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SHORT COMMUNICATION

7,8-Dihydroneopterin-induced Apoptosis in Jurkat T Lymphocytes: A Comparison with Anti-Fas- and Hydrogen Peroxide-mediated Cell Death

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ABSTRACT. Activated cell-mediated immunity, associated for example with HIV infection, is accompanied by elevated concentrations of neopterin and 7,8-dihydroneopterin. Recent data have indicated a role of neopterin derivatives in virus activation and apoptotic cell death, processes likely to involve the action of oxygen free radicals. Because T cell death in AIDS is likely to involve the Fas/Fas ligand system and the action of oxygen free radicals and 7,8-dihydroneopterin, we compared the kinetics and sensitivity of apoptotic cell death of human leukemic Jurkat T cells to that of treatments with 7,8-dihydroneopterin, anti-Fas, and H_2O_2. Upon incubation with 5 mM 7,8-dihydroneopterin and 50 μM hydrogen peroxide over a period of 24 hr, bimodal kinetics were observed with peaks at 5.5 hr (7,8-dihydroneopterin, 13.1%; H_2O_2, 11.4%) and at 24 hr (7,8-dihydroneopterin, 11.2%; H_2O_2, 13.2%). In contrast, anti-Fas (20 ng/mL)-induced apoptosis increased steadily over time, peaking at 11 hr (43.2%). Interestingly, anti-Fas-induced apoptosis was suppressed upon co-incubation with 7,8-dihydroneopterin and H_2O_2 by 62% and 68%, respectively. We also compared the sensitivity to drug treatments of apoptosis induced by 7,8-dihydroneopterin, anti-Fas antibodies, and H_2O_2. 7,8-dihydroneopterin-mediated, and similarly anti-Fas- and H_2O_2-mediated, apoptosis was not inhibited by a broad range of pharmacological inhibitors, such as actinomycin D, cycloheximide, cyclosporin A, and various protein kinase inhibitors. On the contrary, inhibitors with antioxidant abilities, such as pyrrolidinedithiocarbamate, significantly blocked 7,8-dihydroneopterin-, H_2O_2- as well as anti-Fas-mediated apoptosis. These results imply that 7,8-dihydroneopterin-, H_2O_2-, and anti-Fas-mediated cell death might involve related redox sensitive signal transduction pathways. BIOCHEM PHARMACOL **56**;9:1181–1187, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. pteridines; 7; 8-dihydroneopterin; apoptosis; reactive oxygen intermediates; Fas; hydrogen peroxide

Activated cell-mediated immunity is associated with elevated concentrations of T cell-derived IFN-γ,§ leading to the expression of proinflammatory cytokines and enhancing macrophage capacity to secrete reactive oxygen intermediates [1, 2]. In addition, IFN-γ is the central stimulator for the biosynthesis of neopterin and 7,8-dihydroneopterin from guanosine triphosphate [3–5]. The production of neopterin hereby closely correlates with IFN-γ concentrations [6] and the activation of cell-mediated immunity, e.g. in viral infections including HIV infection, in autoimmune disorders, and in certain types of cancer [7–9]. Recent results have propounded a potential role for neopterin derivatives in oxygen radical-mediated processes [10–13], and 7,8-dihydroneopterin and neopterin were shown to be involved in redox signaling leading to apoptosis and the activation of transcription factors nuclear factor κB and activation protein 1 [13–16].

Various pathologic mechanisms have been attributed with the loss of T lymphocytes by apoptosis [17], e.g. crosslinking of the CD4 molecule by the HIV-1 envelope protein [18–22] or an increased expression of the apoptosis-related membrane antigen Fas [23–25]. Recently, it became more apparent that in HIV infection programmed cell death may also be linked to "oxidative stress" [26–30]. We, therefore, speculated that neopterin derivatives directly interfere with the cells' ability to maintain an appropriate oxidant-antioxidant balance and the interaction between HIV replication and activated cell-mediated immunity [31]. Here, we sought a deeper understanding of the biochemical mechanisms leading to the apoptosis of T lymphocytes exposed to 7,8-dihydroneopterin by comparing it with H_2O_2- and anti-Fas-induced apoptosis.

