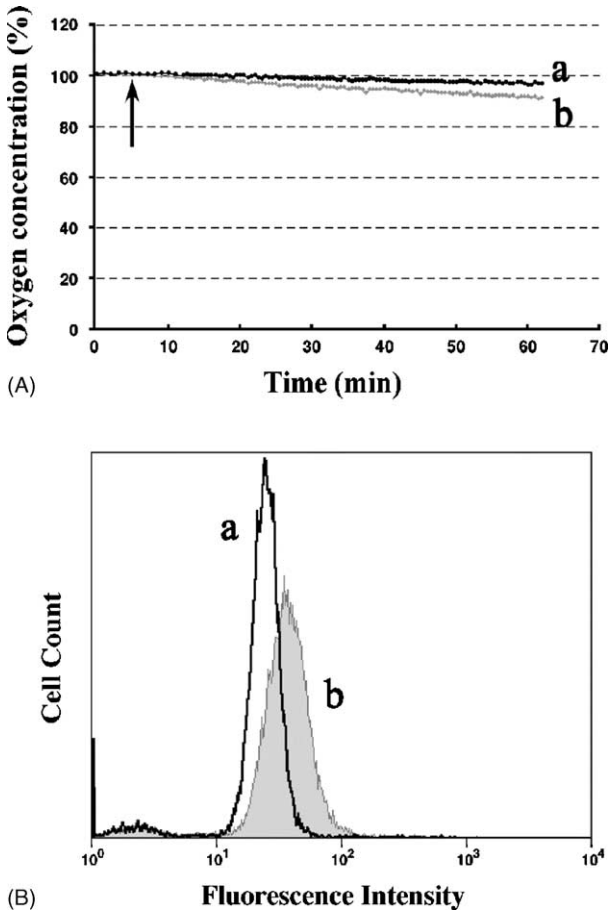

Fig. 1. Effects of 6-formylpterin on O_2 consumption and ROS generation in T cells. (A) The time course of the changes in oxygen concentration measured by oxygen electrodes is shown when (a) PBS or (b) 6-formylpterin (final 2 mM) were added to the chamber containing T cells (2×10^7 in culture medium) and 5 μM carboxy-DCFH-DA. Arrow indicates the addition of (a) PBS or (b) 6-formylpterin to the medium. The oxygen concentration of the medium equilibrated with room air was initially assigned to be 100%. (B) Immediately after the above-mentioned study, the T cells were collected and the intracellular ROS were detected by flow cytometry. The amounts of intracellular ROS in the T cells incubated with (a) medium alone and (b) 6-formylpterin are indicated. The findings of a representative experiment are shown.

3.2. Intracellular ROS and cell death in the resting and the activated T cells

In the microscopic study, the increase in DCF fluorescence was observed in the T cells incubated with 6-formylpterin for 24 hr, but not in the T cells incubated with H_2O_2 (Fig. 2A). In the flow cytometry, an apparent increase in DCF fluorescence was observed in a dose-dependent manner when the resting T cells were incubated with 6-formylpterin for 24 hr, while exogenously administered 5–50 μM H_2O_2 did not induce any increases in DCF fluorescence (data not shown). The pterin derivatives other than 6-formylpterin did not induce apparent increases in DCF fluorescence (Fig. 2B). Further, the production-analysis by RP-HPLC showed that the all pterin derivatives remained unaffected within the 24-hr incubation (data not shown).

Flow cytometric analysis of cell death in the resting T cells using Annexin V and PI revealed that the incubation of the cells with up to 500 μM 6-formylpterin did not induce any cell death (data not shown).

The dose-dependent increase in DCF fluorescence by 6-formylpterin was observed in the PHA/PMA-stimulated T cells, and this increase was negated by NAC (Fig. 3). Although the PMA/PHA-stimulated T cells showed a higher cell death rate compared with the resting T cells, up to 500 μM 6-formylpterin no longer raised the cell death rate in the stimulated T cells. The co-incubation of NAC with 6-formylpterin increased the cell death rate slightly (Fig. 4).

