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Pentoxifylline protects L929 fibroblasts from TNF-α toxicity via the induction of heme oxygenase-1

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Abstract

Tumor necrosis factor-α (TNF-α) is recognized as a principal mediator of a variety of inflammatory conditions. Pentoxifylline (PTX), which can inhibit cellular TNF-α synthesis, also attenuates the toxic effect of TNF-α. However, the mechanism underlying PTX-induced cytoprotection is unknown. Heme oxygenase 1 (HO-1) is an enzyme which degrades heme into biliverdin, free iron, and carbon monoxide (CO). This enzyme has recently been shown to have anti-inflammatory and cytoprotective effects. In this study, we investigated whether protection by PTX against TNF-α-mediated toxicity could be related to its ability to induce HO-1 expression and HO activity in L929 cells. PTX in the range of 0.1–1.0 mM significantly induced HO-1 expression and the resulting HO activity. Pre-incubation of L929 cells with either PTX or the HO activator hemin resulted in the protection of the cells against TNF-α-mediated toxicity. Zinc protoporphyrin, a specific HO competitive inhibitor, abrogated the protective effect of PTX. Hemoglobin, a scavenger of CO, reversed the protective effect of PTX. A cytoprotection comparable to PTX was observed when the cells were treated with the CO-releasing compound tricarbonyldichlororuthenium(II) dimer. These results suggest that HO-1 expression and the ensuing formation of the HO metabolite CO may be a novel pathway by which PTX protects L929 cells from TNF-α-mediated toxicity.

Keywords: Pentoxifylline; TNF-α; Heme oxygenase; Carbon monoxide; Cytoprotection

Pentoxifylline (PTX) is a xanthine derivative that inhibits phosphodiesterases, resulting in the elevation of intracellular cyclic adenosine monophosphate (cAMP) [1]. It has been widely used as an agent for the treatment of cerebrovascular disorders, occlusive arterial diseases, and septic shock [1–3]. The major pharmacological action of PTX is the inhibition of tumor necrosis factor-α (TNF-α) production [1–3]. It is believed that the mechanism for the inhibition of TNF-α production by PTX involves the elevation of intracellular cAMP [1–3]. In addition of its inhibition of TNF-α synthesis, PTX has been found to attenuate the cytotoxic action of TNF via cAMP-independent pathway [4,5]. However, the mechanism underlying PTX-induced cytoprotection is still unknown.

Heme oxygenases (HOs) are ubiquitous enzymes that catalyze the initial and rate-limiting steps in the oxidative degradation of heme to bilirubin; using NADPH and molecular oxygen, HOs cleave a mesocarbon of the heme molecule, producing equimolar quantities of biliverdin, iron, and carbon monoxide (CO) [6]. Biliverdin is reduced to bilirubin by biliverdin reductase and the free iron is either used in intracellular metabolism or sequestered in ferritin. CO may act as a cellular messenger and has been implicated in the regulation of vascular tone and neurotransmission [7]. To date, three HO isoenzymes have been identified; of these, HO-2 and HO-3 are constitutively expressed, whereas HO-1 is inducible [8]. HO-1 is involved in the oxidative stress response, being highly induced and conferring protective

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effects in such conditions both in vivo and in vitro. The mechanism(s) by which HO-1 confers protection against oxidative stress is not clearly understood. It is generally believed that the catalytic by-products derived from the catalysis of heme by HO, namely biliverdin, bilirubin, ferritin accumulation from released free iron, and CO, may mediate the physiological effects of HO [6–8]. Both biliverdin and bilirubin possess antioxidant properties [7]. Furthermore, the iron released during heme catabolism can stimulate ferritin synthesis [9]. It has been suggested that increased levels of ferritin reduce the cellular oxidant potential by further decreasing the intracellular concentration of free iron [9]. Recent reports have also observed that CO at low doses can provide protection against oxidant stress or TNF-α-mediated toxicity [10,11].