MATERIALS AND METHODS

Cell Culture

Jurkat TAg cells, a T lymphoblastic cell line, provided by Dr. G. Crabtree, were maintained in RPMI 1640

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§ *Abbreviations*: IFN-γ, interferon-γ; NAC, N-acetylcysteine; PDTC, pyrrolidinedithiocarbamate; and PI, propidium iodide.

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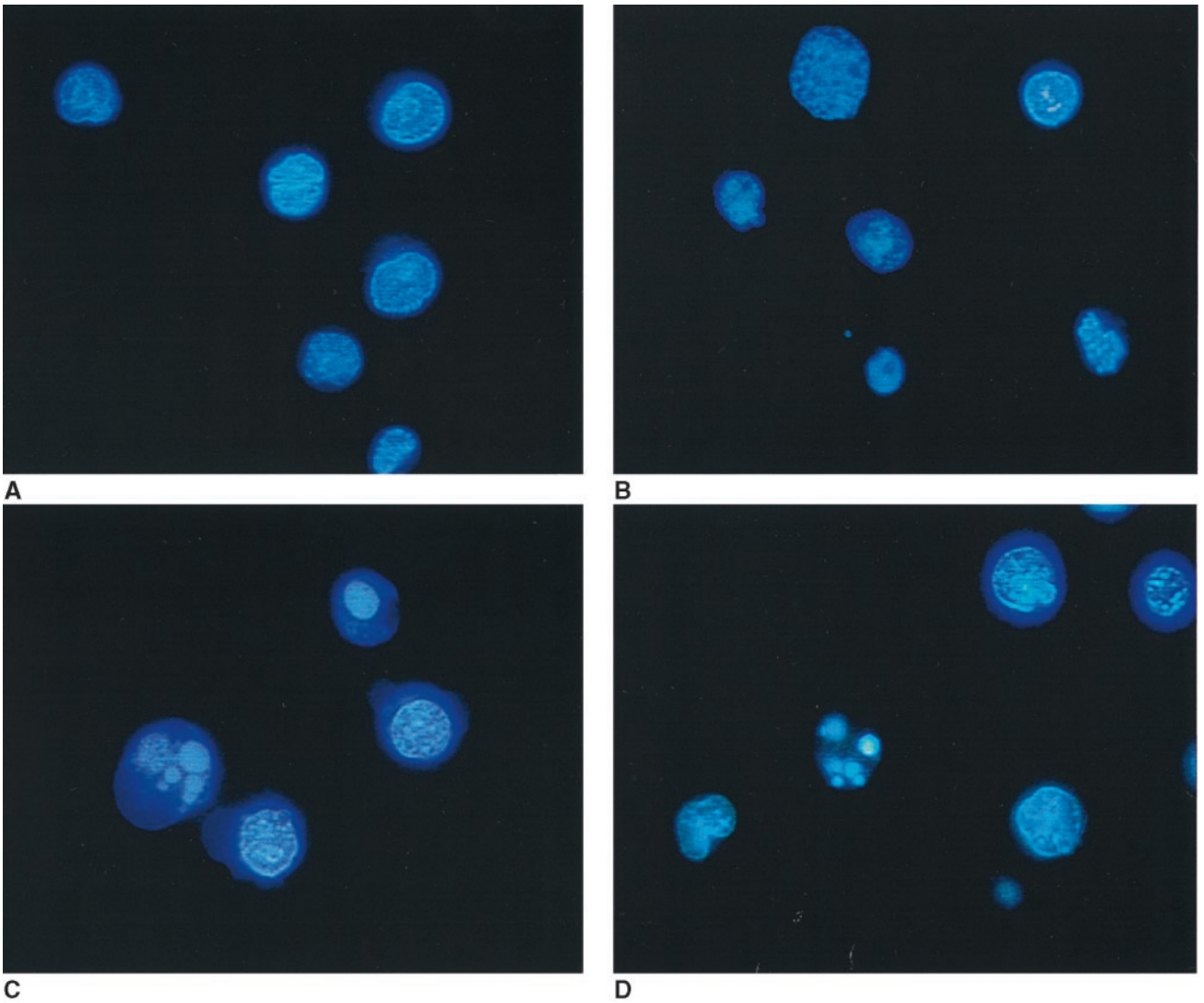