3.3. Translocation of NF-κB to the nucleus in the activated T cells

The NF-κB-DNA binding of the nuclear extracts obtained from the PMA/PHA-stimulated T cells was enhanced compared with that obtained from the resting T cells. The incubation of the cells with up to 500 μM 6-formylpterin did not affect this enhancement at all (Fig. 5). The *in vitro* NF-κB-DNA binding assay is performed under reducing condition and merely shows the presence or absence of NF-κB subunit in the nuclear extracts. Therefore, this result showed that 6-formylpterin did not affect the NF-κB translocation in the activated T cells.

3.4. NF-κB-dependent transcription

When the pNF-κB-Luc transfected T cells were stimulated with PMA and PHA for 6 hr, the relative luciferase activity increased by greater than 10-fold (10.0 ± 2.5, $N = 5$) compared with that when the cells were not stimulated. The incubation of the cells with 6-formylpterin suppressed this increase in a dose-dependent manner, and this suppression was negated by NAC (Fig. 6). This result showed that 6-formylpterin suppressed the NF-κB-dependent transcription and/or posttranscriptional regulation in the activated T cells.

3.5. Cytokine production in the activated T cells

When the T cells were stimulated with PMA and PHA for 24 hr, the levels of measured cytokines (IFN-γ and IL-2) apparently increased compared with those when the cells were not stimulated. The levels of IFN-γ and IL-2 increased from 88 ± 57 pg/mL to $16{,}445 \pm 9{,}941$ pg/mL ($N = 8$), and from 13.7 ± 1.4 pg/mL to 5185 ± 2341 pg/mL ($N = 8$), respectively. The incubation of the cells with 6-formylpterin suppressed this increase in a dose-dependent manner, and this suppression was negated by NAC (Fig. 7).

3.6. Cell proliferation in the activated T cells

When the T cells were stimulated with PMA and PHA for 48 hr, the fluorescence in the CyQUANT assay, which is

