p38 MAPK/PP2Aα/TTP pathway on the connection of TNF-α and caspases activation on hydroquinone-induced apoptosis

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This study investigated tumor necrosis factor-α (TNF-α)-mediated death pathway contribution to hydroquinone (HQ) cytotoxicity in human leukemia U937 cells. HQ-induced apoptosis of human leukemia U937 cells was characterized by the increase in mitochondrial membrane depolarization, procaspase-8 degradation and Bid production. Downregulation of Fas-associated death domain protein (FADD) blocked HQ-induced procaspase-8 degradation and rescued the viability of HQ-treated cells, suggesting the involvement of a death receptor-mediated pathway in HQ-induced cell death. HQ increased expression of TNF-α mRNA stability led to TNF-α protein expression upregulation, whereas HQ suppressed TNF-α-mediated NFκB pathway activation. HQ elicited protein phosphatase 2A catalytic subunit α (PP2Aα) upregulation via p38 mitogen-activated protein kinase (MAPK)-mediated CREB/c-Jun/ATF-2 phosphorylation, and PP2Aα upregulation was found to promote tristetraprolin (TTP) degradation. Suppression of p38 MAPK activation and protein phosphatase 2A (PP2A) activity abrogated TNF-α upregulation and procaspase degradation in HQ-treated cells. Overexpression of TTP suppressed HQ-induced TNF-α upregulation and restored the viability of HQ-treated cells. Moreover, TTP overexpression increased TNF-α mRNA decay in HQ-treated cells. Taken together, our data indicate that HQ elicits TNF-α upregulation via p38 MAPK/PP2A-mediated TTP downregulation, and suggest that the TNF-α-mediated death pathway is involved in HQ-induced U937 cell death. The same pathway was also proven to be involved in the HQ-induced death of human leukemia HL-60 and Jurkat cells.

Introduction

Hydroquinone (HQ) is a major narrow metabolite of the leukemogen benzene (1,2). Previous studies showed that HQ is further converted into bioactive p-benzoquinone in myceloid cells via myeloperoxidase-mediated reaction, leading to myelotoxicity and benzene-related diseases (3,4). Moreover, HQ has been found to induce apoptosis of bone marrow progenitor cells (5). HQ also presents abundantly in cigarette smoke and is suggested to contribute to lung infection of bone marrow progenitor cells (5). HQ also presents abundantly in cigarette smoke and is suggested to contribute to lung infection of bone marrow progenitor cells (5). HQ also presents abundantly in cigarette smoke and is suggested to contribute to lung infection of bone marrow progenitor cells (5). HQ also presents abundantly in cigarette smoke and is suggested to contribute to lung infection of bone marrow progenitor cells (5). HQ also presents abundantly in cigarette smoke and is suggested to contribute to lung infection of bone marrow progenitor cells (5). HQ also presents abundantly in cigarette smoke and is suggested to contribute to lung infection of bone marrow progenitor cells (5).

Materials and methods

Hydroquinone (HQ), actinomycin D, SB202190 (p38 MAPK inhibitor), MG132 (proteasome inhibitor), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), N-acetylcysteine (NAC) and anti-β-actin antibody were obtained from Sigma-Aldrich (St Louis, MO), and rhodamine-123 was the product of Molecular Probes (Eugene, OR). Okadaic acid (PP2A inhibitor), Akt inactivation inhibits LPS-induced cytokine gene expression and NFκB phosphorylation in macrophage-like RAW264.7 cells. However, HQ-induced ERK/AP-1 activation stimulates the production of granulocyte-macrophage colony-stimulating factor in TF-1 leukemia cells (10). On the other hand, HQ differently inhibits LPS-induced proliferation of T cells and B cells isolated from bone marrow and splenocyte (7). These observations suggest that the HQ effect on hematological cells should be cell-type specific, and that the HQ effect on cytokine production, proliferation and/or apoptosis mostly elucidates its toxic mechanism on hematological cells in vivo. Moreover, HQ-induced cytotoxicity is dependent on HQ concentration and time of treatment (7,11–15).

Several studies have shown that HQ induces apoptosis in human lymphoblastic leukemia Jurkat cells, human promyelocytic HL-60 cells, human neutrophils and eosinophils (11–15). Nevertheless, the mechanism responsible for HQ-induced apoptosis is ambiguously delineated. Inayat-Hussain and Ross (11) reported that HQ-induced death in HL-60 cells and Jurkat cells is mediated through caspase-independent and caspase-dependent pathways, respectively. Although caspases-8, -9 and -3 activation are noted with HQ-treated HL-60 and Jurkat cells, HQ-induced cell death was proven not related to the Fas/Fasl-mediated death pathway (11, 16, 17). Because TNF-α could induce cell death via both caspase-dependent and caspase-independent pathways (18–21), the TNF-α-mediated death pathway seems to fulfill the HQ cytotoxic mechanism on HL-60 and Jurkat cells. An interesting feature of TNF-α signaling is the existence of crosstalk between the proapoptotic and NFκB-activating pathways (22). HQ-inhibited TNF-α/NFκB activation is suggested to lead to TNF-α-mediated apoptotic death in primary human CD4+ T cells, human CD34+19-hematopoietic progenitor cells and human leukemia TF-1 cells (23, 24). Moreover, previous studies have shown that HQ induces the secretion of TNF-α in microvascular endothelial cells (25) and phorbol-12-myristate-13-acetate/ ionomycin-treated human peripheral blood mononuclear cells (26).

