0026-895X/04/6601-122–128$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 66:122–128, 2004

Vol. 66, No. 1
3234/1159349
Printed in U.S.A.

Roles of Heme Oxygenase-1 in the Antiproliferative and Antiapoptotic Effects of Nitric Oxide on Jurkat T Cells

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Received January 22, 2004; accepted April 7, 2004

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Nitric oxide (NO) has been shown to exert antiproliferative and antiapoptotic effects on human T cells. Heme oxygenase-1 (HO-1), which degrades heme into biliverdin, free iron (Fe^{2+}), and carbon monoxide (CO), has also been known to have antiproliferative and antiapoptotic effects. Recent evidence suggests that HO-1 is an important cellular target of NO; whether HO-1 expression contributes to the antiproliferative and/or antiapoptotic effects mediated by NO remains to be investigated. In the present study, we examined the effects of NO on HO-1 expression and possible roles of HO-1 in T cell proliferation and apoptosis. Using human Jurkat T cells, we found that the NO donor sodium nitroprusside (SNP) induced HO-1 expression and that preincubation with SNP suppressed T cell proliferation induced by concanavalin A and apoptosis triggered by anti-Fas antibody. Suppressions of T cell proliferation and apoptosis comparable with SNP were also observed when the T cells were preincubated with the HO-1 inducer cobalt protoporphyrin. A phosphorothioate-linked HO-1 antisense oligonucleotide blocked HO-1 expression, and subsequently abrogated the antiproliferative and antiapoptotic effects of SNP. Overexpression of the HO-1 gene after transfection into Jurkat T cells resulted in significant decreases in T cell proliferation and apoptosis. The CO donor tricarbonyldichlororuthenium (II) dimer mimicked the antiproliferative effect of SNP, and the Fe^{2+} donor $FeSO_4$ blocked anti-Fas-induced apoptosis. Taken together, our results suggest that NO induces HO-1 expression in T cells and that suppressions of T cell proliferation and apoptosis afforded by NO are associated with an increased expression of HO-1 by NO.

Activation of T cells through antigen receptor results in the expansion of a limited number of these cells that can be activated in response to specific antigen and enhance an immune response. After activation of T cells, activation-induced cell death, or apoptosis, plays an important role in the termination of an immune response. Thus, a balance of proliferation and apoptosis in T cells controls immunological activity.

Endogenous nitric oxide (NO) is synthesized from L-arginine by a family of NO synthase (NOS) isoenzymes [endothelial NOS, neuronal NOS, and inducible NOS (iNOS)] (Bredt, 1999). In general, constitutive NOS forms, such as endothelial and neuronal NOS, are activated by a transient increase in cytosolic calcium, which promotes the release of NO over several minutes. iNOS is expressed in many cells, including macrophages and hepatocytes after stimulation of immunological or inflammatory reactions, producing large amounts of NO for up to several days (Kroncke et al., 2001). NOS inhibitors, NO-scavenging compounds such as phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), or NO-releasing compounds such as sodium nitroprusside (SNP) are widely used to investigate the contribution of the effects of NO on the overall response to be assessed (Pae et al., 2000). The originally reported functions of NO include inflammation and antimicrobial defense (Hickman-Davis et al., 2001). However, growing evidence reveals that NO can play an important role in T cell-mediated immunity (van der Veen, 2001). NO can regulate T cell proliferation, cytokine

This work was supported by the Ministry of Commerce, Industry and Energy of Korea via the Korea Institute of Industrial Technology Evaluation and Planning.

ABBREVIATIONS: NO, nitric oxide; NOS, nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; PTIO, phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; SNP, sodium nitroprusside; HO, heme oxygenase; CO, carbon monoxide; *z*-VAD, benzyloxycarbonyl-Val-Ala-Asp; CoPP, cobalt protoporphyrin; RuCO, tricarbonyldichlororuthenium (II) dimer; PI, propidium iodide; Con A, concanavalin A; Def, desferrioxamine; Hb, hemoglobin; FITC, fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; ODN, oligonucleotide; MAPK, mitogen-activated protein kinase.

production, apoptosis and signaling activity in vivo and in vitro (Bronte et al., 2003).

Heme oxygenase (HO) is the enzyme catalyzing the degradation of the heme group that produces carbon monoxide (CO), biliverdin, and free iron. Biliverdin is converted to bilirubin by biliverdin reductase, and iron is used in metabolism or sequestered by ferritin (Alcaraz et al., 2003). So far, three isoforms of HO have been fully characterized. HO-2 and HO-3 are constitutive isozymes, whereas HO-1 is induced by a variety of stimuli, many of them related to oxidative stress, in various types of cells, including human T cells (Pae et al., 2003). The exact functional role of HO-1 expression is not fully understood. However, growing evidence shows that HO-1 can exert antiproliferative and antiapoptotic effects (Morse and Choi, 2002; Durante, 2003).

