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SHORT COMMUNICATION

Cytotoxicity Induced by the Combination of Valproic Acid and Tumor Necrosis Factor-α

IMPLICATION FOR VALPROIC ACID-ASSOCIATED HEPATOTOXICITY SYNDROME

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ABSTRACT. A previous study showed that valproic acid (VPA) and tumor necrosis factor-α (TNF-α) exhibit synergistic toxicity (lethality) in Sprague–Dawley and Wistar rats. The present study investigated a possible mechanism for this synergy using an *in vitro* system. Incubation of human U937 cells with 1 mM VPA or with 0.001 ng/mL of TNF-α alone had a negligible effect on cytotoxicity (less than 7%). However, the combination of the two drugs significantly increased the cytotoxicity up to 34%. Chronic treatment of U937 cells with VPA or TNF-α for 48 hr reduced protein kinase C (PKC) activity. Further, the PKC selective inhibitor Gö6976 potentiated VPA-induced cytotoxicity and TNF-α-induced cytotoxicity, whereas the PKC activator phorbol-12-myristate-13-acetate provided a significant protection against the cytotoxicity associated with VPA or TNF-α. These results suggest that the synergism in cytotoxicity exhibited by the combination of VPA and TNF-α may be mediated through attenuation of PKC activity. BIOCHEM PHARMACOL **58**;3:455–459, 1999.

KEY WORDS. VPA; TNF-α; PKC; cytotoxicity; VPA-associated hepatotoxicity syndrome

VPA§ was first introduced in the United States in 1978 as an antiepileptic drug [1]. Since then, rare, but sometimes fatal, hepatotoxicity cases coincident with the use of VPA have been reported [2–5]. Animal models for this syndrome have focused mostly on the effects of VPA and its metabolites on liver metabolism [6, 7]. However, VPA hepatotoxicity is not correlated with the serum concentrations of metabolites of VPA in humans [8] or in animals [9]. Furthermore, a fatty liver, which results from inhibition of fatty acid β-oxidation in mitochondria by several VPA derivatives, does not necessarily lead to the death of hepatocytes [10]. The lethality associated with VPA hepatotoxicity syndrome remains unexplained. Based on literature reports [1, 4, 5, 11–14], we have suggested that there may be a direct link between the consequences of infection and the lethality component of the VPA-associated hepatotoxicity syndrome. In support of this hypothesis, we demonstrated that a single dose of VPA enhanced the lethality of TNF-α in Sprague–Dawley and Wistar rats [15]. However, the mechanism of this enhancement has not been elucidated.

TNF-α is a cytokine produced primarily by macrophages in response to infection and other environmental challenges [16]. Binding of TNF-α to its receptors elicits a wide spectrum of organismal and cellular responses, including fever, shock, tissue injury, anorexia, induction of other cytokines, cell proliferation, and apoptosis [17]. Two TNF-α receptors with molecular masses of 75 kDa (p75) and 55 kDa (p55) have been identified [16]. TNF-α cytotoxicity is induced by the p55 receptor [16]. Upon binding to this type of receptor, TNF-α activates two major signal transduction pathways associated with cytotoxicity via death domain-containing clusters of the receptor, the apoptosis pathway and the c-Jun N-terminal protein kinase (JNK)-involved mitogen-activated protein kinase (MAPK) pathway [18]. It has been shown that TNF-α can transiently stimulate PKC activity within 6 min in U937 cells [19]. PKC may act as a constituent upstream of or parallel to JNK, leading to transcription factor AP-1 activation, in turn mediating induction of other genes (IL-6, IL-8, ICAM-1, and E-selectin, for example) that are important for inflammation [18, 20–22]. It also has been reported that PKC-dependent protein phosphorylation induces resistance to TNF-mediated cytotoxicity and inhibition of PKC-potentiated cytotoxicity of TNF-α [23]. On the other hand, TNF-α causes long-term down-regulation of cytosolic PKC activity in U937 cells [19], and down-regulation or inhibition of PKC promotes apoptosis pathways, including the Bcl-2/$p21^{ras}$-dependent pathway [24, 25] and the ceramide-dependent pathway induced by TNF-α [26–28].

A previous report suggests that a decrease in PKC might be the molecular basis of antimanic effects of VPA [29]. In view of the evidence for the involvement of PKC in

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§ *Abbreviations*: VPA, valproic acid; TNF-α, tumor necrosis factor-α; PKC, protein kinase C; and PMA, phorbol-12-myristate-13-acetate.

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TNF-α-induced cytotoxicity and the effect of VPA on PKC activity, the present study was initiated to investigate whether the synergistic toxicity between VPA and TNF-α observed *in vivo* can also be demonstrated *in vitro*, and whether it is mediated by attenuating PKC activity.