Recently, Shimada et al. (27) found that inhalation of HQ enhances TNF-α release by mice tracheal epithelial cells, which mediates the HQ-induced parasympathetic airway muscle contraction in mice. This finding suggests that HQ toxicity could be mediated through TNF-α in vivo. If HQ induces simultaneously increased TNF-α expression and suppression of TNF-α/NFκB activation on hematological cells, it is plausible that HQ treatment induces apoptotic death. Noticeably, in addition to genetic transcription, an increase in TNF-α mRNA stability has been reported to upregulate TNF-α expression (28). Moreover, ADAM10- or ADAM17-mediated ectodomain shedding of membrane TNF-α affects the production of secreted TNF-α (29). Thus, the change in TNF-α expression should be considered to occur at transcriptional, translational and/or posttranslational levels. Previous studies showed that human leukemia U937 cells are sensitive to TNF-α-induced apoptosis (30, 31). Our recent studies revealed that ADAM10 and ADAM17 are actively expressed in U937 cells (32). Thus, the HQ effect on TNF-α expression and TNF-α-mediated death pathway activation in U937 cells was investigated in this study. To corroborate the common HQ effect, the TNF-α expression regulation mechanism was also conducted on HQ-treated HL-60 and Jurkat cells.

Abbreviations: MV, cytomegalovirus; ELISA, enzyme-linked immunosorbent assay; FADD, Fas-associated death domain protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HQ, hydroquinone; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MK2, p38 MAPKAP kinase 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; N, N-acetylcysteine; NFκB, nuclear factor-kappaB; PP2Aα, protein phosphatase 2A; PP2Aαc, protein phosphatase 2A catalytic subunit; ROS, reactive oxygen species; TTP, tumor necrosis factor-α receptor; siRNA, small interfering RNA; TTP, tristetraprolin.
Z-IETD-fmk (caspase-8 inhibitor), Z-DEVAD-fmk (caspase-3 inhibitor), anti-caspase-3 antibody and anti-caspase-8 antibody were purchased from Calbiochem (San Diego, CA). Anti-ADAM17 (H-300), anti-PP2Ac (N-25), anti-tristetraprolin (TTP), anti-TNF-α (25B28), anti-CREB1 (C-21), anti-phospho-CREB1 (Ser133), anti-Fas (N-18), anti-FasL (Q-20), anti-FADD, anti-phospho-c-Fos (Ser374), anti-phospho-ATF-2 (Thr71) and anti-phospho-c-Jun (Ser73) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase-9, anti-PARP, anti-TNFFR2, anti-NFkB(p65), anti-phospho-NFkB(p65), anti-1xBet, anti-phospho-1xBet, anti-IKKα, anti-phospho-IKKα, anti-p38 MAPK, anti-phospho-p38 MAPK, anti-ERK, anti-phospho-ERK, anti-JNK, anti-phospho-JNK anti-c-Fos, anti-c-Jun, anti-TNF-α and anti-Bcl-2 antibodies were the products of Cell Signaling Technology (Beverly, MA). Anti-Bcl-xl, anti-Bcl-2 antibody (CT) was purchased from Anaspec (San Jose, CA). Anti-Bcl-xL, anti-Bid and anti-cytochrome c antibodies were the products of BD Pharmingen (Rockville, MD). Recombinant human TNF-α, anti-TNFFR1 antibody, monoclonal anti-human extracellular TNF-α–fluorescein and carboxyfluorescein-conjugated mouse IgG1, isotype control were obtained from R&D Systems (Minneapolis, MN). Horseradish peroxidase-conjugated secondary antibodies were obtained from Pierce (Rockford, IL). Cell culture supplies were purchased from Gibco/Life Technologies (Carlsbad, CA). Unless otherwise specified, all other reagents were of analytical grade.

Cell culture

Human acute myeloid leukemic U937 cells and human lymphoblastic leukemic Jurkat cells (Clone E6-1, TIB-152) obtained from American Type Culture Collection (Rockville, MD) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco BRL), 2 mM l-glutamine, penicillin (100 units/ml) and streptomycin (100 µg/ml) at 37°C in a humidified atmosphere with 95% air and 5% CO₂. Human promyelocytic HL-60 cells (BCRC 60027) obtained from Bioresource Collection and Research Center (Hsinchu, Taiwan) were grown in RPMI 1640 medium supplemented with 20% fetal calf serum (Gibco BRL), 4 mM l-glutamine, penicillin (100 units/ml) and streptomycin (100 µg/ml) in a humidified atmosphere with 9% air and 5% CO₂. Human promyelocytic HL-60 cells were harvested by incubating with 0.01% human IgG in phosphate-buffered saline. One million cells were washed in 1 ml of cold PBS twice and then suspended in 100 µl PBS and 1 µl fluorescent parameter flow cytometry was performed by staining cells with monoclonal anti-human extracellular TNF-α–fluorescein (BD Pharmingen Technical (San Jose, CA)).

RNA preparation and reverse transcriptase–PCR

Total RNA was isolated from untreated control cells and HQ-treated cells by the guanidinium thiocyanate procedure (R&D Systems). The stained cells were analyzed using Beckman Coulter Epics XL flow cytometer (Coulter Epics XL, Beckman Coulter).

