Full Paper

Clioquinol Increases the Expression of VGF, a Neuropeptide Precursor, Through Induction of c-Fos Expression

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Abstract. Clioquinol was used extensively in the mid-1900s as an amebicide to treat indigestion and diarrhea. It was eventually withdrawn from the market because it was linked to subacute myelo-optic neuropathy (SMON) in Japan. However, the pathogenesis of SMON has not yet been elucidated in detail. As reported previously, we performed a global analysis on human neuroblastoma cells using DNA chips. The global analysis and quantitative PCR demonstrated that the mRNA level of VGF (nonacronymic), the precursor of neuropeptides involved in pain reactions, was significantly increased when SH-SY5Y and IMR-32 neuroblastoma cells were treated with clioquinol. Promoter analyses in SH-SY5Y cells revealed that a region responsive to clioquinol exists between –1381 and –1349 of the human *VGF* gene, which contains an activator protein (AP)-1 site-like sequence. The introduction of mutations at this site significantly reduced clioquinol-induced transcriptional activation. Clioquinol induced the expression of the AP-1 family transcription factors, c-Jun and c-Fos. Electrophoresis mobility shift assays demonstrated that c-Jun and c-Fos could bind to the AP-1 site at –1374/–1368 in SH-SY5Y cells treated with clioquinol. RNA interference against c-Fos significantly suppressed clioquinol-induced VGF mRNA expression. These results suggest that the clioquinol-induced expression of c-Fos mediates the induction of VGF expression.

Keywords: clioquinol, subacute myelo-optic neuropathy (SMON), VGF, AP-1, c-Fos

Introduction

Clioquinol (5-chloro-7-endo-8-quinolinol), a Cu²⁺/Zn²⁺/Fe²⁺ chelator/ionophore, was used extensively as an amebicide to treat indigestion and diarrhea in the mid-1900s. It was withdrawn from the market because its use was epidemiologically linked to the incidence of subacute myelo-optic neuropathy (SMON) in Japan (1). SMON is characterized by the subacute onset of sensory and motor disturbances in the lower extremities and visual impairment preceded by abdominal symptoms (2, 3). Although pathological studies demonstrated axonopathy of the spinal cord and optic nerves (4), the underlying mechanisms of clioquinol toxicity have yet to be elucidated. Clioquinol was found to suppress nerve

growth factor-induced Trk autophosphorylation and neurite outgrowth in PC12 cells (5). When injected into young rats, it attenuated long-term potentiation in the dentate gyrus (6). Another study demonstrated that the cytotoxicity of clioquinol was mediated by oxidative stress (7), inhibition of the 20S proteasome (8), or relocation of cytoplasmic X-linked inhibitor of apoptosis protein to the nucleus (9).

To clarify the molecular mechanisms underlying clioquinol-induced neurotoxicity, we performed a global analysis on human neuroblastoma cells using DNA chips and demonstrated that clioquinol induced DNA double-strand breaks, leading to the activation of ATM and downstream p53 signaling. This pathway may at least partly contribute to clioquinol-induced neurotoxicity (10).

A local injection of clioquinol induced mechanical hyperalgesia and cold allodynia via the activation of TRPA1 in mice (11). In the global analysis, we found that the expression of VGF, a neuropeptide precursor

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involved in pain reactions, was increased by clioquinol. This led us to undertake the investigation of the molecular basis of clioquinol-induced expression of VGF. We report here that the clioquinol-induced expression of VGF was mediated by the induction of c-Fos, a member of the activator protein (AP)-1 transcription factors.

Materials and Methods

Materials

Clioquinol was purchased from Merck (Darmstadt, Germany). Antibodies against c-Jun (60A8) and c-Fos (9F6) were purchased from Cell Signaling Technology (Danvers, MA, USA). The antibody against β -actin (AC-15) was obtained from Sigma (St. Louis, MO, USA). [γ - 32 P]-ATP was purchased from MP Biomedicals (Solon, OH, USA).

Cell culture

Human SH-SY5Y cells, purchased from the European Collection of Cell Cultures, were cultured in Ham's F12: Eagle's medium with Earle's salts (1:1) supplemented with non-essential amino acids and 15% fetal bovine serum (FBS). IMR-32 cells from the JCRB Cell Bank were cultured in Eagle's medium with Earle's salts supplemented with non-essential amino acids and 10% FBS.

Quantitative PCR

Quantitative PCR was performed as described previously (10). Gene expression was quantified using standard curves that were generated using serially diluted plasmid reference samples and normalized to the expression level of hypoxanthine phosphoribosyltransferase (HPRT). The specificity of the PCR products was confirmed by gel electrophoresis and dissociation curve analysis. The sequences of primers for the VGF detection were as follows: 5'-GGAAGTGCAGATTTCAGTCCA-3' for sense, 5'-CGGGCTCTCCAGATTCATC-3' for antisense.