The objective of this study was to examine whether the protective effect of PTX against TNF-α-mediated toxicity in L929 cells was mediated by its HO-1 expression. We demonstrated that HO-1 expression and the resulting HO activity were responsible for the protection afforded by PTX against the toxic effect of TNF-α. We also examined the protective effect of CO, a major catalytic by-product of the HO-1 catalysis of heme, on TNF-α-mediated toxicity.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco-BRL (Grand Island, NY). Human TNF-α was purchased from R&D Systems (Minneapolis, MN). A polyconal HO-1 antibody was obtained from StressGen (Victoria, Canada). Zinc protoporphyrin IX (ZnPP), hemin, and Bilirubin IX were obtained from Porphyrin Products (Logan, UT). 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was from Alexis Biochemicals (La Jolla, CA). Tricarbonyldichlororuthenium(II) dimer (RuCO), iron, crystal violet, PTX, and hemoglubin (Hb) were obtained from Sigma Chemical (St. Louis, MO).

Cell culture. L929 cells were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). The cells were maintained at 5×10^5 cells/ml in DMEM supplemented with 10% heat-inactivated FBS, penicillin G (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

Viability assay. Cell viability was determined using the crystal violet assay. Briefly, cells were seeded in 12-well plates at 2×10^4/well. After 48 h incubation, the media were replaced and cells were treated with PTX and/or TNF-α. Upon completion of the treatment, media were removed and cells were washed twice with ice-cold phosphate-buffered saline (PBS). Cells were fixed with methanol for 5 min and then stained for 10 min with 0.1% crystal violet solution. Excess stain was removed and the fixed cells were washed twice for 5 and 10 min with PBS. Dye was eluted with 0.1 trisodium citrate in 50% ethanol for 1 h. Optical density at 630 nm was measured using a microtiter plate reader.

Western blotting. L929 cells were lysed in electrophoresis buffer (125 mM Tris (pH 6.8), 12.5% glycerol, 2% SDS, and 1 mM dithiothreitol), boiled, and sonicated. The lysate was centrifuged at 14,000*g* for 15 min at 4 °C, the supernatant was collected, and SDS–PAGE was performed. The separated blots were electrophoretically transferred to nitrocellulose membranes and blocked overnight at 4 °C in TBS containing Tween 20 (0.1%) and nonfat milk (5%). Blots were then incubated with HO-1 (1:1000) or actin (1:1000) antibody for 1 h. Membranes were then washed in TBS-T and incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit (1:500) or goat anti-mouse (1:500 dilution) antibody. After further washing with TBS-T, blots were developed using the ECL method (Amersham, Arlington Heights, IL).

Measurement of HO activity. Cell lysate was used for the measurement of HO activity and assayed using NADPH generating system. The amount of bilirubin generated was determined by scanning spectrophotometer (Lambda 17 UV/Vis; Perkin–Elmer Cetus Instruments, Norwalk, CT) and defined as the difference between 460 and 530 nm (extinction coefficient, 40 mM^{-1} cm^{-1} for bilirubin). Results are expressed as pmoles of bilirubin per milligram of protein per 1 h.

Statistical analyses. The data are presented as means ± SEM for a number of experiments. Statistical significance ($p < 0.05$) between the experimental groups was determined by means of ANOVA and Student's *t* test.

Results

PTX induces HO-1 expression and HO activity

We examined the effects of PTX on HO-1 expression and HO activity in L929 cells. As shown in Fig. 1A, exposure of the cells to PTX (0.1–1.0 mM) for 12 h resulted in concentration-dependent increases in HO activity. Western blot analysis revealed that enhanced HO activity by PTX directly correlated with HO-1 protein levels (Fig. 1B). The concentration of PTX that produced maximal increase in HO activity (0.5 mM) was chosen to determine the effect of PTX on HO activity and HO-1 expression over time. Treatment of the cells

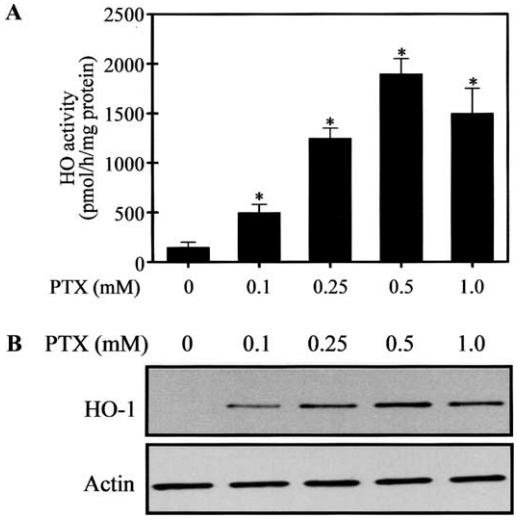

Fig. 1. Dose-dependent effects of PTX on HO activity and HO-1 expression in L929 cells. Cells were incubated with indicated doses of PTX for 12 h. (A) HO activity was determined via bilirubin formation as described under Materials and methods. All data shown are means ± SEM of three independent observations in separate cell culture wells. $*p < 0.05$ compared with untreated control. (B) Western blot analysis was performed as described under Material and methods. The blots are representative of at least three separate experiments.