Although HO-1 expression by NO has been reported in various types of cells, including endothelial cells (Sawle et al., 2001), vascular smooth muscle cells (Hartsfield et al., 1997), fibroblasts (Alcaraz et al., 2000), macrophages (Alcaraz et al., 2001), and hepatocytes (Choi et al., 2003), whether HO-1 expression could also be induced by NO in human T cells remains to be established. Moreover, whether HO-1 expression could make a significant contribution to suppressions of T cell proliferation and apoptosis afforded by NO has not been addressed. The aim of the present study, therefore, was to examine the effect of NO on HO-1 expression and to assess the antiproliferative and antiapoptotic functions of HO-1 in human Jurkat T cells.

Materials and Methods

Reagents and Antibodies. RPMI 1640 supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA), and 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT) was used as complete medium in all cultures. Anti-CD3 (clone UCHT1) and anti-CD28 (clone CD28.2) antibodies were purchased from Immunotech (Westbrook, ME) and BD Biosciences PharMingen (San Diego, CA), respectively. Recombinant interleukin-2 was obtained from R&D Systems (Minneapolis, MN). Benzyloxycarbonyl-Val-Ala-Asp (*z*-VAD) was purchased from BioVision (San Diego, CA). Anti-HO-1, anti-procaspase-8, anti-procaspase-3 and anti-β-actin primary antibodies and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cobalt protoporphyrin (CoPP) was obtained from Porphyrin Products (Logan, UT). Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), tricarbonyldichlororuthenium (II) dimer (RuCO), propidium iodide (PI), concanavalin A (Con A), desferrioxamine (Def), $FeSO_4$, and hemoglobin (Hb) were purchased from Sigma-Aldrich (St. Louis, MO). Annexin V-FITC and anti-CD95 antibody were obtained from Molecular Probes (Eugene, OR) and R&D Systems, respectively. Sense and antisense oligonucleotides were supplied by Invitrogen and transfected with the Superfect transfection reagent (QIAGEN, Hilden, Germany). The other reagents were from Sigma-Aldrich.

Isolation of $CD4^+$ T Cells from Peripheral Blood and Generation of T Lymphoblast Cells. Peripheral blood mononuclear leukocytes were isolated from healthy blood by Ficoll-Paque density gradient centrifugation. After three washes in HBSS (Invitrogen), $CD4^+$ T cells were isolated from peripheral blood mononuclear leukocytes using the MACS negative depletion system (Miltenyi Biotec, Auburn, CA). No contamination with $CD8^+$ T cells, B cells, monocytes, or NK cells was detected. T lymphoblast cells were generated as follows: $CD4^+$ T cells were treated with 50 mg/ml of phytohemagglutinin (Sigma-Aldrich) for 15 min in complete medium, the mitogen was then washed out, and cells were resuspended in complete medium supplemented with 30 U/ml of recombinant human interleukin-2. Fresh medium was added each 48 h, and cultures were carried out for a maximum of 6 days.

HO-1 Sense and Antisense Oligonucleotide (ODN) Treatment. The sense/antisense ODNs for HO-1 were directed against the flanking translation initiation cordon in the human HO-1 cDNA (Wagener et al., 1999). The antisense sequence was 5′-CGC CTT CAT GGT GCC-3′, whereas the sense sequence was 5′-GGC ACC ATG AAG GCG-3′. ODNs were phosphorothioated on the first three bases on the 3′ end. The T cells were transfected using the Superfect transfection reagent following the manufacturer's instructions. In brief, the cells were seeded in culture plates before transfection. The proportions used were 1 μg of DNA/5 μl of transfection reagent/well. The T cells were incubated for 4 h with the ODNs; the medium was then replaced with fresh medium containing 10% serum.

Jurkat T Cells and HO-1 Transfection. The human acute T cell leukemia Jurkat clone E6-1 was obtained from the American Type Culture Collection (Manassas, VA). HO-1 cDNA was a kind gift from Dr. Augustine M. K. Choi (University of Pittsburgh, Pittsburgh, PA). HO-1 was cloned into pcDNA3 (Invitrogen). Jurkat T cells (5×10^6) were transfected with 10 μg of constructs by electroporation at 270V, 950 μF in serum-free RPMI 1640 using a Gene pulser (Bio-Rad) followed by culture in RPMI 1640 supplemented with 10% FBS for 48 h and plating on 96-well plates at 5×10^5 cells/well in the presence of 1.25 mg/ml G418. Single stable clone of transfectant was isolated and expanded.

T Cell Activation and Proliferation. Jurkat T cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS at 37°C in 5% CO_2. The NO donor SNP and the HO-1 inducer CoPP were preincubated for 12 h before stimulation with Con A. The CO donor RuCO, Hb, bilirubin, and Fe^{2+} were preincubated for 1 h before being added to plates and stimulated with Con A. In some experiments, HO-1 antisense ODN and HO-1 sense ODN were preincubated at least for 4 h before additions of SNP or CoPP to culture plates. After 3 days of culture, Con A-induced proliferation was assessed by [^{3}H] thymidine (0.5 μCi/well; Amersham Biosciences, Piscataway, NJ) uptake for the next 16 h.