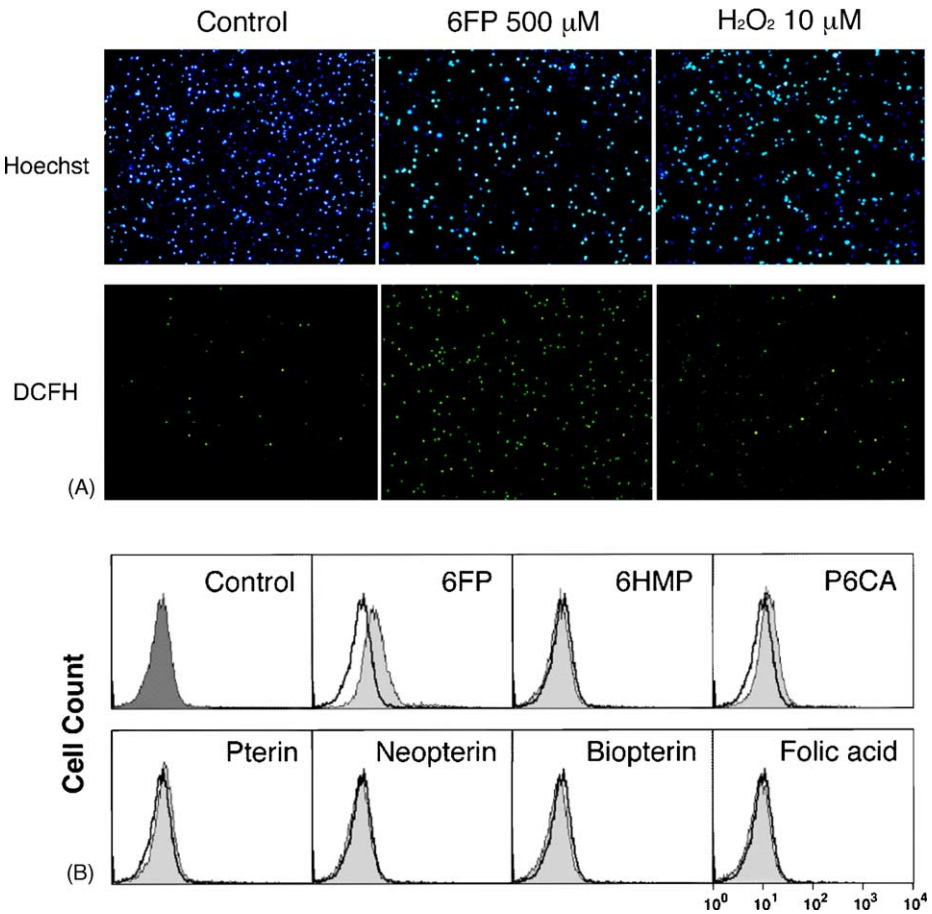

Fig. 2. Effects of 6-formylpterin, H_2O_2, and other pterin derivatives on ROS generation in T cells. (A) T cells were incubated with medium alone (Control), 500 μM 6-formylpterin (6FP 500 μM), or 10 μM H_2O_2 (H_2O_2 10 μM) for 24 hr. The amounts of intracellular ROS were then visualized using a fluorescence microscope with a probe, carboxy-DCFH-DA (DCFH). The total cell distribution was depicted using another probe, Hoechst 33342 (Hoechst). (B) T cells were incubated with medium alone (Control), 500 μM 6-formylpterin (6FP), 6-hydroxymethylpterin (6HMP), pterin-6-carboxylic acid (P6CA), pterin, neopterin, biopterin and folic acid) for 24 hr, and then the cells were incubated with 2 μM DCFH-DA for an additional 30 min. The finding of a representative one of five experiments is shown.

proportional to the change in cell number, increased to approximately double (2.1 ± 0.5, $N = 9$) that when the cells were not stimulated. The incubation of the cells with 6-formylpterin suppressed this increase in a dose-dependent manner, and this suppression was negated by NAC (Fig. 8).

4. Discussion

In the present study, we demonstrated that 6-formylpterin brought about the elevation of intracellular ROS without inducing cell death both in the resting and in the activated T cells and further, in the activated T cells, 6-formylpterin suppressed the NF-κB activation, cytokine production, and cell proliferation.

The generation of intracellular ROS by 6-formylpterin was shown using the probe, carboxy-DCFH-DA. This probe is widely used to measure intracellular H_2O_2 formation [19,20]. Recently, it has been shown that the use of this probe to measure H_2O_2 production in cells is problematic, because the probe could be oxidized by any intracellular oxidant, such as ferryl species, peroxynitrite, etc. [21]. In neutrophils, we documented the formation of H_2O_2 by 6-formylpterin using electron paramagnetic resonance (EPR) spectroscopy with a spin trap [10]. However, in T cells, we failed to document the formation of not only H_2O_2 but also superoxide anions and hydroxyl radicals using EPR (data not shown). Since oxygen consumption and concomitant ROS generation were observed, the formation of the oxygen-centered radicals should occur in T cells, but the original radicals of the generated ROS are not clear from this alone.

The flow cytometric analysis showed that neither exogenously administered H_2O_2 nor the pterin derivatives other than 6-formylpterin (6-hydroxymethylpterin, pterin-6-carboxylic acid, pterin, neopterin, biopterin, and folic acid) brought about the elevation of intracellular ROS. Further, the production-analysis showed that the pterin derivatives including 6-formylpterin remained unaffected within the 24-hr incubation. These results jointly indicate that the intracellular ROS generation is a specific effect of 6-formylpterin. It was already shown that, among the conjugated