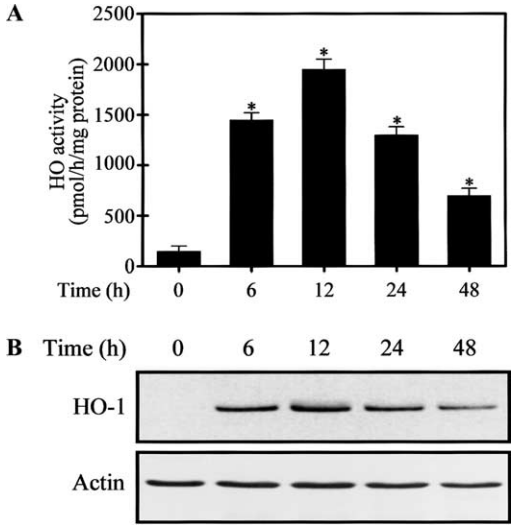

Fig. 2. Time-dependent effects of PTX on HO-1 expression and HO activity in L929 cells. Cells were incubated with 0.5 mM PTX for indicated times. (A) HO activity was determined via bilirubin formation as described under Material and methods. All data shown are means ± SEM of three independent observations in separate cell culture wells. $*p < 0.05$ compared with untreated control. (B) Western blot analysis was performed as described under Material and methods. The blots are representative of at least three separate experiments.

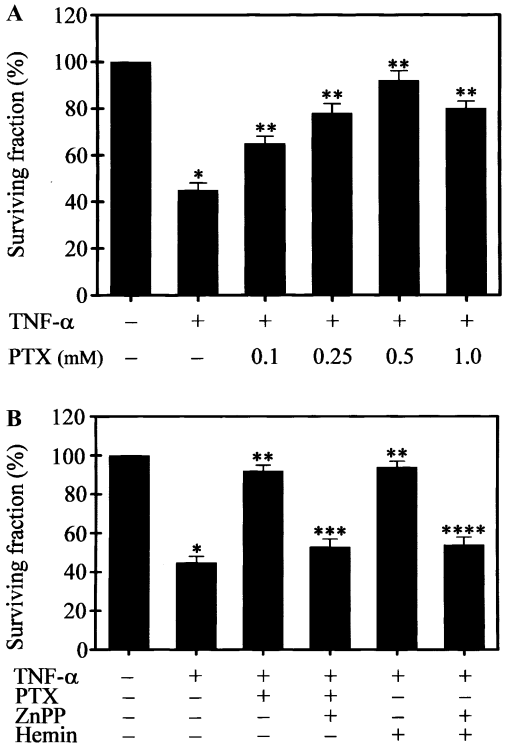

Fig. 3. PTX-induced HO-1 reduces TNF-α-mediated toxicity. (A) Cytoprotective effect of PTX on TNF-α-mediated toxicity in L929 cells. Cells were pre-incubated with indicated doses of PTX for 12 h. Then, 20 ng/ml TNF-α was given to the cells and incubation was continued for 48 h, followed by a cytotoxicity assay as described under Material and methods. (B) Effects of the HO inhibitor ZnPP on cytoprotection induced by PTX or the HO activator hemin. Cells were pre-incubated with 0.5 mM PTX or 10 μM hemin for 12 h in the absence or presence of 20 μM ZnPP. Then, 20 ng/ml TNF-α was given to the cells and incubation was continued for 48 h, followed by a cytotoxicity assay as described under Material and methods. All data shown are means ± SEM of three independent observations in separate cell culture wells. $*p < 0.05$ compared with untreated control, $**p < 0.05$ compared with TNF-α, $***p < 0.05$ compared with PTX + TNF-α, and $****p < 0.05$ compared with hemin + TNF-α.

with PTX resulted in time-dependent increases in HO activity, with a maximal enzymatic activity at 12 h (Fig. 2A). This effect correlated with time-dependent increases in HO-1 expression (Fig. 2B). PTX in the range of 0.1–1.0 mM had no significant effect on cell viability under these conditions (data not shown).