Western Blot Analysis. Cells were solubilized in ice-cold 1% Triton X-100 lysis buffer supplemented with protease and phosphatase inhibitors as described previously (21). After 30 min on ice, the lysates were clarified by centrifugation, and the protein concentration was determined with the Pierce bicinchoninic acid protein assay reagent. Proteins (20 μg) were resolved by SDS-PAGE (10% acrylamide), transferred to nitrocellulose membranes, and probed with specific antibody (diluted 1:1000), followed by incubation with secondary horseradish peroxidase-conjugated antibody (1:100,000). Bands were detected using the Luminol chemiluminescent detection reagents (New England Biolabs, Beverly, MA). Blots were exposed to autoradiographic film (DuPont Merck Chemistry Department, Wilmington, DE) for 1 to 2 min for detection.

Flow Cytometric Analysis. Cells were suspended in HBSS containing 5% FBS, fixed by the drop-wise addition of ice-cold 70% ethanol to a final 50% concentration, and held on ice for 1 h. After extensive washing, the cells were suspended in HBSS containing 50 μg/ml of PI and 50 μg/ml of Annexin V-FITC, and incubated for 1 h at room temperature. Stained cells were analyzed by flow cytometry on a FACSVantage with CellQuest software (BD Biosciences, Franklin Lakes, NJ).

Statistics. Data were expressed as mean ± S.E.M. of the individual titer. Levels of significant differences between groups were determined by the Student's *t* test. P values < 0.01 were considered statistically significant.

Results

Effects of NO on HO-1 Expression and T Cell Proliferation. The NO donor SNP was used at doses (0.05–0.3 mM) that did not produce direct cytotoxicity on human Jur-

kat T cells (not shown). At first, HO-1 expression by SNP was detected in nonstimulated T cells (Fig. 1A). Incubation of T cells with SNP for 12 h resulted in a dose-dependent increase in HO-1 expression (Fig. 1A). HO-1 expression by SNP was almost completely inhibited by the NO scavenger PTIO (Fig. 1A), indicating that HO-1 expression was caused by NO released spontaneously from SNP and not by breakdown products of SNP. We addressed whether SNP preincubation could suppress T cell proliferation in association with HO-1 expression. For this purpose, Jurkat T cells were preincubated with SNP for 12 h and then stimulated 72 h with the mitogen Con A. Preincubation of the T cells with SNP resulted in a dose-dependent suppression of Con A-stimulated proliferation (Fig. 1B); this effect was paralleled by increased HO-1 expression (Fig. 1A). Suppression of T cell proliferation by SNP preincubation was abrogated by PTIO (Fig. 1B), suggesting that NO released from SNP mediated antiproliferative effect. A phosphorothioate-linked HO-1 antisense ODN showed specificity in blocking HO-1 expression, whereas HO-1 sense ODN was without effect under these conditions (Fig. 2A). Suppression of T cell proliferation by SNP preincubation was also abrogated by HO-1 antisense ODN (Fig. 2B), demonstrating that HO-1 expression by NO preincubation could be involved in NO-mediated antiproliferative effect. A suppression of T cell proliferation comparable with SNP preincubation was observed when the T cells were preincubated with the HO-1 inducer CoPP (Fig. 3). In control experiments, HO-1 antisense ODN showed a significant specificity in blocking HO-1 expression induced by CoPP (Fig. 3A). HO-1 antisense ODN, PTIO, or CoPP alone had no significant effect on HO-1 expression and cell viability under these conditions (data not shown). The expression of HO-1 by either SNP or CoPP was also demonstrated in primary human $CD4^+$ T cells (Fig. 4A). Similar to those observed in Jurkat T cells, HO-1 antisense ODN markedly blocked HO-1 expression and again significantly reversed the antiproliferative effect of SNP preincubation in $CD4^+$ T cells stimulated with anti-CD3 and anti-CD28 antibodies (Fig. 4B).

Based on our present findings that the suppression of T cell

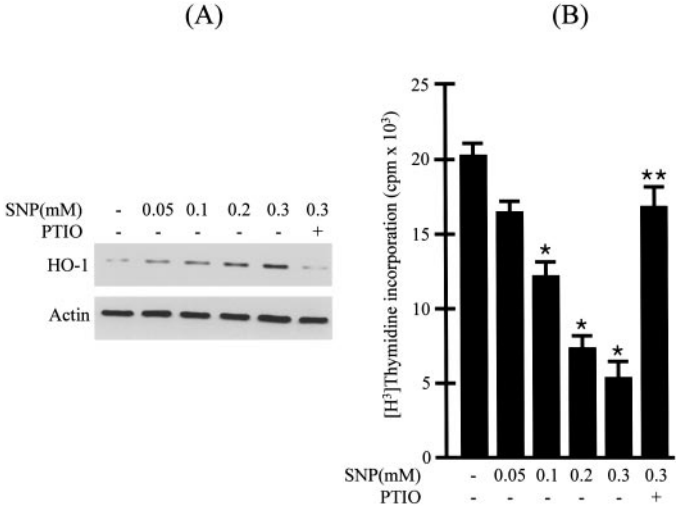

Fig. 1. Effects of NO on HO-1 expression and T cell proliferation. Jurkat T cells were preincubated 12 h with medium or indicated concentrations of SNP in the presence or absence of 1 mM of PTIO. The T cells, then, were either harvested for Western blot analysis (A) or stimulated 72 h with 1 μM of Con A for proliferation assay (B). Western blot analysis (A) and [^{3}H]thymidine incorporation assay (B) were performed as described under *Materials and Methods*. *, $P < 0.01$ with respect to Con A; **, $P < 0.01$ with respect to Con A + SNP (0.3 mM).