FIG. 1. Hoechst/PI staining of apoptotic Jurkat T lymphocytes mediated by (A) control, (B) 7,8-dihydroneopterin, (C) H_2O_2, and (D) anti-Fas. Cells were incubated with control, 7,8-dihydroneopterin (5 mM), H_2O_2 (50 μM), or anti-Fas antibodies (20 ng/mL) for 5.5 hr. Thereafter, cells were washed in PBS and incubated in Hoechst staining solution (10 μg/mL PBS) and PI (5 μg/mL) and analyzed on an Olympus BX 50 microscope. Pictures were taken with an Olympus U-CMAD-2 camera.

(Biochrom), supplemented with 10% heat-inactivated fetal calf serum (Biochrom) and 100 units/mL of penicillin; 0.1 mg/mL of streptomycin; and 2 mM L-glutamine (Serva) in a humidified atmosphere containing 5% CO_2.

Chemicals

7,8-dihydroneopterin was purchased from Schircks Lab, H_2O_2 from Merck, and anti-Fas antibodies (CH-11) from Oncor. Inhibitors used in this study were GF 109203X (Calbiochem) stock 1 mM in DMSO, Sigma; staurosporin (Calbiochem), stock 2.1 mM in DMSO; herbimycin A (Calbiochem), stock 174 μM in DMSO; tyrphostin A25 (Calbiochem), stock 10 μg/mL in DMSO; cyclosporin A (Calbiochem), stock 1 mM in ethanol; actinomycin D (Calbiochem), stock 1 mg/mL in methanol; cycloheximide (Sigma), stock 10 mg/mL in ethanol; EGTA, 5 mM (Sigma); EDTA, 2 mM (Merck); NAC (Sigma); and PDTC (Sigma). PI staining solution contained 50 μg PI/mL (Sigma), 0.1% Triton X-100 (Serva), and 0.1% tri-sodiumcitrate dihydrate (Merck) in distilled water.

Evaluation of Cellular Apoptosis

Apoptotic cells were characterized by their morphology and by the quantification of their intranuclear contents of fluorescing DNA by Hoechst and PI staining [32,33]. Prior to assays, cells were washed once in PBS (Serva) and then plated at a density of 4×10^4 cells per mL of complete medium. Cells were pre-incubated with inhibitors for 0–6 hr. Thereafter, cells were washed twice in PBS prior to incubation with 7,8-dihydroneopterin, anti-Fas antibodies, and H_2O_2. Thereafter, cells were centrifuged and resuspended in PI staining solution and assayed on a FACScan (Becton Dickinson).