MATERIALS AND METHODS

Culture

The human monocytic leukemia cell line U937 was obtained from the American Type Culture Collection (ATCC code CRL-1593.2) and cultured in RPMI 1640 (GIBCO BRL) with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37° in a 5% CO_2 incubator. Cells were harvested and centrifuged. The cell pellets were then resuspended in fresh medium.

Cytoxicity Assay

VPA- or TNF-α-induced cytotoxicity was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; Sigma) assay [30]. Cells (90 μL) were plated at 1.5×10^4 per well in the presence or absence of PKC modulators. After incubation with various concentrations of VPA (0.01 to 15 mM; Sigma) or TNF-α (0.001 to 1000 ng/mL; Genzyme) at 37° for 48 hr, 10 μL of MTT (5 mg/mL in RPMI 1640 medium; Sigma) was added to each well. Following a further 4-hr incubation at 37°, 100 μL of 0.04 N HCl in isopropanol was added. After the dark blue formazan had been dissolved, the absorbance of each well was measured with a microplate reader using a test wavelength of 570 nm. In the cases where PKC modulators such as the PKC inhibitor Gö6976 (50 nM; Calbiochem) and the PKC activator PMA (20 nM; Calbiochem) were used, cells were first incubated with PKC modulators at 37° for 30 min. Then, various concentrations of VPA or TNF-α were added, and incubation was continued at 37° for 48 hr in the presence of modulators. To assess the cytotoxic interaction between VPA and TNF-α, cells were first incubated with different concentrations of TNF-α for 24 hr and then treated with 1 mM VPA at 37° for another 48 hr in the presence of TNF-α. Control cells were treated with 1 mM VPA or 0.001 ng/mL of TNF-α alone for 72 hr, and then were subjected to the MTT assay.

PKC Activity Assay

U937 cells were treated in 75-cm^2 flasks with different concentrations of VPA or TNF-α for 48 hr. About 20×10^6 cells were harvested. The total amount of cells was calculated according to the correction by [^{3}H]methionine incorporation, and total PKC activity was measured using a PKC kit purchased from GIBCO BRL. In brief, after incubation with VPA or TNF-α, 30 mL of cells was transferred into a tube, washed three times, and resuspended in 10 mL of methionine-deficient RPMI 1640 medium containing 5 μCi of [^{3}H]methionine (NEN Dupont) per tube. Six hours later, the cells were harvested, and the pellets were washed with PBS. Then, following the procedure provided by the PKC kit, the pellets were subjected to cell lysis, partial protein purification, and PKC activity assay. PKC activity was determined by picomoles of ^{32}P incorporation in the substrate of PKC per minute per 10^6 cells.

Data Analysis and Statistics

Results are expressed as means ± SD obtained from three independent experiments. The effects of VPA and TNF-α on PKC activity were evaluated by Student's paired *t*-test for grouped data. Interaction between VPA and TNF-α in inducing cytotoxicity (see Fig. 1) and the effects of PKC modulators on VPA- or TNF-α-induced cytotoxicity (see Fig. 2) were evaluated using logistic regression analysis.

RESULTS AND DISCUSSION

Based on the hypothesis that there may be a link between infection and the development of VPA-associated hepatotoxicity syndrome, we have shown that VPA enhances the lethality of TNF-α in Sprague–Dawley and Wistar rats [15]. In the present study, U937 cells were incubated with different concentrations of VPA or TNF-α at 37° for 48 hr. In both cases, cytotoxicity increased with concentration (data not shown). The IC_{50} for VPA was about 5 mM. Despite having relatively high numbers of TNF-α receptors per cell, U937 cells were resistant to the cytotoxic effect of TNF-α. Cytotoxicity was 40–50% even at very high concentrations of TNF-α (100–1000 ng/mL).

Low concentrations of VPA and TNF-α, which have negligible effects individually on cytotoxicity, were used to evaluate their synergy. The cytotoxicity induced by VPA at 1 mM was 6.5 ± 2.5%, and the cytotoxicity induced by TNF-α at 0.001 ng/mL was 5.5 ± 3.3%. After incubation with 0.001 ng/mL of TNF-α for 24 hr at 37°, followed by 1 mM VPA for another 48 hr, cells exhibited cytotoxicity up to 33.7 ± 2.3% (Fig. 1). Thus, it appears that VPA, within its therapeutic range (0.07 to 1.1 mM), together with TNF-α, synergistically induced cytotoxicity in U937 cells ($P < 0.0001$ by logistic regression analysis).