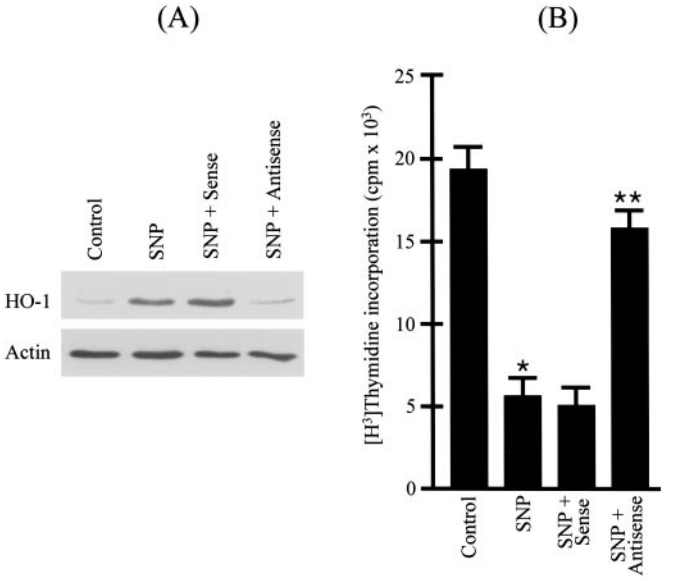

Fig. 2. Effects of HO-1 antisense ODN on NO-induced HO-1 expression and T cell proliferation. Jurkat T cells were preincubated 12 h with medium (control) or 0.3 mM of SNP after 4-h treatment with 10 μg/ml of HO-1 sense ODN or 10 μg/ml of HO-1 antisense ODN. The T cells, then, were either harvested for Western blot analysis (A) or stimulated 72 h with 1 μM of Con A for proliferation assay (B). Western blot analysis (A) and [^{3}H]thymidine incorporation assay (B) were performed as described under *Materials and Methods*. *, $P < 0.01$ with respect to Con A; **, $P < 0.01$ with respect to Con A + SNP.

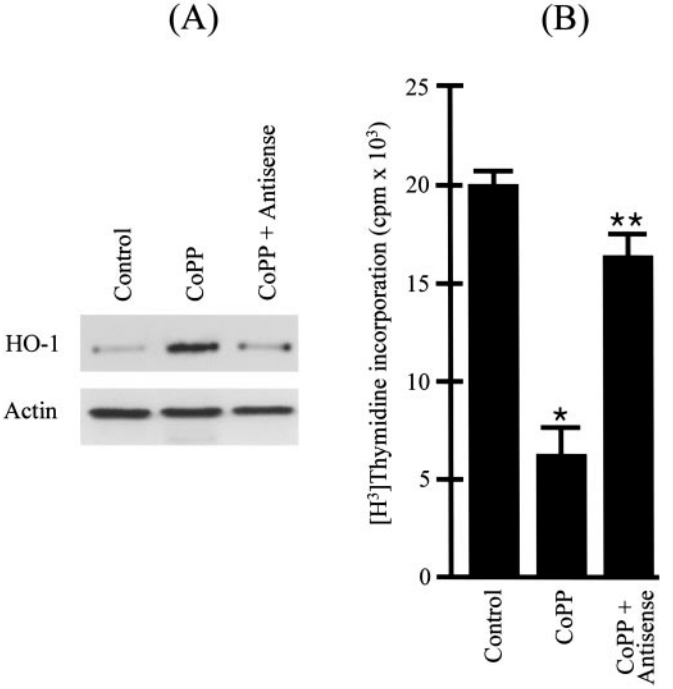

Fig. 3. Effects of CoPP on HO-1 expression and T cell proliferation. Jurkat T cells were preincubated 12 h with medium (control) or 20 μM of CoPP in the presence or absence of 10 μg/ml of HO-1 antisense ODN. The T cells were then either harvested for Western blot analysis (A) or stimulated 72 h with 1 μM of Con A for proliferation assay (B). Western blot analysis (A) and [^{3}H]thymidine incorporation assay (B) were performed as described under *Materials and Methods*. *, $P < 0.01$ with respect to Con A; **, $P < 0.01$ with respect to Con A + CoPP.

proliferation by SNP preincubation was associated with HO-1 expression, we accordingly examined whether overexpression of HO-1 could also suppress T cell proliferation. Overexpression of the HO-1 after transfection of HO-1 gene into Jurkat T cells resulted in the enhanced expression of HO-1 protein (Fig. 5A). In accordance with our data obtained from Figs. 1 to 3, HO-1 overexpression resulted in decreases in T cell proliferation (Fig. 5B).