PTX-induced HO-1 reduces TNF-mediated toxicity

We determined whether HO-1 expression and the resulting HO activity were responsible for the protection afforded by PTX against the toxic effect of TNF-α. As shown in Fig. 3A, incubation of L929 cells with TNF-α (20 ng/mL) for 48 h resulted in a marked toxicity and reduction of cell viability by 45%. However, pre-incubation of the cells with PTX for 12 h diminished TNF-α-mediated toxicity in a concentration-dependent manner. Protection by PTX was abrogated in the presence of the HO inhibitor ZnPP and a similar protective effect was observed when the cells were pre-incubated with hemin, an established activator of HO-1 expression (Fig. 3B). Again, ZnPP abrogated the protective action of hemin (Fig. 3B). PTX, ZnPP, or hemin alone had no significant effects on cell viability under these conditions (data not shown).

Protective effect of HO-1 may be mediated via CO

The mechanism by which PTX-induced HO-1 confers protection of the cells against TNF-α-mediated toxicity is not clear. We hypothesized that CO, a major catalytic by-product of the HO-1 catalysis of heme, might mediate this protective effect. L929 cells were pre-incubated for 12 h with PTX alone or PTX plus the CO scavenger Hb and then treated with TNF-α. Hb reversed the protective effect of PTX on TNF-α-mediated toxicity and a protection comparable to PTX was observed when L929 cells were treated with the CO-releasing compound RuCO (Fig. 4A). We then examined whether inhibiting guanylyl cyclase, a major target enzyme of CO, would ablate the protective effect of PTX-induced HO-1 or CO. Incubation of PTX- or RuCO-treated L929 cells with ODQ, an inhibitor of guanylyl cyclase, abrogated the protective effects of PTX and RuCO on TNF-α-mediated toxicity (Fig. 4B). In addition, the other HO-1 by-products, bilirubin and free iron, failed to relieve TNF-α-mediated toxicity in L929 cells (data

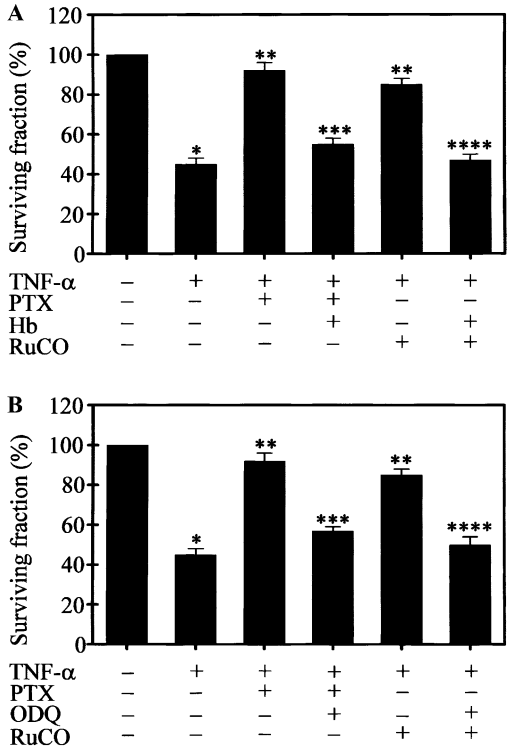

Fig. 4. Cytoprotective effect of HO-1 is mediated via CO. (A) Effects of the CO-scavenger Hb on cytoprotection induced by PTX or the CO-releasing compound RuCO in L929 cells. Cells were pre-incubated with 0.5 mM PTX or 10 μM RuCO in the absence or presence of 50 μg/ml Hb for 12 h. Then, 20 ng/ml TNF-α was given to the cells and incubation was continued for 48 h, followed by a cytotoxicity assay as described under Material and methods. (B) Effects of ODQ on cytoprotection induced by PTX or RuCO. Cells were pre-incubated with 0.5 mM PTX or 10 μM RuCO for 12 h in the absence or presence of 20 μM ODQ. Then, 20 ng/ml TNF-α was given to the cells and incubation was continued for 48 h, followed by a cytotoxicity assay as described under Material and methods. All data shown are means ± SEM of three independent observations in separate cell culture wells. $*p < 0.05$ compared with untreated control, $**p < 0.05$ compared with TNF-α, $***p < 0.05$ compared with PTX + TNF-α, and $****p < 0.05$ compared with RuCO + TNF-α.

not shown). Hb or RuCO alone had no significant effect on cell viability under these conditions (data not shown).