Reporter constructs and luciferase assay

Human genomic DNA was isolated from SH-SY5Y cells with a PUREGENE DNA Isolation Kit (Gentra SYSTEMS, Minneapolis, MN, USA). The 5'-flanking and non-coding regions of the human *VGF* gene were amplified by PCR using KOD -Plus- (TOYOBO, Osaka) and cloned into the NanoLuc vector pNL1.2 (Promega, Madison, WI, USA). The 2.3-kb 5'-flanking and non-coding regions (12) were cloned into the EcoRV/HindIII site of pNL1.2. A series of 5'-deletion constructs were made by cleavage with restriction enzymes or amplification by PCR. All constructs were subjected to sequencing

analyses to verify the orientation and fidelity of the insert. NanoLuc plasmids (0.4 μ g/well) and the firefly luciferase vector pGL4.23 (0.1 μ g/well; Promega) were co-transfected into SH-SY5Y cells with TransIT-LT1 Reagent (Mirus, Madison, WI, USA). These cells were cultured for 24 h, and then stimulated with 20 μ M clioquinol for 24 h. This concentration of clioquinol was previously shown to induce significant cytotoxicity (10). NanoLuc activity in cell lysates was determined and normalized with firefly luciferase activity.

Electrophoresis mobility shift assay (EMSA)

EMSA was performed as described previously (13). A double-stranded probe containing the AP-1 site at -1374/-1368 of the *VGF* gene was prepared by annealing complementary synthetic oligonucleotides. The sense sequence was 5'-TCTGGCGTGTGTCATTGTCAT-3'. The sequence of the mutated probe was 5'-TCTGGC GTGTtgcATTGTCAT-3'. The probe was labeled at the 5'-end with [γ - 32 P]-ATP and T4 polynucleotide kinase. Nuclear extracts of SH-SY5Y cells were prepared as described previously (14). The nuclear extracts and labeled probe were incubated at 25°C for 30 min, resolved in a 4% polyacrylamide gel, and analyzed by a Fujix BAS 5000 Bio-imaging Analyzer (Fuji Film, Tokyo).

Gene silencing of c-Jun or c-Fos

Control or Validated Stealth siRNAs against c-Jun (JUNVHS40918) or c-Fos (FOSVHS41046) were purchased from Life Technologies Japan, Tokyo. Using Lipofectamine RNAiMAX reagent (Life Technologies Japan), siRNAs were transfected to SH-SY5Y cells by the reverse transfection method at a final concentration of 50 nM. These cells were cultured for 24 h and then stimulated with 20 μ M clioquinol for 24 h. The efficiency of siRNA was verified by western blot analyses as described previously (10). Quantitative PCR was performed as described above.

Statistical analyses

Values were expressed as the mean $\pm$ S.E. Statistical analysis was performed by Student's *t*-test. A one-way ANOVA followed by Bonferroni's *t*-test was applied to multiple treatment groups.

Results

Clioquinol increased VGF mRNA levels

A global analysis with DNA chips was performed using SH-SY5Y cells grown in the presence or absence of 50 μ M clioquinol for 24 h, as described previously (10). Among the 2429 genes up-regulated by clioquinol

Fig. 1. Clioquinol increased the expression of VGF mRNA. Cells were grown in the presence or absence of 50 μ M clioquinol for 24 h. Transcription levels were measured by quantitative PCR and normalized to the level of hypoxanthine phosphoribosyltransferase (HPRT) mRNA. Levels were expressed as “fold of DMSO”. Open bars, DMSO. Closed bars, clioquinol. * $P < 0.05$ vs. DMSO (N = 3).

(GEO database accession code: GSE32173), we focused on neuron-specific genes. The expression of VGF, a neuropeptide precursor, was up-regulated when cells were treated with clioquinol. The up-regulation of mRNA expression was verified by quantitative PCR in SH-SY5Y and IMR-32 cells (Fig. 1).

The AP-1 site-like sequence at -1374/-1368 was essential for transcriptional activation of the human VGF promoter by clioquinol

We isolated the promoter region of the human *VGF* gene and examined its transcriptional activity in SH-SY5Y cells. Approximately 2.3 kb of the 5'-flanking region and 5'-noncoding region were subcloned into a luciferase vector and deletion mutants were constructed. As demonstrated in Fig. 2A, clioquinol-induced transcriptional activity of the *VGF* promoter was markedly decreased by the deletion to -1260, but not to -1609. Therefore, the region between -1609 and -1260 was analyzed in more detail. Clioquinol-induced transcriptional activity of the *VGF* promoter was dramatically decreased by deletion up to -1349, but not to -1381 (Fig. 2B). Since an AP-1 site-like sequence was found at -1374/-1368, a mutation was introduced to this region. The introduction of a mutation at the AP-1 site-like sequence significantly suppressed clioquinol-induced transcriptional activity (Fig. 2C), suggesting the essential role of the AP-1 site at -1374/-1368.