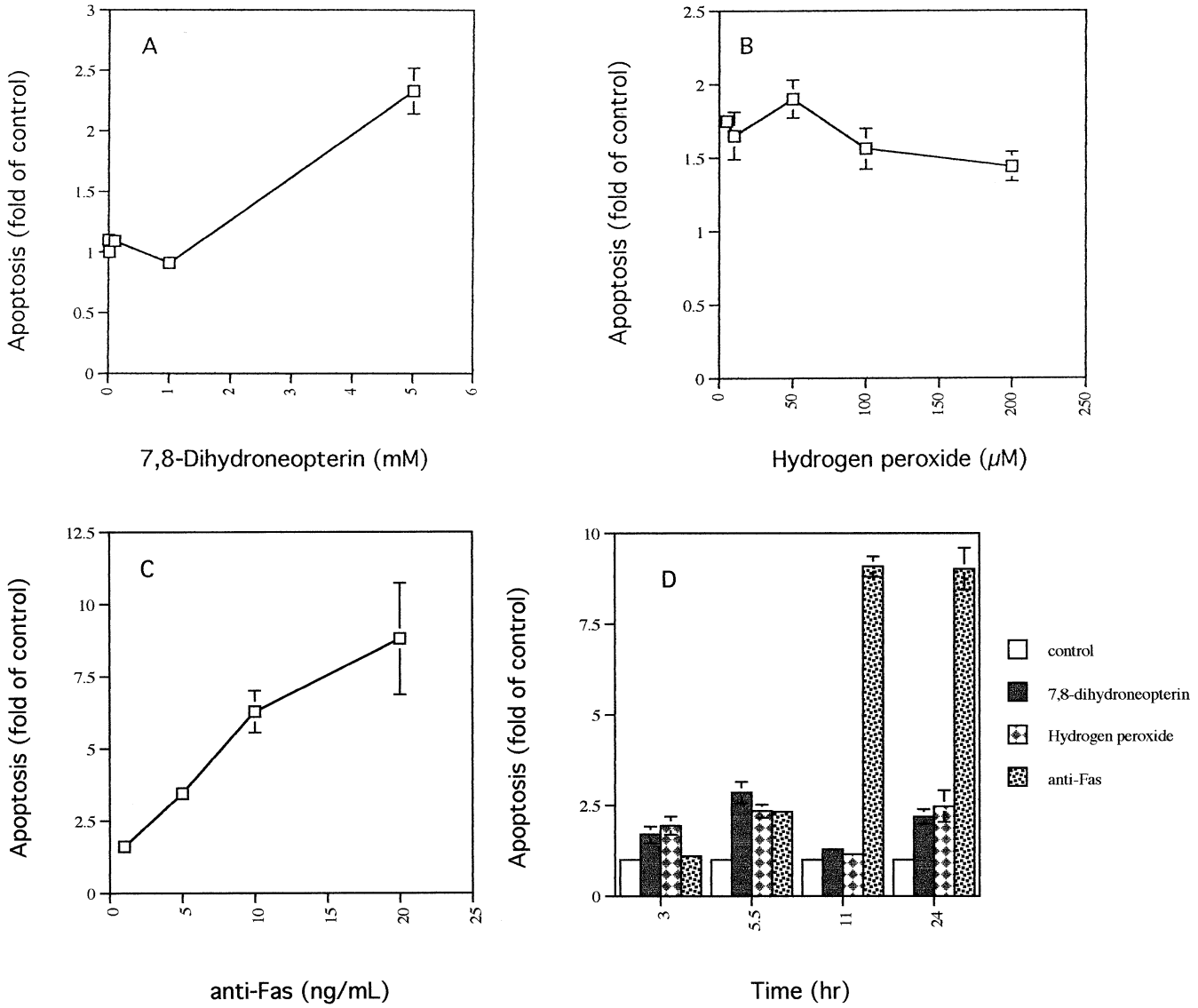

FIG. 2. Kinetics and sensitivity of 7,8-dihydroneopterin-, H_2O_2-, and anti-Fas-mediated apoptosis. Jurkat cells (4×10^4 cells/mL) were incubated with (A) 7,8-dihydroneopterin (*P*-values: 5 mM < 0.001, 1 mM not significant, 0.1 mM < 0.01), (B) H_2O_2 (*P*-values: 10 μM < 0.01, 50 μM < 0.001, 100 μM < 0.01, 200 μM < 0.01), and (C) anti-Fas antibodies (*P*-values: 5 ng/mL < 0.001, 10 ng/mL < 0.001, 20 ng/mL < 0.01) at various concentrations and times (D) for 3, 5.5, 11 and 24 hr (*P*-values: 3 hr: 7,8-dihydroneopterin < 0.05, H_2O_2 < 0.05, anti-Fas not significant; 5.5 hr: 7,8-dihydroneopterin < 0.01, H_2O_2 <0.01, anti-Fas < 0.001; 11 hr: 7,8-dihydroneopterin < 0.001, H_2O_2 not significant, anti-Fas < 0.001; 24 hr: 7,8-dihydroneopterin < 0.01, H_2O_2 < 0.05, anti-Fas < 0.001; not significant). Thereafter, cells were centrifuged and resuspended in PI staining solution and assayed on a FACScan. Apoptosis in typical experiments was: (A) 4.9% (control), 5.2% (100 μM of 7,8-dihydroneopterin), 4.5% (1 mM 7,8-dihydroneopterin), 16.7% (5 mM 7,8-dihydroneopterin); (B) 5.8% (control), 6.6% (10 μM H_2O_2), 9.1% (50 μM H_2O_2), 6.4% (100 μM H_2O_2), 6.4% (200 μM of H_2O_2); (C) 2.8% (control), 12.6% (5 ng/mL of anti-Fas), 21.9% (10 ng/mL of anti-Fas), 34.1% (20 ng/mL of anti-Fas); and (D) 3 hrs: 7.6% (control), 5.9% (20 ng/mL of anti-Fas), 7.8% (5 mM 7,8-dihydroneopterin), 9.6% (50 μM H_2O_2); 5.5 hr: 5.7% (control), 9.7% (20 ng/mL of anti-Fas), 10.2% (5 mM 7,8-dihydroneopterin), 18.9% (50 μM H_2O_2); 11 hr: 4.6% (control), 35.5% (20 ng/mL of anti-Fas), 6.4% (5 mM 7,8-dihydroneopterin), 6.6% (50 μM H_2O_2); and 24 hr: 4.1% (control), 42.2% (20 ng/mL of anti-Fas), 6.5% (5 mM 7,8-dihydroneopterin), 6.7% (50 μM H_2O_2).