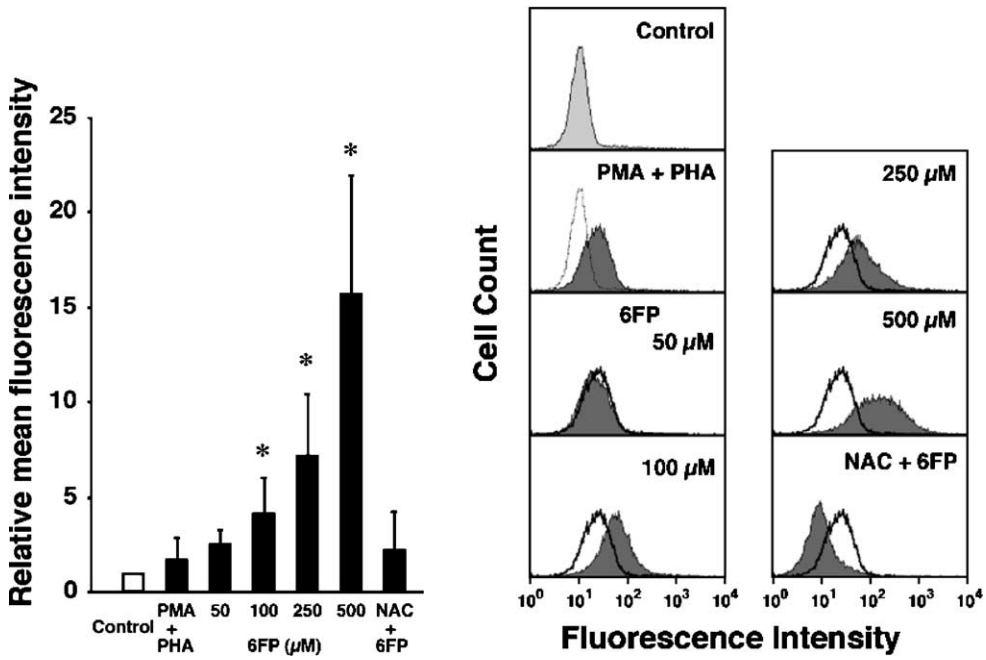

Fig. 3. Intracellular H_2O_2 generation by 6-formylpterin in the T cells stimulated with PHA/PMA and the counteraction by NAC. Purified T cells (1×10^6 mL^{-1}) were stimulated with 1 μg/mL phytohemagglutinin (PHA) in conjunction with 5 ng/mL phorbol myristate acetate (PMA) and incubated with 6-formylpterin (6FP, 50, 100, 250, and 500 μM) or 500 μM 6-formylpterin with 10 mM *N*-acetyl-L-cystein (NAC) for 24 hr, and then the cells were incubated with 2 μM DCFH-DA for an additional 30 min. The relative mean fluorescence intensity was calculated when the mean fluorescence intensity obtained from the unstimulated cells (Control) was assigned to 1, and the values are shown as means ± SD (N = 6). $^*P < 0.05$: significantly different from the values obtained from the cells stimulated with PHA/PMA but not incubated with 6-formylpterin and NAC. The finding of a representative experiment is shown in the right half of the graph.