Clioquinol increased the expression of c-Jun and c-Fos

A global analysis with DNA chips indicated that clioquinol increased the expression of mRNAs for c-Jun and c-Fos, members of the AP-1 transcription factors

Fig. 2. Clioquinol-induced transcriptional activation of the *VGF* promoter was dependent on the AP-1 site. A: The clioquinol-responsive element existed between -1609 and -1260 of the *VGF* promoter. The 2.3-kb 5'-flanking and non-coding regions and a series of 5'-deletion constructs were cloned into pNL1.2. NanoLuc plasmids (0.4 μ g/well) and the firefly luciferase vector (0.1 μ g/well) were co-transfected into SH-SY5Y cells. Cells were cultured for 24 h, and then stimulated with 20 μ M clioquinol for 24 h. B: The clioquinol-responsive element existed between -1381 and -1349 of the *VGF* promoter. C: The introduction of mutations at the AP-1 site-like sequence (5'-TGTGTCA-3' to 5'-TGTtgCA-3') abolished clioquinol-induced transcriptional activation. NanoLuc activity in cell lysates was determined and normalized to firefly luciferase activity, and was denoted as the fold-increase induced by clioquinol. Bars represent means $\pm$ S.E. of 3 experiments. * $P < 0.05$.

(GEO database accession code: GSE32173). Therefore, the induction of c-Jun and c-Fos was verified by western blot analysis. As shown in Fig. 3, stimulation with 50 μ M clioquinol for 24 h increased c-Jun protein levels in both SH-SY5Y and IMR-32 cells. Although c-Fos was not detected in control cells, its expression was markedly induced by clioquinol in both cells.

Clioquinol increased binding of c-Jun and c-Fos to the AP-1 site in the VGF promoter

To verify whether c-Jun and c-Fos bind to the AP-1 site in the *VGF* promoter, EMSA was performed using nuclear extracts obtained from SH-SY5Y cells (Fig. 4). An intense band was observed with a probe containing

the AP-1 site of the *VGF* promoter when cells were stimulated with clioquinol. The band was markedly diminished in the presence of an excess amount of the unlabeled probe. In contrast, the band persisted in the presence of an excess amount of the mutated probe. Preincubation of the nuclear extract with an anti-c-Jun antibody or anti-c-Fos antibody, but not by a control IgG, generated super-shifted bands. These results suggest that c-Jun and c-Fos, the expression and binding of which to the *VGF* promoter was increased by clioquinol, are involved in the inducible expression of VGF.

Gene silencing of c-Fos attenuated the promoter activity of the VGF gene

To examine whether c-Jun and c-Fos are involved in the clioquinol-induced expression of VGF, siRNA against c-Jun or c-Fos was transfected to SH-SY5Y cells. As shown in Fig. 5A, anti-c-Fos siRNA almost completely suppressed the clioquinol-induced expression of c-Fos. Although it did not affect basal expression, anti-c-Jun siRNA suppressed the inducible expression of c-Jun. Quantitative PCR revealed that anti-c-Fos siRNA significantly suppressed the clioquinol-induced expression of VGF mRNA (Fig. 5B). Anti-c-Jun siRNA also slightly suppressed the induction of VGF mRNA. These results suggest that the clioquinol-induced expression of c-Fos mediates the induction of VGF expression.

Fig. 3. Clioquinol increased the expression of c-Jun and c-Fos. Cells were grown in the presence or absence of 50 μ M clioquinol for 24 h, and whole cell lysates were prepared. Western blot analysis was performed as described previously. After the detection of c-Jun (43 kDa) or c-Fos (62 kDa), blots were stripped and β -actin (42 kDa) was detected. The experiment was repeated 3 times and representative results are shown.

Fig. 4. c-Jun and c-Fos bound to the AP-1 site-like sequence of the *VGF* promoter. EMSA detected nuclear proteins that strongly bound to the AP-1 site in the presence of 20 μ M clioquinol. The super-shift of the band in the presence of an anti-c-Jun or anti-c-Fos antibody is depicted. Binding specificity was evaluated with a 100-fold excess of an unlabeled oligonucleotide. Nuclear extracts were preincubated in the presence or absence of an antibody (0.24 μ g). The experiment was repeated 3 times and representative results are shown.