Effects of Exogenous CO on T Cell Proliferation. We examined the effect of CO, one of the HO-1 reaction products, on T cell proliferation. Jurkat T cells were stimulated with Con A after 1-h preincubation with the CO donor RuCO. T cell proliferation was dose dependently reduced by RuCO (Fig. 6A). Hb, a CO scavenger, abrogated the antiproliferative effects of RuCO (Fig. 6A), suggesting that CO released from RuCO mediated antiproliferative effect. Unlike the antiproliferative effect of CO, neither bilirubin (20 μM) nor free iron (20 μM) had a significant effect on T cell proliferation (not shown). RuCO was used at noncytotoxic doses and Hb alone had no significant effect on cell viability under these conditions (data not shown).

Because of the important role of caspases in T cell proliferation (Alam et al., 1999; Kennedy et al., 1999), we studied the effects of RuCO on caspase-8 and caspase-3, which have been known to be activated during T cell proliferation. We demonstrated that RuCO significantly inhibited Con A-induced caspase-8 and caspase-3 activations at 24 h (Fig. 6B). In addition, the pan-caspase inhibitor, *z*-VAD, exerted antiproliferative activity in Con A-activated Jurkat T cells (Fig. 6C), supporting the involvement of caspase activation in T cell proliferation.

Effects of NO, HO-1 Overexpression, and Fe^{2+} on Fas-Triggered Apoptosis. Because HO-1 can show beneficial effects by inhibiting proliferation and apoptosis (Otterbein et al., 2003), we next addressed whether SNP preincubation could also inhibit Fas-mediated apoptosis in Jurkat T cells in association with HO-1 expression. For this end, the T cells were preincubated with SNP for 12 h, and apoptosis was triggered by anti-Fas agonistic antibody. Apoptosis was then assessed by flow cytometry analysis using Annexin V/PI double staining. As shown in Fig. 7A, Fas ligation significantly increased the percentage of Annexin V-positive events within 12 h of treatment; nevertheless, preincubation of the T cells with SNP resulted in a dose-dependent inhibition of Fas-mediated apoptosis. Both PTIO and HO-1 antisense ODN reversed the antiapoptotic effect exerted by SNP preincubation (Fig. 7B). Antiapoptotic effect comparable with SNP was also observed when the T cells were preincubated with CoPP (Fig. 7B), further suggesting that HO-1 expression could be responsible for the antiapoptotic effect of NO. The antiapoptotic effect of SNP preincubation was also demonstrated in human $CD4^+$ T lymphoblast cells (Fig. 7C). As observed in Jurkat T cells, HO-1 antisense ODN significantly reversed the antiapoptotic effect of SNP preincubation in $CD4^+$ T lymphoblast cells (Fig. 7C).

In another experimental set, we found that Jurkat T cells transfected with HO-1 gene were resistant to Fas-mediated apoptosis and that this resistance was abolished by chelating free iron with the iron chelator Def (Fig. 8A). Furthermore, exogenous addition of free iron rendered Jurkat T cells resistant to Fas-mediated apoptosis (Fig. 8B). Neither RuCO (20 μM) nor bilirubin (20 μM) had a significant effect on Fas-mediated apoptosis in T cells (not shown).

Discussion

The present study shows, for the first time, that NO can induce HO-1 expression in human T cells and that suppres-

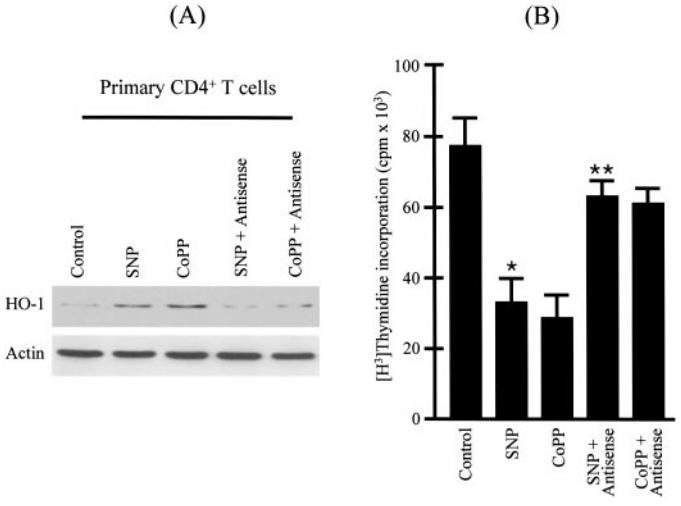

Fig. 4. Effects of NO on HO-1 expression and $CD4^+$ T cell proliferation. Primary $CD4^+$ T cells were preincubated 12 h with medium or 0.3 mM of SNP, or 20 mM of CoPP in the presence or absence of 10 μg/ml of HO-1 antisense ODN. The T cells, then, were either harvested for Western blot analysis (A) or stimulated for 4 days with 1 μg/ml of immobilized anti-CD3 antibody and 1 μg/ml of soluble anti-CD28 antibody for proliferation assay (B). Western blot analysis (A) and [^{3}H]thymidine incorporation assay (B) were performed as described under *Materials and Methods*. *, $P < 0.01$ with respect to anti-CD3/anti-CD28; **, $P < 0.01$ with respect to anti-CD3/anti-CD28 + SNP.