In parallel, cells were incubated with 7,8-dihydroneopterin (5 mM), anti-Fas antibodies (20 ng/mL), or H_2O_2 (50 μM) for 5.5 hr as described above. Next, cells were washed in PBS and incubated in Hoechst staining solution (10 μg/mL PBS) and PI (5 μg/mL) and analyzed on an Olympus BX 50 fluorescence microscope. Pictures were taken with an Olympus U-CMAD-2 camera.

RESULTS AND DISCUSSION

Following incubation with 7,8-dihydroneopterin, H_2O_2, and anti-Fas antibodies, Jurkat T lymphocytes exhibited cell death by apoptosis, which is consistent with earlier findings in the literature [13, 15, 34, 35]. Striking morphological changes included the formation of apoptotic bodies

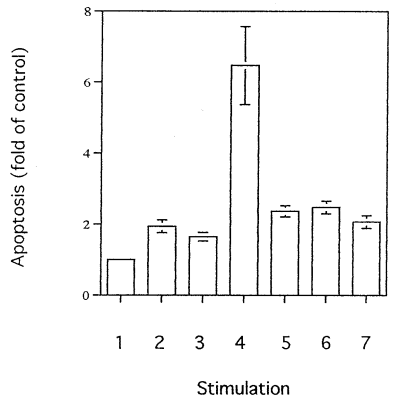

FIG. 3. Effect of the co-incubation of 7,8-dihydroneopterin with H_2O_2 or with anti-Fas on apoptosis of Jurkat cells. Jurkat cells (4×10^4 cells/mL) were incubated with 7,8-dihydroneopterin (5 mM), anti-Fas antibodies (20 ng/mL), and H_2O_2 (50 μM) for 5.5 hr. Thereafter, cells were centrifuged and resuspended in PI staining solution and assayed on a FACScan. [1] medium, [2] 7,8-dihydroneopterin ($P < 0.001$), [3] H_2O_2 ($P < 0.001$), [4] anti-Fas ($P < 0.001$), [5] 7,8-dihydroneopterin and H_2O_2 ($P < 0.001$), [6] 7,8-dihydroneopterin and anti-Fas ($P < 0.001$), and [7] H_2O_2 and anti-Fas ($P < 0.001$). Apoptosis in a typical experiment was: 1.4%, control; 4.0%, 7,8-dihydroneopterin; 4.8%, H_2O_2; 20.2%, anti-Fas; 3.8%, 7,8-dihydroneopterin and H_2O_2; 5.8%, 7,8-dihydroneopterin and anti-Fas; and 6.4%, H_2O_2 and anti-Fas.

and nuclei with condensed chromatin as exhibited by Hoechst-staining (Fig. 1a, b and c). In parallel experiments, cell death was assayed by flow cytometry that allowed simultaneous monitoring of PI uptake and cell size (Fig. 1d, e and f).