Fig. 5. Gene silencing of c-Fos attenuated the clioquinol-induced expression of VGF mRNA. **A:** The expression of c-Jun and c-Fos in SH-SY5Y cells transfected with anti-c-Jun or anti-c-Fos siRNA. siRNAs were transfected to SH-SY5Y cells at a final concentration of 50 nM. These cells were cultured for 24 h and then stimulated with 20 μ M clioquinol for 24 h. Whole cell lysates (10 μ g) were subjected to western blot analysis. Membranes were stripped and re-hybridized with an anti- β -actin antibody. **B:** The transfection of anti-c-Fos siRNA significantly suppressed VGF mRNA levels. Transcription levels were measured by quantitative PCR and normalized to the level of HPRT mRNA. Levels were expressed as “fold of DMSO”. Bars represent means $\pm$ S.E. (N = 3). * P < 0.05 vs. control siRNA.

Discussion

The main findings of this study are that 1) VGF mRNA levels were significantly increased by the clioquinol treatment; 2) clioquinol-induced transcriptional activation of the *VGF* promoter was dependent on the AP-1 site-like sequence at -1374/-1368; 3) clioquinol induced the expression of the AP-1 transcription factors, c-Jun and c-Fos; 4) clioquinol enhanced the binding of c-Jun and c-Fos to the AP-1 site-like sequence at -1374/-1368 in the *VGF* promoter; and 5) RNA interference against c-Fos significantly suppressed clioquinol-induced VGF mRNA expression. These results suggest that the clioquinol-induced expression of c-Fos mediates the

induction of VGF expression.

SMON is characterized by the subacute onset of sensory and motor disturbances in the lower extremities and visual impairment preceded by abdominal symptoms (2, 3). Although pathological studies demonstrated axonopathy of the spinal cord and optic nerves (4), the underlying mechanisms of clioquinol toxicity have yet to be elucidated. As we described previously, the concentration of clioquinol used in this study (20–50 μ M) is compatible with its plasma level in SMON patients (15, 16).

VGF is a neuropeptide precursor, the gene of which was originally identified to be nerve growth factor (NGF)-responsive (17). Its transcriptional regulation has mainly been focused on induction by NGF; and the proximal promoter region containing the cAMP-response element, CCAAT element, E-box, and G(S)G element was shown to be essential for its expression (18–20). We demonstrated for the first time that the expression of VGF is induced by clioquinol in a manner that is dependent on the distal AP-1 site in the promoter.

The expression of VGF is limited to neurons in the central and peripheral nervous systems and to various endocrine cells. VGF-derived peptides were shown to exhibit various biological activities (21). Among them, an increase in the mRNA and protein levels of VGF was shown in dorsal root ganglia following sciatic nerve transection (22) or in the surrounding area after spinal cord injury (23). Thus, elevations in VGF levels have been implicated in neuropathic pain. Several C-terminal peptides derived from VGF were previously reported to cause pain responses. A peripheral injection of TLQP-21 (residues 557 to 576 of VGF) was shown to increase the pain-related licking response in a mouse model (24). The intrathecal application of TLQP-62 (C-terminal 62 amino acids of VGF) to naive rats was found to cause long-lasting mechanical and cold behavioral allodynia (25). AQEE-30 and LQEQ-19, corresponding to the last 30 and 19 amino acids of VGF, respectively, were also shown to evoke thermal hyperalgesia in rats (26). Thus, the induction of VGF may be involved in clioquinol-induced mechanical hyperalgesia and cold allodynia (11).

We focused on c-Jun and c-Fos among members of the AP-1 transcription factors because their expression levels were markedly increased in SH-SY5Y cells. In RNA interference experiments, the contribution of c-Fos to the induction of VGF expression appeared to be greater than that of c-Jun (Fig. 5B), and this was attributed to differences in the efficiencies of siRNAs (Fig. 5A). Thus, the contribution of c-Jun to the induction of VGF expression cannot be excluded because c-Fos is known to require Jun family proteins for its

functional expression through the formation of heterodimers. The induction levels of c-Jun and c-Fos were much lower in IMR-32 cells than in SH-SY5Y cells (Fig. 3). This coincides with the induction level of VGF mRNA being markedly lower in IMR-32 cells than in SH-SY5Y cells (Fig. 1). The induction of c-Fos by clioquinol was also reported *in vivo*. An i.p. injection of clioquinol to rats and mice was shown to induce c-Fos expression in the hippocampus and telencephalon (27). Further analyses are required on the level of c-Fos in SMON patients administered with clioquinol.

In conclusion, clioquinol, the causative compound of SMON, induced the expression of VGF, the precursor of neuropeptides involved in pain reactions, by inducing c-Fos expression.

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