Flow cytometry analyses of TNF-α surface expression

After specific treatment, non-specific antibody-binding sites were blocked by preincubating with 100 µg/ml of human IgG in phosphate-buffered saline. One fluorescent parameter flow cytometry was performed by staining cells with monoclonal anti-human extracellular TNF-α–fluorescein and carboxyfluorescein-conjugated mouse IgG1, isotype control according to the manufacturer’s protocol (R&D Systems). The stained cells were analyzed using Beckman Coulter Epics XL flow cytometer (Coulter Epics XL, Beckman Coulter).

Determination of soluble TNF-α by enzyme-linked immunosorbent assay

After HQ treatment, the culture media of U937 cells were collected and centrifuged at 12,000 r.p.m. for 10 min and the clarified supernatants were collected. Enzyme-linked immunosorbent assay (ELISA) for soluble TNF-α (sTNF-α) was performed according to the manufacturer’s protocol (R&D Systems). Absorbance was read at wavelength 450 nm using a plate reader, and protein concentrations were calculated using a standard curve generated each time an assay was performed.

RNA preparation and reverse transcriptase–PCR

Total RNA was isolated from untreated control cells and HQ-treated cells using the RNeasy minikit (QIAGEN, Valencia, CA) according to the instructions of the manufacturer. Reverse transcriptase reaction was performed with 2 µg of total RNA using M-MLV reverse transcriptase (Promega, Madison, WI) according to the manufacturer’s recommendations. A reaction without reverse transcriptase was performed in parallel to ensure the absence of genomic DNA contamination. After initial denaturation at 95°C for 10 min, PCR amplification was performed using GoTaq Flexi DNA polymerase (Promega, Madison, WI) followed by 35 cycles at 94°C for 30 s, 65°C for 20 s and 72°C for 30 s. After a final extension at 72°C for 5 min, PCR products were resolved on 2% agarose gels and visualized by ethidium bromide transillumination under UV light. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to check the efficiency of complementary DNA (cDNA) synthesis and PCR amplification. Primer sequences were as follows: Fas-forward, 5'-CAAGAGGTGAGGACTAGAGGAA-3'; Fas-reverse, 5'-GACAAAGCCACCAAGTTA-3'; FasL-forward, 5'-TCTCAGAGAATTCCTGCTTGTCTT-3'; FasL-reverse, 5'-AGAAGCTTCCCCTTCTCCAGA-3'; TNFα-forward, 5'-ATGAGCACTGAAAGCATGATC-3'; TNFα-reverse, 5'-CACCACTCCAGAAC-3'; PP2Ac-forward, 5'-CATGAATGATTAATT-3'; PP2Ac-reverse, 5'-CTTCCTGTTGTCACAAGG-3'.

Measurement of TNF-α mRNA stability

Actinomycin D (10 µg/ml) was added directly to cell cultures that were already treated with HQ without removal of the original stimulant. Cells were collected at 0.5 and 1 h after the addition of actinomycin D. The level of miRNA was measured by real-time PCR and results were normalized to that of GAPDH. Primer sequences were as follows: TNF-α-forward, 5'-CCACGGACCCCTTCTGCTTAC-3'; TNF-α-reverse, 5'-GCTGTCAGCTTACCG-3'; GAPDH-forward, 5'-GAAATCTCCACCATCCATCTCCAGG-3'; GAPDH-reverse, 5'-GAGCCCCAGGTTCTCCAGTGT-3'. Quantitative PCR was performed using iQ5 System (Bio-Rad, Hercules, CA). Reactions were performed using GoTag qPCR Master mix (Promega). Thermocycling conditions were at 95°C for 2 min followed by 40 cycles of amplification at 95°C for 15 s and 60°C for 60 s. Specificity was verified by melting curve analysis and agarose gel electrophoresis.

DNA transfection

The pcDNA-PP2Acct plasmid was a generous gift from Dr D. Pimm (International Center for Genetic Engineering and Biotechnology, Italy). A plasmid encoding the full-length human TTP cDNA containing a hemagglutinin epitope tag under control of the cytomegalovirus (CMV) promoter (pcMV-TTP-HA) was generously provided by Dr P. Blumberg (National Institute of Environmental Health Sciences, Durham, NC). The pcMV-MEK1 (expressed the constitutively active MEK1) vector was a generous gift from Dr W.C. Hung (National Health Research Institutes, Taiwan). The luciferase construct, pGL-TNF-α, containing the promoter region between –1016 and 223 of the human TNF-α gene was prepared in our laboratory (33). Transected TNF-α promoter clones, pGL-TNF-α-1057 (–834 to 223) and pGL-TNF-α-479 (–256 to 223), were generated using PCR methods. The region including –1104 to –83 of PP2Acct gene promoter ligated with the pGL3-basic luciferase vector was a generous gift from Dr G.C. Tsokos (Harvard Medical School, Boston, MA). Transected PP2Acct promoter clones, pGL-P2Aαct-227 (–309 to –83) and pGL-P2Aαct-120 (–202 to –83), were generated using PCR methods. Mutation at CREB site at positions from –242 to –233 (GTGACGGTAC–AAAAATACAA) of PP2Acct promoter was prepared using QuickChang site-directed mutagenesis kit (Stratagene). The plasmids were transfected into U937 cells using 4D-Nucleofector (Lona Colegene AG, Germany).

A luciferase activity was determined using the dual-luciferase reporter assay system according to the manufacturer’s recommendation (Promega). The relative luciferase activity was calculated as the ratio between the firefly luciferase and the control Renilla luciferase activity.