Discussion

PTX has been shown to attenuate the toxic effect of TNF-α via cAMP-independent pathway in L929 mouse fibroblast cells [4,5]. However, the mechanism underlying PTX-induced protection of the cells remains to be established. In the present study, we have demonstrated that PTX induces the protective protein HO-1 expression and HO activity in L929 cells. Moreover, according to our data, HO-1 expression and the resulting HO activity are responsible for the protection afforded by PTX against the toxic effect of TNF-α.

We mainly focused on investigating whether protection by PTX against TNF-α-mediated toxicity could be related to its ability to induce HO-1 expression and the resulting HO activity in L929 cells. Interestingly, PTX in the range of 0.1–1.0 mM significantly induced HO-1 expression and the resulting HO activity in a concentration- and time-dependent manner (Figs. 1 and 2). Pre-incubation of L929 cells with either PTX or the HO activator hemin resulted in protection against TNF-α-mediated toxicity (Fig. 3A). The involvement of HO-1 in the protective action of PTX was confirmed using pharmacological approaches. Hence, a specific HO competitive inhibitor, ZnPP, abrogated the protective effect of PTX on TNF-α-mediated toxicity (Fig. 3B). Furthermore, Hb (a scavenger of CO) and RuCO (a CO-releasing compound) were used to determine whether CO released from heme degradation by HO-1 was responsible for the protective action of PTX. Hb reversed the protective effect of PTX against TNF-α-mediated toxicity and a protection comparable to PTX was observed when L929 cells were treated with RuCO (Fig. 4A). According to these data, we suggest that HO-1 expression and the ensuing formation of the protective HO metabolite CO may be a novel pathway by which PTX protects the cells from TNF-α-mediated toxicity.

The mechanism by which PTX-induced HO-1 or CO can exert a protective function is not clear yet. It is believed that CO exerts biological effects via a guanyl cyclase or cGMP pathway in the vascular and neuronal systems [12,13]. This pathway may also be involved in the protective effects of PTX-induced HO-1 and CO, based on our observations that ODQ, a guanylyl cyclase inhibitor, ablated the protective effects of PTX and CO (Fig. 4B). Alternative signal pathway(s) may also be involved in this cytoprotection that important mediators such as nuclear factor-κB and TNF death receptor pathways which have been shown to be important in modulating the toxic effects of TNF-α. Rigorous studies are needed to investigate that the plausible signaling pathway(s) by PTX-induced HO-1 or CO regulates its protective effect.

Recent studies indicate that HO-1 has significant physiological mediator functions in circulation. The exogenous administration of CO relaxes blood vessels isolated from various vascular sources and animal species [14]. Moreover, expression of HO-1 causes a marked decrease in blood pressure in hypertensive rats whereas HO-1 inhibition increases blood pressure, suggesting that endogenous CO subserves a tonic vasodepressor function [15,16]. HO-1 derived CO also blocks the synthesis of growth factors from vascular cells and directly inhibits vascular smooth muscle cell proliferation [17,18]. In addition, expression of HO-1 in vivo suppresses a variety of inflammatory responses, including endotoxic shock and graft rejection [6]. It is interesting that PTX, which has been proven to be an

inducer of HO-1 in this study, is an anti-inflammatory agent used to treat vascular diseases and inflammatory responses [1].

In conclusion, the present results suggest that HO-1 expression and increased HO activity by PTX could contribute to its cellular defense against TNF-α-mediated toxicity in L929 cells. Our data also suggest that the protective effect of HO-1 may be mediated via CO. Further studies are needed to clarify the precise mechanisms by which PTX can induce HO-1 expression in the cells.

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