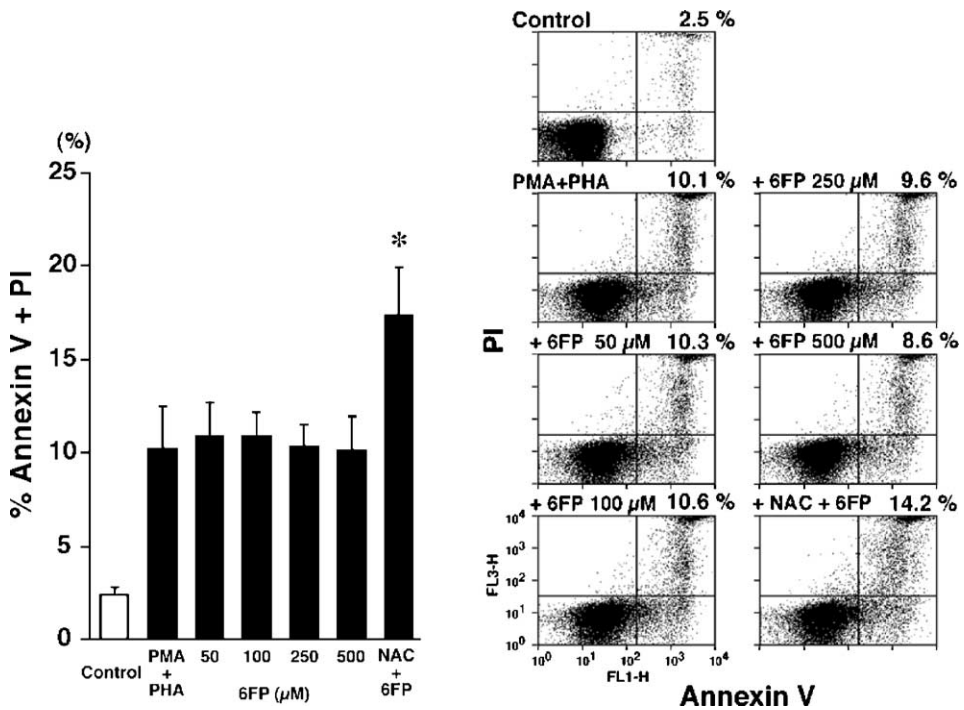

Fig. 4. Effects of 6-formylpterin on cell death of the T cells stimulated with PHA/PMA. Purified T cells (1×10^6 mL^{-1}) were stimulated with 1 μg/mL PHA in conjunction with 5 ng/mL PMA and incubated with 6-formylpterin (6FP, 50, 100, 250, and 500 μM) or 500 μM 6-formylpterin with 10 mM NAC for 24 hr, and then the cells were stained with FITC-labeled Annexin V and PI as described in Section 2. Annexin V staining corresponds to early apoptosis, PI staining cells alone represent dead cells, and Annexin V and PI positive cells corresponds to late apoptosis. The percentages of Annexin V and PI positive cells are shown as means ± SD (N = 6). $^*P < 0.05$: significantly different from the values obtained from the cells stimulated with PHA/PMA but not incubated with 6-formylpterin and NAC. The finding of a representative experiment is shown in the right half of the graph.

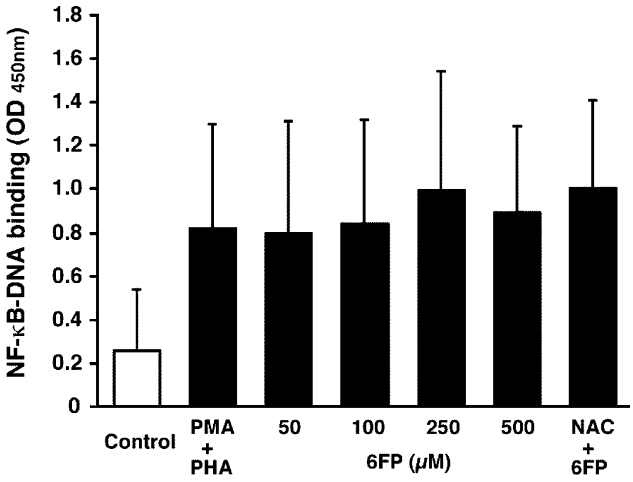

Fig. 5. Effects of 6-formylpterin on the translocation of NF-κB to the nucleus in the T cells stimulated with PHA/PMA. Purified T cells (1×10^6 mL^{-1}) were stimulated with 1 μg/mL PHA in conjunction with 5 ng/mL PMA and incubated with 6-formylpterin (6FP, 50, 100, 250, and 500 μM) for 2 hr. The cells were harvested and nuclear extracts were prepared. NF-κB-DNA binding was determined as described in Section 2. The optical densities are shown as means ± SD (N = 4).

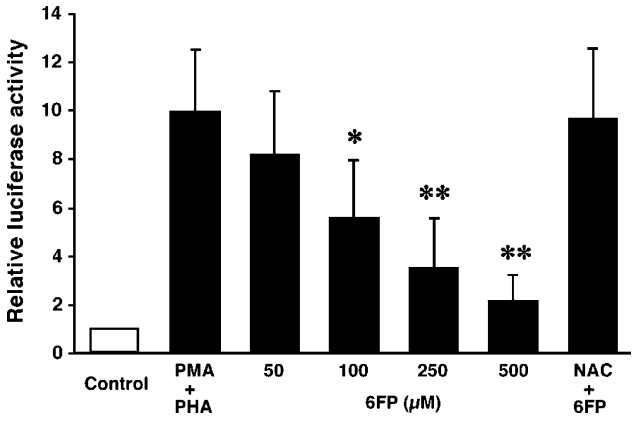

Fig. 6. Inhibitory effects of 6-formylpterin on the NF-κB activation in the T cells stimulated with PHA/PMA and its reversal by NAC. Purified T cells were transfected with the pNF-κB-Luc containing five copies of NF-κB-binding motif as described in Section 2. After transfection, the cells (1×10^6 mL^{-1}) were stimulated with 1 μg/mL PHA in conjunction with 5 ng/mL PMA and incubated with 6-formylpterin (6FP, 50, 100, 250, and 500 μM) or 500 μM 6-formylpterin with 10 mM NAC for 6 hr. Then, the cells were harvested and the luciferase activity of the lysates was determined. The relative luciferase activity was calculated when the luciferase activity obtained from the unstimulated cells (Control) was assigned to 1, and the values are shown as means ± SD (N = 5). $^{*}P < 0.05$ and $^{**}P < 0.01$: significantly different from the values obtained from the cells stimulated with PHA/PMA but not incubated with 6-formylpterin and NAC.

pterins (6-formylpterin, 6-hydroxymethylpterin, pterin-6-carboxylic acid, pterin, neopterin, and biopterin), only 6-formylpterin reacted with NAD(P)H and that only 6-formylpterin induced increases in intracellular ROS and apoptosis in HL-60 cells [4].