RNA interference

c-Jun small interfering RNA (siRNA) (catalog no. sc-29223), ATF-2 siRNA (catalog no. sc-2867), CREB1 siRNA (catalog no. sc-29281), FADD siRNA (catalog no. sc-35352) and negative control siRNA (catalog no. sc-37007) were purchased from Santa Cruz Biotechnology. For the transfection procedure, cells were grown to 60% confluence and siRNA was transfected using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation assays were performed in essentially the same manner described previously (34). Primers used for amplifying the PP2Acct promoter were 5'-CAGTTAACGACCGCTGCTACCGG-3' (forward) and 5'-GCCCGTTCCCTGTTGACTCTTG-3' (reverse). PCR was performed using the cycling parameters: 94°C for 30 s, 60°C for 30 s and 72°C for 30 s for a total of 30 cycles. PCR products (–394/–83 region of PP2Acct promoter) were separated on a 2% agarose gel stained with ethidium bromide and were visualized under UV light.

Other tests

Measurement of cell viability using MTT assay, annexin V–fluorescein isothiocyanate/propidium iodide double staining, measurement of mitochondrial membrane potential, measurement of intracellular reactive oxygen species (ROS) production and western blot analyses were performed in essentially the same manner as described previously (34,35).

Statistical analysis

All data are presented as mean ± SD. Results were compared using one-way analysis of variance followed by Tukey’s multiple comparison test. Differences were considered significant at two-tailed P < 0.05. All statistical analyses were performed with GraphPad Prism Version 5.01 (GraphPad software, La Jolla, CA).

Ref: 819
Results

HQ-induced cell death via activation of death receptor-mediated pathway

Upon exposure to HQ, U937 cells showed a concentration-dependent and time-dependent decrease in cell viability (Figure 1A). A reduction of ~50% in cell viability was observed after treatment with 10 µM HQ for 24 h. In the absence of HQ, U937 cells were viable with low propidium iodide and annexin V staining (lower left quadrants of the dot plots, Supplementary Figure S1A, available at Carcinogenesis Online). HQ treatment caused an increase in cells stained with annexin V and annexin V/propidium iodide, suggesting that HQ treatment led to apoptosis and late apoptosis of U937 cells. Immunoblotting analyses revealed the degradation of procaspase-3, procaspase-8, procaspase-9 and poly (ADP ribose) polymerase (caspase-3 substrate) after HQ treatment (Figure 1B). Figure 1C shows that caspase inhibitors rescued the viability of HQ-treated U937 cells, confirming that caspases were involved in HQ-induced cell death. These results supported the notion that HQ induced apoptosis in U937 cells.

Increasing evidence suggests that altered mitochondrial function is linked to apoptosis and a decreasing mitochondrial transmembrane potential is associated with mitochondrial dysfunction (36). Rhodamine fluorescence was employed to determine the mitochondrial membrane potential (ΔΨm) in U937 cells following treatment with 10 µM HQ. As shown in Supplementary Figure S1B, available at Carcinogenesis Online, flow cytometry analysis showed that >98% of untreated control U937 cells were functionally active with high rhodamine-123 signals. HQ treatment induced a loss of ΔΨm in U937 cells, suggesting that the mitochondrial pathway was involved in HQ-induced apoptosis. During mitochondrion-mediated apoptosis, cytochrome c is released from the mitochondria into the cytosol, where it promotes caspase activation (37). As shown in Figure 1D, HQ induced the release of cytochrome c into cytosol. In the mitochondrial pathway, caspase-8 converts the Bid from the inactive form (22 kDa) into the active form (15 kDa), which is called truncated Bid (tBid). tBid has been reported to be associated with the mitochondrial outer membrane and causes disruption to ΔΨm, thus resulting in cytochrome c release (17). Cleavage of Bid to produce tBid was observed in HQ-treated cells, reflecting that the cytotoxicity of HQ was mediated through the caspase-8/mitochondria-dependent death pathway. Moreover, HQ treatment induced downregulation of Bcl-2 and Bcl-xL in U937 cells.

Death receptors of the TNF family such as Fas and TNFR1 recruit FADD and procaspase-8 to the receptor. Recruitment of procaspase-8 through FADD leads to its autocleavage and activation and in turn activates effector caspases such as caspase-3 in causing cell death (16). Downregulation of FADD expression with siRNA markedly abrogated the HQ-induced degradation of procaspases-8/-3 and the production of tBid (Figure 1E). Compared with control siRNA, FADD siRNA rescued the viability of HQ-treated cells (Figure 1F). These findings suggest that HQ-induced apoptosis in U937 cells was related to death receptor-mediated pathway activation.

HQ induced TNF-α upregulation via increased TNF-α mRNA stability

As shown in Figure 2A, immunoblotting analyses displayed a concentration-dependent increase in TNF-α protein expression after HQ treatment, whereas HQ insignificantly affected Fas, Fasl, TNFR1 and TNFR2 protein expression in U937 cells. Unlike that of Fas, Fasl, TNFR1 and TNFR2 mRNA, the TNF-α mRNA level was increased by HQ treatment as evidenced by RT–PCR assay (Figure 2A). Real-time PCR analyses also revealed an increase in TNF-α mRNA level in HQ-treated U937 cells (Figure 2B). Flow cytometry analyses showed that HQ treatment induced an increase in cell surface TNF-α expression (Figure 2C). ELISA assay revealed that HQ treatment led to an increase in soluble TNF-α (sTNF-α) detected in the culture medium of U937 cells (Figure 2D). A number of studies showed that sTNF-α liberation from its membrane-bound form is mediated by ADAM10 and ADAM17, resulting in sTNF-α secretion into the culture medium (29). As shown in Figure 2E, quantification of protein expression from western blot analyses showed that HQ treatment did not affect ADAM10 and ADAM17 protein expression. Promoter assay revealed that HQ treatment did not significantly enhance the luciferase activity of TNF-α promoter constructs (Figure 2F). mRNA stability analyses following transcriptional inhibition by actinomycin D showed increased TNF-α mRNA stability after HQ treatment (Figure 2G), revealing that HQ posttranscriptionally upregulated TNF-α expression in U937 cells.