Next, we compared the kinetics and sensitivity of apoptotic cell death of human leukemic Jurkat T cells to treatments with 7,8-dihydroneopterin, anti-Fas, and H_2O_2. Bimodal kinetics were observed after the treatment of cells with 7,8-dihydroneopterin and H_2O_2 (Fig. 2D). 7,8-dihydroneopterin induced a maximum of apoptotic cells (13.1%) at a concentration of 5 mM and 5.5 hr (Figs. 2A and D). H_2O_2 (50 μM) induced maximum apoptosis at 24 hr (13.2%) (Fig. 2B and D). In contrast, anti-Fas (20 ng/mL) differed from 7,8-dihydroneopterin- and H_2O_2-induced apoptosis by kinetics. Anti-Fas apoptosis increased steadily and peaked at 11 hr (43.2%) (Fig. 2C and D).

We then incubated Jurkat cells with combinations of 7,8-dihydroneopterin, H_2O_2, and anti-Fas antibodies. Optimal concentrations of 7,8-dihydroneopterin, H_2O_2, and anti-Fas antibodies were chosen from the results shown in Figs. 2A, B, and C and data in the literature [27,36,15]. Interestingly, Fas-induced apoptosis was suppressed upon co-incubation with 7,8-dihydroneopterin and H_2O_2 by 62% and 68%, respectively (Fig. 3). In view of recent data [37] showing the beneficial effects of reactive oxygen intermediates, namely their induction of cell resistance to Fas-mediated apoptosis, one might speculate that H_2O_2 and 7,8-dihydroneopterin suppress anti-Fas-mediated apoptosis by a similar mechanism.

We also compared the sensitivity of Jurkat T lymphocytes to the drug treatments of apoptosis induced by 7,8-dihydroneopterin, anti-Fas, and H_2O_2 (Fig. 4). 7,8-dihydroneopterin-mediated, and similarly anti-Fas- and H_2O_2-mediated, apoptosis was not inhibited by actinomycin D or cycloheximide. In accordance with reports on anti-Fas-mediated apoptosis [38], this lack of inhibition might suggest the independence of H_2O_2- and 7,8-dihydroneopterin-mediated apoptosis of mRNA transcription and *de novo* protein synthesis. This result corresponds to earlier observations that increases in the expression of immediate-early genes and enhanced binding of transcriptional factors to DNA with oxidative stress did not require new protein synthesis. (The possibility of post-translational modifications mediated by phosphorylation/dephosphorylation reactions was also discussed by the authors.) [39]. Likewise, in the present study, 7,8-dihydroneopterin-, H_2O_2-, and anti-Fas-mediated apoptosis was not inhibited by the calcineurin inhibitor cyclosporin A or by the two protein kinase inhibitors staurosporine and GF 109203X. An insensitivity of anti-Fas-mediated apoptosis to cyclosporin A and GF 109203X was described earlier by Hunter and Karin [38]. These same authors, however, showed an effective inhibition of apoptosis by staurosporin. In the present study, H_2O_2-mediated apoptosis was inhibited by EGTA, a bivalent cation chelator (37%). This result complements an earlier report [40] that found an increase in Ca^{2+} influx upon incubation with H_2O_2. Interestingly, 7,8-dihydroneopterin-mediated apoptosis appears to be unaffected by the chelation of extracellular Ca^{2+}, although significant increases in intracellular Ca^{2+} were observed after incubation of the myelomonocytic cell line THP-1 with 7,8-dihydroneopterin [41].