In the present study, in accordance with the PHA/PMA stimulation following the prestimulation by PHA alone in the NF-κB reporter gene assay, the T cells were always stimulated by PHA and PMA in the other assays. This stimulation induced intense T cell responses, such as the NF-κB activation, cytokine production and cell prolifera-

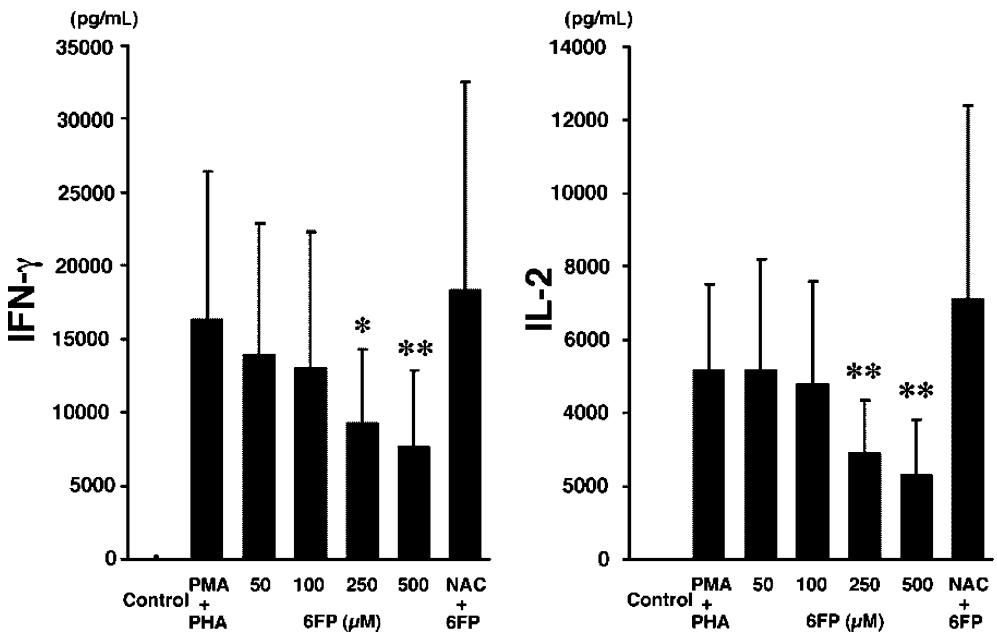

Fig. 7. Inhibitory effects of 6-formylpterin on the production of cytokines (IFN-γ and IL-2) in T cells stimulated with PHA/PMA and the action of NAC on them. Purified T cells (1×10^6 mL^{-1}) were stimulated with 1 μg/mL PHA in conjunction with 5 ng/mL PMA and incubated with 6-formylpterin (6FP, 50, 100, 250, and 500 μM) or 500 μM 6-formylpterin with 10 mM NAC for 24 hr, and then the levels of cytokines (IFN-γ and IL-2) in culture supernatants were measured by sandwich ELISA as described in Section 2. The values are shown as means ± SD (N = 8). $^{*}P < 0.05$ and $^{**}P < 0.01$: significantly different from the values obtained from the cells stimulated with PHA/PMA alone. Mean levels of IFN-γ and IL-2 in Controls were 88 ± 57 pg/mL and 13.7 ± 1.4 pg/mL, respectively.