HQ-induced PP2Acα upregulation elicited TNF-α upregulation

Previous studies showed that HQ-induced cell death or hematopoietic effect is associated with MAPK-mediated pathways (10,13). The activation of MAPKs was thus examined in HQ-treated cells. In contrast to that of phosphoINK, the phospho-p38 MAPK level was notably increased in HQ-treated cells (Figure 3A). Alternatively, phosphorylated ERK was reduced after HQ treatment for 24 h. Sun et al. (38) suggested that protein phosphatase 2A (PP2A) regulated the TNF-α mRNA stability by modulating the phosphorylation state of members in the p38 MAPK/ MAPKAP kinase 2 (MK2)/tristetraprolin (TTP) pathway. The core heterodimer of PP2A consists of a 36 kDa catalytic subunit (PP2A-C) and a 65 kDa regulatory subunit (PP2A-A). This heterodimer interacts with a variable third subunit (PP2A-B isoforms) that influences cellular substrate specificity (39). There are two catalytic subunit isoforms of PP2A, PP2Acα and PP2Acβ, that share 97% identity in their primary amino acid sequences (40). The specific function of these two subunits remains unknown, but PP2Acα is assumed to be the predominant type owing to the embryonic lethality of PP2Acα−/− knockout mice and because PP2Acα is expressed at higher levels than PP2Acβ (41,42). To elucidate the PP2A effect on HQ-induced TNF-α upregulation, PP2Ac expression was examined. Figure 3B shows that HQ induced PP2Ac upregulation. Pretreatment with SB202190 (p38 MAPK inhibitor) abolished HQ-induced PP2Ac and TNF-α upregulation. Moreover, SB202190 attenuated HQ-induced ERK inactivation (Figure 3C). Transfection of constitutively active MEK1 led to ERK phosphorylation, but abrogated p38 MAPK phosphorylation and PP2Ac upregulation in HQ-treated cells (Supplementary Figure S2, available at Carcinogenesis Online). This is in line with our previous results showing crosstalk between ERK and p38 MAPK in U937 cells (35). Pretreatment with okadaic acid (PP2A inhibitor) attenuated the ability of HQ to induce TNF-α upregulation and procaspases-8/-9 degradation (Figure 3D). Moreover, either SB202190 or okadaic acid reduced TNF-α mRNA level in HQ-treated cells (Figure 3E). Overexpression of PP2Acα led to TNF-α upregulation compared with control vector-transfected cells (Figure 3F). Consistently, the level of TNF-α mRNA in pCDNA-PP2Acα-transfected cells was higher than that in control vector-transfected cells (Figure 3G). Taken together, these results suggested a causal relationship between PP2Acα upregulation and TNF-α upregulation in HQ-treated cells. Figure 3H shows that pretreatment with okadaic acid rescued the viability of HQ-treated cells, whereas overexpression of PP2Acα exacerbated the cytotoxicity of HQ toward U937 cells.
PP2Ac promoter supported PP2Ac expression. Consistently, previous studies suggested that CREB at positions –240 to –233 was involved in genetically regulating PP2Ac expression (43). HQ treatment led to an increase in pGL-PP2Acα-1022 and pGL-PP2Acα-227 promoter luciferase activity. However, deletion of the –309 and –202 segment abrogated the HQ ability to upregulate PP2Acα promoter luciferase activity, suggesting that the elements that respond to HQ treatment were localized within positions –309 to –202. Analyses of the PP2Acα promoter using web software PROMO 3.0 (http://alggen.lsi.upc.es/recerca/frame-recerca.html) showed that the region within –309 to –202 of PP2Acα promoter contained c-Jun, CREB and ATF-2 transcription factor-binding sites. CREB, c-Jun and ATF-2 binding sites are found to colocalize on positions –242 and –233 of PP2Acα promoter. Moreover, a putative c-Jun binding site at positions –253 to –247 is neighboring the CREB/c-Jun/ATF-2-binding site. Given that MAPKs were involved in AP-1 and CREB activation (44), the levels of phospho-c-Jun, -ATF-2 and -CREB were measured. Figure 4B shows that HQ treatment increased c-Jun, ATF-2 and CREB phosphorylation. Pretreatment with SB202190 abolished phosphorylation of c-Jun, ATF-2 and CREB (Figure 4C). Knockdown of c-Jun, ATF-2 and CREB suppressed HQ-induced PP2Acα upregulation and TNF-α upregulation (Figure 4D). Consistently, HQ insignificantly affected PP2Acα promoter luciferase activity in c-Jun, ATF-2 or CREB siRNA-transfected cells compared with that of control siRNA-transfected cells (Figure 4E). Mutation on CREB/c-Jun/ATF-2 at positions –242 to –232 reduced PP2Acα promoter luciferase activity and abrogated the HQ ability to increase PP2Acα promoter luciferase activity (Figure 4A), suggesting that the putative CREB/c-Jun/ATF-2 binding site at positions –242 to –233 was a response element for HQ treatment. Chromatin immunoprecipitation assay showed that HQ increased the CREB, c-Jun and ATF-2 binding to PP2Acα promoter (Figure 4F). These findings suggested that p38 MAPK-mediated phosphorylation of c-Jun, ATF-2 and CREB were involved in transcriptional regulation of PP2Acα gene.