To study the impact of tyrosine phosphorylation, we used the inhibitors herbimycin A and tyrphostin A25. While we did not detect any inhibiting effect of tyrphostin A25, anti-Fas-mediated apoptosis was significantly inhibited by herbimycin A (29%). This result corresponds to earlier data [38], which showed an effective inhibition of Fas-induced DNA fragmentation by herbimycin A and genistein. In contrast, another group of researchers [42] claimed that Fas-mediated apoptosis does not involve src kinases or CD45. As opposed to results in the literature that show a stimulation of tyrosine kinase activity and an increase in tyrosine phosphorylation by oxidants (reviewed in [43]), we were not able to detect an inhibiting effect of the tyrosine kinase inhibitors herbimycin and tyrphostin on apoptosis mediated by H_2O_2 and 7,8-dihydroneopterin. Our results, however, are in accordance with earlier findings [44] that millimolar concentrations of H_2O_2 are necessary to induce substantial increases in protein tyrosine kinase activity and protein tyrosine phosphorylations.

Earlier studies indicated a potential role of neopterin derivatives in oxygen free radical-mediated processes (for a review see [31]). Above normal levels of reactive oxygen intermediates are referred to as oxidative stress and may result in apoptosis [28, 30, 45]. Oxidative stress-associated apoptosis can be blocked by compounds with antioxidant abilities. In this study, we tested the effects of NAC, a thiol