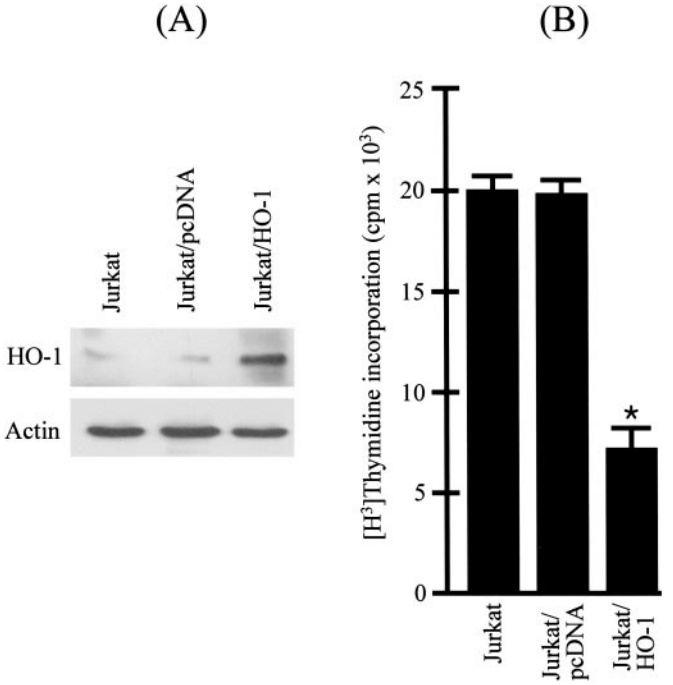

Fig. 5. Effects of HO-1 overexpression on T cell proliferation. Jurkat T cells were stably transfected with either empty vector (Jurkat/pcDNA) or HO-1 gene (Jurkat/HO-1), and either harvested for Western blot analysis (A) or stimulated 72 h with 1 μM of Con A for proliferation assay (B). Western blot analysis (A) and [^{3}H]thymidine incorporation assay (B) were performed as described under *Materials and Methods*. *, $P < 0.01$ with respect to empty vector.

sions of T cell proliferation and apoptosis afforded by NO pretreatment are causally associated with HO-1 expression by NO.

It is generally accepted that freshly isolated human T cells do not produce NO. Despite this fact, NO might function as a paracrine factor in maintaining T cell immunity. In a murine knockout model for iNOS, the reduced NO-producing capacity was shown to be correlated to the enhanced responses of T cells (Wei et al., 1995), alternatively suggesting that T cells are easily exposed to NO in vivo. The paracrine factor NO is probably produced by antigen-presenting cells, which are reported to express iNOS (Hoffman et al., 2002), and inherently interacts with naive T cells in vivo. In our study, we used the NO donor SNP as a source of NO, the capacity of which to spontaneously release NO in the in vitro cultures of human Jurkat T cells was confirmed by using the NO scavenger PTIO. NO released from SNP at noncytotoxic doses induced HO-1 expression in nonstimulated T cells (Figs. 1A and 4A), and preincubation of Jurkat T cells or primary $CD4^+$ T cells with SNP suppressed cellular proliferation and apoptosis (Figs. 1, 4, and 7). Moreover, the HO-1 inducer CoPP mimicked SNP-induced effects (Figs. 3 and 7). To find out whether suppressions of T cell proliferation and apoptosis afforded by NO pretreatment could be associated with HO-1 expression, the present study used an antisense ODN to HO-1. HO-1 antisense ODN blocked HO-1 expression (Fig. 2A) and subsequently abrogated the antiproliferative and ant-apoptotic effects of a NO donor (Figs. 2 and 7) and a HO-1 inducer (Figs. 3 and 7). Furthermore, overexpression of HO-1 after transfection of HO-1 gene into Jurkat T cells resulted in significant decreases in T cell proliferation and apoptosis (Figs. 5 and 8). These results demonstrate that suppressions of T cell proliferation and apoptosis afforded by NO pretreatment are associated with increased expression of HO-1 by NO.

It has been reported that the coincubation of T cells with NO potently suppressed cellular proliferation (Kosonen et al., 1997; Kosonen et al., 1998; Mahidhara et al., 2003) and apoptosis (Bernassola et al., 2001; Umansky et al., 2001; Beltran et al., 2002; Zech et al., 2003). Reports in the literature concerning the effects of NO treatment on T cells describe antiproliferative and antiapoptotic effects, probably through cGMP-dependent or -independent mechanisms (Kosonen et al., 1998), reversible disruption of Janus kinase-3/signal transducer and activator of transcription-5 (Bingisser et al., 1998; Simoncic et al., 2002), and, more recently, caspase inactivation by *S*-nitrosylation (Mahidhara et al., 2003; Zech et al., 2003). In this study, we demonstrated as well that the antiproliferative and antiapoptotic effects of NO on T cells are dependent of the schedule of NO treatment; HO-1 expression is involved in antiproliferative and antiapoptotic effects of NO pretreatment.