PP2A-elicited TTP degradation upregulated TNF-α expression

In view of the findings showing that TTP is critically involved in TNF-α mRNA turnover (22,45–47), TTP expression was thus examined in HQ-treated cells. Figure 5A shows that HQ downregulated TTP protein expression. However, HQ did not affect TTP mRNA level as evidenced by RT–PCR analyses (data not shown). Pretreatment with MG132 (proteasome inhibitor) attenuated HQ-induced TTP downregulation and TNF-α upregulation, but insignificantly affected HQ-induced PP2Acα upregulation (Figure 5B). Figure 5C shows that okadaic acid suppressed
HQ-induced TTP downregulation, whereas overexpression of PP2Acα reduced TTP expression. As shown in Figure 5D and E, overexpression of TTP suppressed HQ-induced TNF-α upregulation and rescued the viability of HQ-treated cells. Figure 5F shows that TTP overexpression suppressed the HQ-induced increase in TNF-α mRNA stability.

Collectively, these observations suggested that HQ-induced TNF-α upregulation was mediated through PP2A-elicited TTP downregulation.

HQ suppressed TNF-α mRNA stability

Previous studies showed that HQ inhibited TNF-α-induced activation of NFκB in primary human CD4+ T cells (23), human CD34+19 hematopoietic progenitor cells and human leukemia TF-1 cells (24), leading to TNF-α-mediated apoptotic death pathway activation. Thus, the HQ effect on TNF-α-induced NFκB activation in U937 cells was examined. Supplementary Figure S3, available at Carcinogenesis Online, shows that co-treatment with HQ abolished TNF-α-induced NFκB activation, IκB phosphorylation and IKKα phosphorylation in U937 cells, indicating that HQ suppressed TNF-α-induced NFκB activation in U937 cells.

TNF-α upregulation was involved in HQ-induced death of HL-60 and Jurkat cells

In view of previous studies showing that HQ-induced death in HL-60 cells and Jurkat cells (11), TNF-α, PP2Acα and TTP expression in HQ-treated HL-60 cells and Jurkat cells were thus examined. HQ treatment reduced the viability of HL-60 and Jurkat cells in a concentration-dependent manner (data not shown). As shown in Supplementary Figure S4A, available at Carcinogenesis Online, treatment with 25 µM HQ led to an ~35 and 40% reduction in viability in HL-60 and Jurkat cells, respectively. The single 25 µM HQ concentration was used to explore the HQ effect on TNF-α, PP2Acα and TTP expression. Supplementary Figure S4B, available at Carcinogenesis Online, reveals that HQ-induced TNF-α mRNA stability in Jurkat and HL-60 cells. Supplementary Figure S4C, available at Carcinogenesis Online, shows that HQ induced upregulation of TNF-α and PP2Acα, but downregulated TTP in HQ-treated HL-60 and Jurkat cells. Moreover, HQ elicited p38 MAPK activation in HL-60 and Jurkat cells. Supplementary Figure S4D and E, available at Carcinogenesis Online, shows that okadaic acid and SB202190 pretreatment abolished HQ-induced TNF-α upregulation.
and increased the TNF-α mRNA level. SB202190 also suppressed HQ-induced PP2Acα upregulation. SB202190 and okadaic acid restored the viability of HQ-treated HL-60 and Jurkat cells (Supplementary Figure S4F, available at Carcinogenesis Online). Supplementary Figure S4G, available at Carcinogenesis Online, shows that TTP overexpression suppressed the HQ-induced increase in TNF-α protein expression and mRNA level. Moreover, HQ inhibited TNF-α-induced NFκB activation in HL-60 and Jurkat cells (Supplementary Figure S4H, available at Carcinogenesis Online). These findings reveal that TNF-α upregulation is involved in HQ-induced death in HL-60 and Jurkat cells, and that PP2A-mediated TTP downregulation leads to TNF-α upregulation in HQ-treated HL-60 and Jurkat cells.