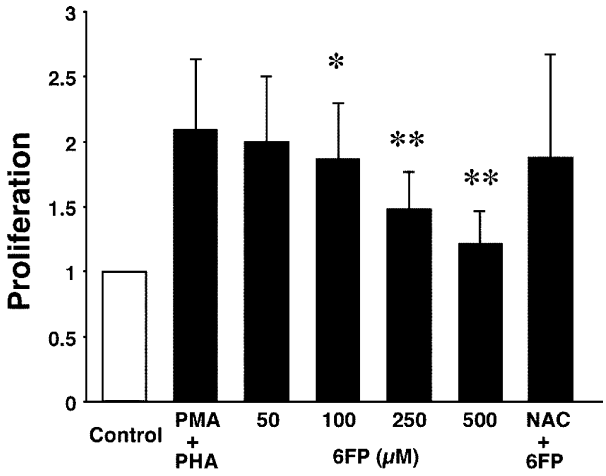

Fig. 8. Inhibitory effects of 6-formylpterin on the proliferation of the T cells stimulated with PHA/PMA and its reversal by NAC. Purified T cells (1×10^6 mL^{-1}) were stimulated with 1 μg/mL PHA in conjunction with 5 ng/mL PMA and incubated with 6-formylpterin (6FP, 50, 100, 250, and 500 μM) or 500 μM 6-formylpterin with 10 mM NAC for 48 hr, and then the cell numbers were measured using the CyQUANT assay as described in Section 2. The folds of control fluorescence was calculated when the fluorescence of the unstimulated cells (Control) was assigned to 1, and the values are shown as means ± SD (N = 9). $^{*}P < 0.05$ and $^{**}P < 0.01$: significantly different from the values obtained from the cells stimulated with PHA/PMA but not incubated with 6-formylpterin and NAC.

tion. In the PHA/PMA-stimulated T cells, the dose-dependent increase in DCF fluorescence was observed, which was considered to reflect the intracellular ROS generation by 6-formylpterin, and the complete negation of this increase by NAC was considered to be due to the quenching of the generated ROS by NAC as an antioxidant. In inverse proportion to the increase in DCF fluorescence, the T cell responses were all suppressed by 6-formylpterin in a dose-dependent manner, and these suppressions were all negated by NAC. Therefore, the ROS generated by 6-formylpterin was considered to induce the suppression of the T cell responses. The possibility of cell death as a mechanism underlying these suppressions was eliminated by the analysis of cell death using Annexin V and PI.

NF-κB is required for IL-2 and IFN-γ transcription [22,23]. Therefore, the suppression of the production of these cytokines is attributed to that of the NF-κB activation by 6-formylpterin. Further, NF-κB activation has a fundamental role in T cell proliferation [24]. Thus, the suppression of cell proliferation is also attributed to that of NF-κB activation by 6-formylpterin. In the present study, the activation of NF-κB was evaluated in two different stages of the NF-κB-dependent signaling pathway. One is the translocation of NF-κB to the nucleus examined by NF-κB-DNA binding assay, and the other is the NF-κB-dependent transcription examined by NF-κB reporter gene assay. The former was not affected by 6-formylpterin, and the latter was suppressed by 6-formylpterin in a dose-dependent manner. These results showed that 6-formylpterin did not affect the translocation of NF-κB into the nucleus, but interfered with the NF-κB-dependent transcription and/or posttranscriptional regulation in the nucleus. It is reported that, in the cytosol, a pro-oxidant signal is required to activate NF-κB but that, in the nucleus, NF-κB must be maintained in a reduced state for DNA binding [25]. Since 6-formylpterin intracellularly generates ROS as a pro-oxidant, it is speculated that the generated ROS permeates into the nucleus and converts the redox state in the nucleus from a reduced state to an oxidant state, which interrupts the DNA binding of NF-κB, that is, NF-κB-dependent transcription. The suppression of the NF-κB-dependent transcription by 6-formylpterin was negated by NAC, which further supports this speculation.

It was once reported that degradation of folic acid to 6-formylpterin is a property of tumor cells, but not normal cells in culture and that 6-formylpterin is found in the urine of cancer patients only [3]. Later, however, it was demonstrated that normal human skin fibroblasts in culture readily degrade folic acid to 6-formylpterin [26]. Further, it was reported that an enzyme bound to the human erythrocyte membrane has folic acid oxidase activity and yields 6-formylpterin [27]. Although 6-formylpterin is thought not usually to occur *in vivo*, it may thus possibly function as an endogenous substance.

In conclusion, 6-formylpterin intracellularly generates ROS and suppresses NF-κB activation, cytokine production, and cell proliferation in human peripheral blood T cells without inducing cell death. The underlying mechanism of this suppression was speculated to be that the generated ROS rendered the redox state in the nucleus an oxidant state, which inhibited the DNA binding of NF-κB. These findings support the generalized notion in human immunology that oxidative stress suppresses the immune responses, and further suggest that the pro-oxidant has the potential for application as an immunosuppressant.

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