The important physiological function of HO-1 has been confirmed by observations in HO-1-knockout mice (Poss and Tonegawa, 1997) and a HO-1-deficient human case (Kawashima et al., 2002). HO-1-knockout mice develop a progressive long-term inflammatory state, which is of interest in that iNOS-knockout mice show exaggerated T cell responses, and a human lacking HO-1 enzymatic activity died of an inflammatory syndrome. HO-1 exerts its antiproliferative and antiapoptotic properties via the production of bilirubin, CO, and free iron under various pathophysiological conditions (Otterbein et al., 2003a). Of three HO-1 reaction products, CO has been shown to have antiproliferative effects (Morse and Choi, 2002). Antiproliferative effect of CO has been linked to its ability to modulate the activity of guanylate cyclase and to increase the levels of cellular cGMP and/or the mitogen-activated protein kinase (MAPK) pathways. In vascular smooth muscle cells, CO exerts its antiproliferative effect via p38 MAPK and cGMP pathway (Otterbein et al., 2003b). The antiproliferative effect of CO in airway smooth muscle cells is mediated by the extracellular signal-regulated kinase MAPK pathway but not by cGMP pathway (Song et al., 2002). In T cells, CO exerts its antiproliferative effect via a caspase-dependent pathway, which is independent of guanylate cyclase/cGMP system and p38 MAPK pathway (Song et al., 2004). In the present study, we have demonstrated that CO blocked low-level caspase-8 and caspase-3 activations in Con A-stimulated Jurkat T cells (Fig. 6B). The blocking effect of CO on caspase-8 and caspase-3 activations might be linked to its antiproliferative effect, because the pan-caspase inhibitor *z*-VAD inhibited T cell proliferation (Fig. 6C). However, the mechanism(s) by which CO can block caspase-8 and caspase-3 activations during T cell stimulation remains to be established. In addition, the free iron, another HO-1 byproduct, mediated the antiapoptotic effect of NO (Fig. 8). The antiapoptotic mechanism of free iron produced by HO-1 has been well documented in our previous paper (Choi et al.,

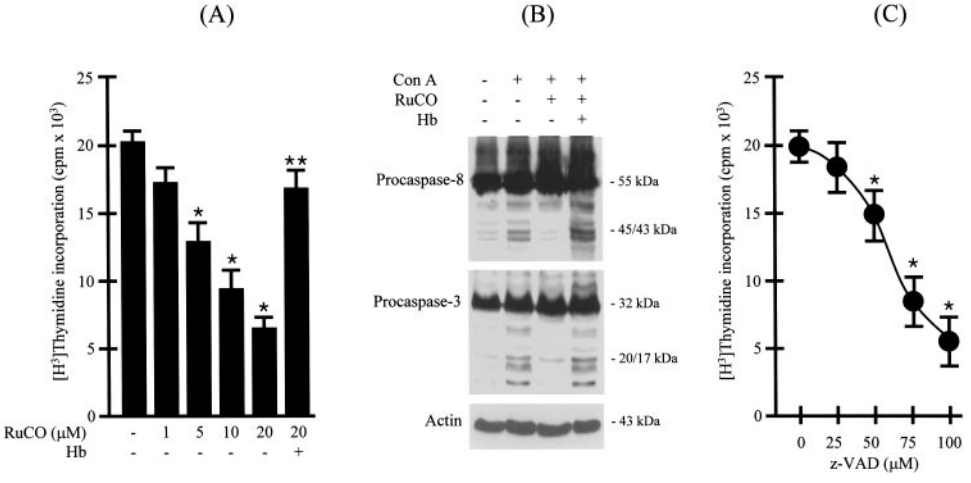

Fig. 6. Effects of CO on T cell proliferation. A, Jurkat T cells were preincubated 1 h with medium or indicated concentrations of RuCO in the presence or absence of 40 μM of Hb and stimulated 72 h with 1 μM of Con A. B, the T cells were preincubated 1 h with medium or 20 mM of RuCO in the presence or absence of 40 μM of Hb, stimulated 24 h with 1 μM of Con A, and harvested for Western blot analysis. C, the T cells were preincubated 1 h with medium or indicated concentrations of z-VAD, and stimulated 72 h with 1 μM of Con A. Western blot analysis (B) and [^{3}H]thymidine incorporation assay (A and C) was performed as described under *Materials and Methods*. *, $P < 0.01$ with respect to Con A; **, $P < 0.01$ with respect to Con A + RuCO (20 μM).