Discussion

Previous studies showed that TTP phosphorylation by p38 MAPK/MK2 pathway leads to phospho-TTP binding with 14-3-3, thus preventing the TTP interaction with PP2A (38,48,49). The results of Hitti et al. (50) and Sun et al. (38) suggested that TTP dephosphorylation by PP2A leads to unphosphorylated TTP binding to the AU-rich element of TNF-α mRNA, which promotes TNF-α mRNA degradation. Meanwhile, dephosphorylated form of TTP is subjected to proteasome degradation (51). Our data consistently showed that HQ-induced PP2Acα upregulation leads to TTP downregulation, whereas MG132 restores TTP protein expression in HQ-treated cells. Moreover, the findings that TNF-α mRNA stability markedly increases along with PP2A-mediated TTP degradation in HQ-treated U937 cells are in line with previous studies showing the role of TTP in TNF-α mRNA degradation (38,50). Brooks et al. (45) suggested that TTP regulates both TTP and TNF-α mRNA stability via targeting 3′-untranslated region of these mRNA. Consequently, LPS treatment induces increased TTP and TNF-α transcription in THP-1 cells, but TTP limits TTP and TNF-α protein expression in LPS-treated THP-1 cells (45). The finding that okadaic acid (PP2A inhibitor) treatment further increases LPS-induced TTP upregulation in MH-S cells, a mouse macrophage cell line (38) reflects that unphosphorylated TTP may elicit TTP mRNA decay and TNF-α mRNA decay. Because TTP dephosphorylation simultaneously evokes TNF-α mRNA decay and proteasome degradation of TTP (50,51), it is evident that both constitute TTP expression and the TTP effect on its own mRNA stability are required for homeostatic TTP and TNF-α expression in unstimulated cells. Our data reveal that HQ treatment does not increase the TTP mRNA level. Thus, it is conceivable that PP2A-mediated TTP degradation should irreversibly deregulate TTP expression, leading to TNF-α upregulation and TTP downregulation in HQ-treated cells. However, the residual TTP expression is also
noted in HQ-treated cells. Noticeably, phosphorylation of TTP by p38 MAPK/MK2 has been proven to stabilize TTP protein and increase TNF-α mRNA stability (38,48,49). Given that HQ elicits p38 MAPK activation in U937 cells, the contribution of p38 MAPK-mediated TTP phosphorylation to TNF-α mRNA stability could not be excluded in HQ-treated U937 cells.

Fig. 4. Analysis of HQ-responsive element in the promoter region of PP2Ac gene. (A) Schematic drawings showing truncated PP2Ac promoter fragments. Numbers indicated the distance in base pairs from the translational start site. (Top of left panel) Nucleotide sequences at positions –260 to –231 of PP2Ac promoter showing CREB, c-Jun and ATF-2 binding sites. (Bottom of left panel) Putative CREB, c-Jun and ATF-2 sites on PP2Ac promoter region. Mutated CREB/c-Jun/ATF-2 site was indicated by underline. (Right panel) Effect of HQ treatment on luciferase activity of the PP2Ac promoter constructs. After transfection with indicated plasmids for 24 h, transfected cells were treated with 10 µM HQ for 24 h and then harvested for measuring luciferase activity (mean ± SD, *P < 0.05). (B) Effect of HQ treatment on the levels of phospho-c-Jun, phospho-CREB and phospho-ATF-2. U937 cells were treated with indicated HQ concentrations for 24 h. (C) Effect of SB202190 on the levels of phospho-c-Jun, phospho-CREB and phospho-ATF-2 in HQ-treated cells. U937 cells were pretreated with 100 nM SB202190 for 1 h and then incubated with 10 µM HQ for 24 h. (D) Effect of CREB siRNA, c-Jun siRNA and ATF-2 siRNA on HQ-induced PP2Ac and TNF-α upregulation. U937 cells were transfected with 100 nM control siRNA, CREB siRNA, c-Jun siRNA or ATF-2 siRNA, respectively. After 24 h posttransfection, the cells were treated with 10 µM HQ for 24 h. (E) Effect of CREB siRNA, c-Jun siRNA and c-Fos siRNA on HQ-elicited increase in luciferase activity of pGL-PP2Ac-1022. After cotransfection with pGL-PP2Ac-1022 and 100 nM siRNA (control siRNA, CREB siRNA, c-Jun siRNA or ATF-2 siRNA) for 24 h, U937 cells were treated with 10 µM HQ for 24 h. Cross-linked chromatin from HQ-treated cells was immunoprecipitated with CREB1, c-Jun or ATF-2 antibodies and analyzed by PCR with specific primers for the region from –394 to –83 of PP2Ac promoter. Input, non-immunoprecipitated cross-linked chromatin.
Our data reveal that okadaic acid or TTP overexpression attenuates HQ-induced TNF-α upregulation and restores the viability of HQ-treated cells (Figures 3 and 5), emphasizing the notion that HQ-induced TNF-α upregulation is closely related to HQ-induced death of U937 cells. Overexpression of PP2Ac upregulates TNF-α expression, but does not markedly reduce the viability of U937 cells (Figure 3H). However, HQ treatment induces death of pcDNA-PP2Ac-transfected cells, reflecting that suppression of NFκB pathway plays a crucial role in the cytotoxicity of HQ. Previous studies show that the HQ inhibitory activity on TNF-α-mediated NFκB activation renders primary human CD4⁺ T cells, human CD34⁺/19 hematopoietic progenitor cells and human leukemia TF-1 cells susceptible to TNF-α-induced apoptosis (23,24). Collectively, TNF-α upregulation and NFκB suppression constitute the key players evoking TNF-α-mediated cell death in HQ-treated cells. Lee et al. (9) suggested that HQ-induced Akt inactivation inhibits NFκB phosphorylation in macrophage-like RAW264.7 cells. The finding that HQ treatment reduced the level of phospho-Akt in U937 cells (data not shown) implies that