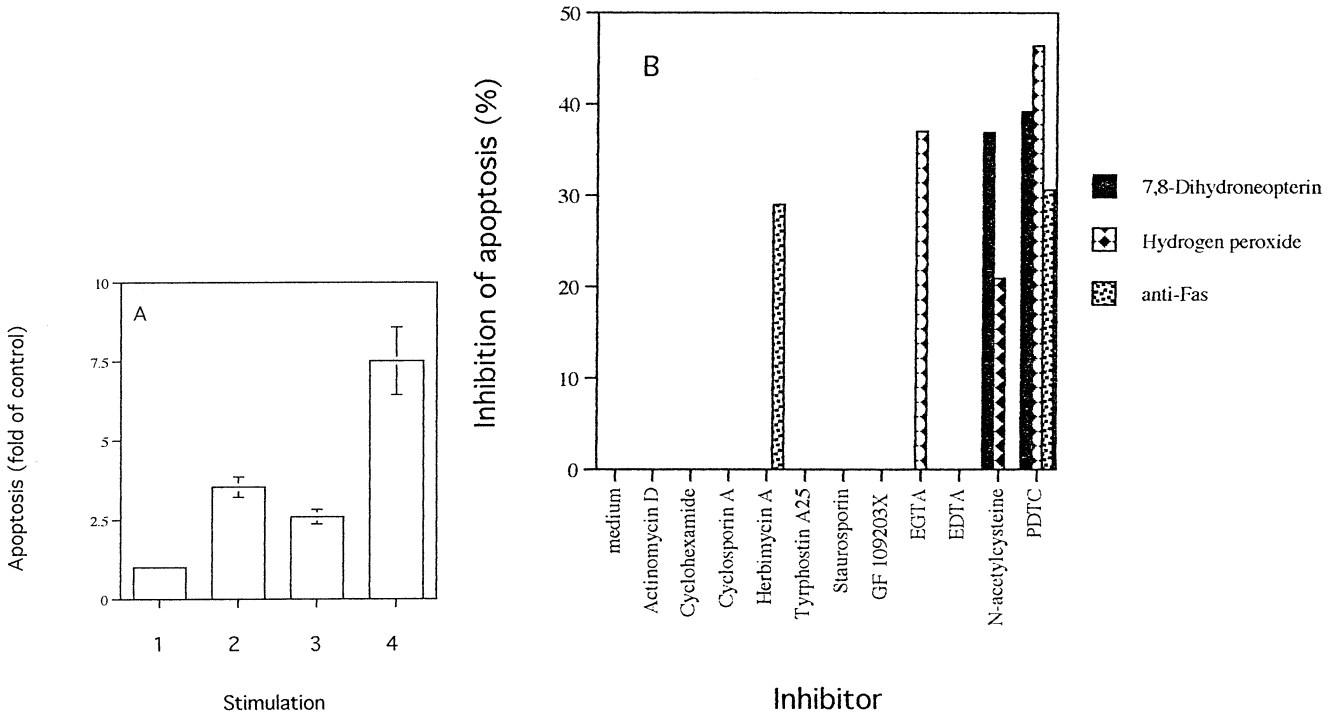

FIG. 4. Effect of pharmacological inhibitors on 7,8-dihydroneopterin-, H_2O_2-, and anti-Fas-mediated apoptosis of Jurkat cells. (A) Apoptosis induced by 7,8-dihydroneopterin, anti-Fas and H_2O_2. Cells were washed once in PBS and resuspended in complete medium at a final concentration of 4×10^4 cells/mL and incubated for 5.5 hr with [1] medium, [2] 7,8-dihydroneopterin (5 mM), [3] H_2O_2 (50 μM), or [4] anti-Fas antibodies (CH-11, 20 ng/mL). Thereafter, cells were centrifuged and resuspended in PI staining solution and assayed on a FACScan. (*P*-values: 7,8-dihydroneopterin < 0.01, H_2O_2 < 0.001, and anti-Fas < 0.001). (B) Inhibition of apoptosis. Cells were plated at a density of 4×10^4 cells/mL and pre-incubated with various inhibitors: actinomycin D (1 μg/mL), cycloheximide (10 μg/mL), cyclosporin A (1 μM), herbimycin (1 μM), tyrphostin A25 (100 μM), staurosporin (1 μM), GF 109203X (5.8 μM), EGTA (5 mM), EDTA (2 mM), NAC (5 mM), and PDTC (100 μM) prior to stimulation with 7,8-dihydroneopterin (5 mM), H_2O_2 (50 μM), or anti-Fas antibodies (20 ng/mL). Thereafter, cells were centrifuged and resuspended in PI staining solution and assayed on a FACS Scan. [*P*-values: 7,8-dihydroneopterin (NAC < 0.01, PDTC < 0.001), H_2O_2 (EGTA < 0.001, NAC < 0.05, PDTC < 0.001), and anti-Fas (herbimycin A < 0.01, NAC not significant, PDTC < 0.01)].

antioxidant and glutathione precursor [29, 46], and PDTC, a metal chelator found to inhibit nuclear factor κB-related gene expression [26, 47]. Whereas PDTC was able to block apoptosis induced by anti-Fas, H_2O_2, and 7,8-dihydroneopterin, coincubation with NAC only led to a significant reduction of H_2O_2-and 7,8-dihydroneopterin-mediated apoptosis (Fig. 4). It is likely that PDTC, because of its numerous possible abilities to interfere with cellular pathways [27, 47], is a more powerful inhibitor of apoptosis. Contradictory to our data on Fas-mediated apoptosis, in other studies [48, 49] it was observed that the apoptotic action of Fas was abolished by the antioxidant NAC. Yet, another group of researchers [37] reached the opposite conclusion, claiming that reactive oxygen intermediates induce cellular resistance to anti-Fas-mediated apoptosis. Their results fit well with earlier data [50] that show that the antioxidants deferoxamine, α-tocopherol, glutathione, and catalase did not protect cells from anti-Fas-mediated apoptosis and our own findings that anti-Fas-mediated cell death was not inhibited by NAC (Fig. 4). Taken together, our finding that H_2O_2 and 7,8-dihydroneopterin inhibit anti-Fas-mediated apoptosis in Jurkat T lymphocytes may be explained by this mechanism (Fig. 3).

Our data fit well with previous observations that neopterin and 7,8-dihydroneopterin may interfere with the ability of a cell to maintain an appropriate oxidant-antioxidant balance. Increasing concentrations of 7,8-dihydroneopterin scavenged reactive oxygen intermediates and quenched H_2O_2-induced chemiluminescence. Interestingly, high doses of 7,8-dihydroneopterin (5 mM) enhanced the formation of reactive oxygen species [13]. From our results, we hypothesize that 7,8-dihydroneopterin-, H_2O_2,- and anti-Fas-mediated cell death might involve related, but not identical, redox sensitive signal transduction pathways.

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