2004). In our previous work, we have demonstrated that Fe^{2+} activates the nuclear factor-κB, which can be activated by Fe^{2+}-mediated reactive oxygen species, and protects Jurkat T cells and primary $CD4^+$ T lymphoblast cells from Fas-triggered apoptosis via nuclear factor-κB–dependent induction of antiapoptotic proteins (Choi et al., 2004). Our data showing that the HO-1 byproducts mediated antiproliferative and antiapoptotic effects in T cells collectively but indirectly support our conclusion that suppressions of T cell proliferation and apoptosis afforded by NO pretreatment are correlated to HO-1 expression by NO.

In summary, we have shown that NO is a regulator of HO-1 expression in human T cells. According to our data, HO-1 expression is responsible for suppressions of T cell proliferation and apoptosis afforded by NO. Thus, HO-1 is an important cellular target of NO, with clinical implications for the prevention of T cell-mediated abnormal immunity by virtue of its role in T cell suppression and protection.

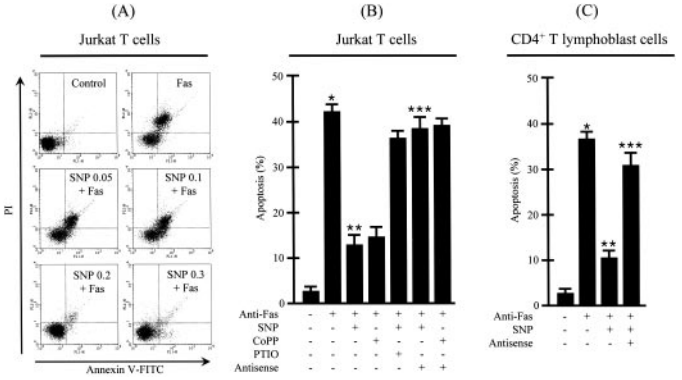

Fig. 7. Effects of NO and HO-1 inducer on Fas-mediated apoptosis. A, Jurkat T cells were preincubated 12 h with medium (control) or indicated concentrations of SNP. The T cells, then, were treated 12 h with 1 μg/ml of anti-Fas antibody, harvested, and stained 20 min with Annexin V-FITC and PI for a flow cytometric analysis. In these representative dot plots, Annexin V-positive (apoptotic) events induced by anti-Fas antibody are progressively reduced by SNP. B, Jurkat T cells were preincubated 12 h with medium, 0.3 μM of SNP, or 20 μM of CoPP in the presence or absence of 1 mM of PTIO or 10 μg/ml of HO-1 antisense ODN, and then treated 12 h with 1 μg/ml of anti-Fas antibody. C, $CD4^+$ T lymphoblast cells were preincubated 12 h with medium or 0.3 mM of SNP in the presence or absence of 10 μg/ml of HO-1 antisense ODN and then treated 12 h with 1 μg/ml of anti-Fas antibody. The percentages of Annexin V-positive (apoptotic) cells were analyzed by using a flow cytometry after double staining with Annexin V-FITC and PI. *, $P < 0.01$ with respect to untreated group; **, $P < 0.01$ with respect to anti-Fas; *** $P < 0.01$ with respect with anti-Fas + SNP.

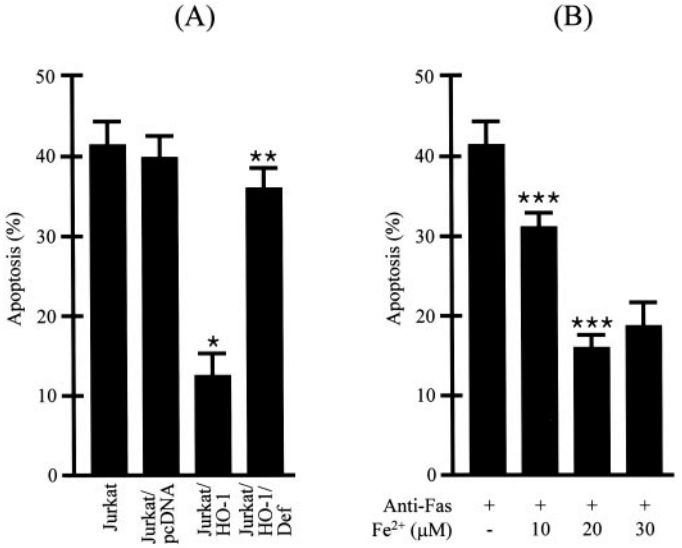

Fig. 8. Effects of HO-1 overexpression and free iron on Fas-mediated apoptosis. A, Jurkat T cells stably transfected with empty vector or HO-1 gene were treated 12 h with 1 μg/ml of anti-Fas antibody in the presence or absence of 0.1 mM of Def, and the percentages of apoptotic cells were analyzed by a flow cytometry. B, Jurkat T cells were preincubated 1 h with indicated concentrations of $FeSO_4$, and then treated 12 h with 1 μg/ml of anti-Fas antibody. The percentages of apoptotic cells were analyzed by using a flow cytometry after double staining with Annexin V-FITC and PI. *, $P < 0.01$ with respect to empty vector; **, $P < 0.01$ with respect to HO-1; ***, $P < 0.01$ with respect with anti-Fas.

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