Fig. 5. HQ-induced PP2Ac upregulation elicited TTP degradation. (A) Effect of HQ on TTP protein expression. U937 cells were treated with indicated HQ concentrations for 24h. (B) Effect of MG132 (proteasome inhibitor) on HQ-induced TTP downregulation. Cells were pretreated with 1 μM MG132 for 1h and then incubated with 10 μM HQ for 24h. (C) Effect of okadaic acid and PP2Ac overexpression on TTP expression in HQ-treated cells. (Left panel) U937 cells were pretreated with 10nM okadaic acid (OA) for 1h and then incubated with 10 μM HQ for 24h. (Right panel) After 24h posttransfection, control vector- and pcDNA-PP2Ac-transfected cells were harvested for western blot analyses. (D) Overexpression of TTP attenuated HQ-induced TNF-α upregulation. After transfection with an empty vector or pCMV-TTP-HA for 24h, transfected cells were treated with 10 μM HQ for 24h. (E) Overexpression of TTP rescued the viability of HQ-treated cells. After transfection with an empty vector or pCMV-TTP-HA for 24h, transfected cells were treated with 10 μM HQ for 24h. Cell viability was determined using MTT. The values represent averages of three independent experiments with triplicate measurement (mean ± SD, *P < 0.05). (F) Effect of HQ treatment on TNF-α mRNA stability. The control vector- and pCMV-TPP-HA-transfected cells were treated with or without 10 μM HQ for 24h and then incubated with 10 μg/ml actinomycin D for the indicated time periods. The level of TNF-α mRNA was analyzed by real-time PCR. The control vector-transfected and pCMV-TTP-HA-transfected cells without actinomycin D treatment were used as control.

Fig. 6. Signaling pathways elucidate TNF-α-mediated death pathway activation in HQ-treated cells. HQ treatment induced p38 MAPK-mediated CREB/c-Jun/ATF-2 activation, leading to PP2Ac upregulation. PP2Ac upregulation further promoted TTP degradation and thus increased TNF-α mRNA stability. Consequently, TNF-α upregulation increased the secretion of soluble TNF-α into the culture medium of HQ-treated cells. Given that HQ suppressed TNF-α-induced NFκB activation, secreted TNF-α elicited apoptosis of U937 cells through caspase-8/mitochondria-mediated death pathway.
HQ-induced NFκB inactivation may be associated with Akt inactivation in U937 cells.

Several lines of evidence indicate that abnormal expression of cytokine such as TNF-α and IL-32 is associated with myelodysplastic syndromes (52–53). Approximately one-third of myelodysplastic syndromes patients ultimately develop acute myeloid leukemia (53). Both fibroblasts and macrophages from bone marrow of myelodysplastic syndromes patients are functionally abnormal, denoting increased apoptosis and high production of both IL-6 and TNF-α (54). Increased TNF-α levels occurring in bone marrow microenvironment after radiation are proved to contribute toward the onset and progression of secondary myelodysplastic syndromes in mice (55). Obviously, an excessive TNF-α production should pathologically affect function of bone marrow-derived cells. Qiu et al. (56) found that myeloid-specific TTP knockout mice are highly and abnormally susceptible to LPS challenge, with elevations of serum TNF-α levels to 110-fold greater to control. Moreover, TNF-α mRNA stability in bone marrow-derived macrophages of the myeloid-specific TTP knockout mice notably increases (56). These observations suggest that TTP plays a crucial role in regulating TNF-α expression in bone marrow-derived cells. Noticeably, myeloid-specific TTP knockout mice show a phenotype of myeloid hyperplasia, suggesting that an excessive production of TNF-α may lead to abnormal differentiation of myeloid cells. On the other hand, HQ administered to mice is proven to block differentiation at the myelocyte stage, resulting in an overproliferation of incompletely differentiated myeloid cells (57).

Taken together, the observation that HQ-induced TTP downregulation elicits TNF-α upregulation should, at least in part, elucidate the role of ROS in the cytotoxicity of HQ in vivo. Recent study also shows that the increment of TNF-α secretion is associated with HQ toxicity in vivo when mice are exposed to HQ (27).

Previous studies suggested that HQ induced ROS generation in lymphocytes, and NAC (ROS scavenger) suppressed HQ cytotoxicity (11,12,15). Thus, the involvement of ROS in the cytotoxicity of HQ was examined in U937 cells. As shown in Supplementary Figure S5A, available at Carcinogenesis Online, HQ induced ROS generation in a concentration- and time-dependent manner. Pretreatment with NAC suppressed ROS generation in HQ-treated U937 cells (data not shown). Moreover, NAC attenuated HQ-induced caspases-8/9/3-3 degradation and restored the viability of HQ-treated cells (Supplementary Figure SSB and C, available at Carcinogenesis Online). Supplementary Figure SSED, available at Carcinogenesis Online, shows that NAC abolished HQ-elicted p38 MAPK phosphorylation, PP2Acct upregulation and TNF-α upregulation. Consistently, pretreatment with NAC abolished HQ-induced increase in TNF-α mRNA stability (Supplementary Figure SSE, available at Carcinogenesis Online). These results suggest that HQ-induced ROS generation is an upstream event for eliciting TNF-α upregulation in HQ-treated U937 cells.

In summary, our data show that HQ treatment induces p38 MAPK-mediated PP2Acct upregulation, thus increasing TTP downregulation and blocking TTP-mediated TNF-α mRNA decay. Consequently, TNF-α upregulation increases the production of sTNF-α in HQ-treated cells. Because HQ suppresses TNF-α-induced NFκB activation, sTNF-α elicits apoptosis of U937 cells through caspase-8/mitochondria-mediated death pathway (Figure 6). The same pathway also applies to elucidate HQ-induced death of HL-60 and Jurkat cells.

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