To investigate the mechanism of this synergistic action, PKC activity was measured in cells after chronic treatment with VPA or TNF-α. PKC activity in U937 cells was determined after exposure to three different concentrations of VPA (0.1, 1, and 3 mM) for 48 hr and was found to decrease in a concentration-dependent fashion; at these three concentrations, the corresponding decreases in PKC activity were 12.7 ± 5.5, 31.7 ± 5.1, and 56.5 ± 19.1% ($P < 0.05$ for 1 and 3 mM as compared with control; $P < 0.05$ for 3 mM as compared with cytotoxicities at 0.1 and 1 mM). Using a similar protocol, it was found that PKC activity also decreased in the presence of TNF-α for 48 hr: at TNF-α concentrations of 0.01, 0.1, and 1 ng/mL, PKC activity decreased by 34.3 ± 28.8, 38.7 ± 13.6, and 54.5 ±

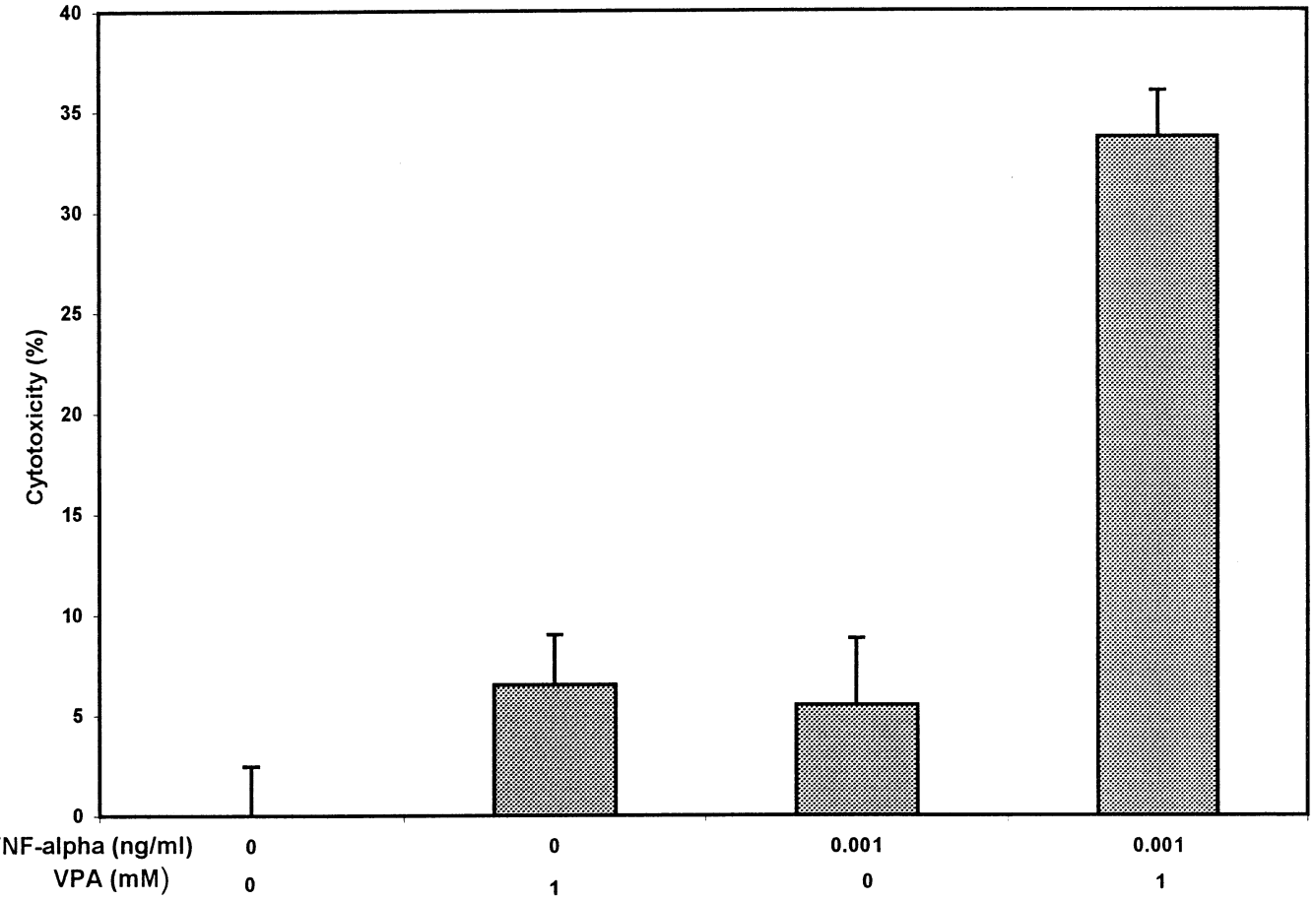

FIG. 1. Cytotoxicity associated with a combination of VPA and TNF-α. U937 cells (1.5×10^4) were preincubated with 0.001 ng/mL of TNF-α at 37° for 24 hr, and then were exposed to 1 mM VPA in the presence of TNF-α for 48 hr. Control cells were treated with no drug or 1 mM VPA or 0.001 ng/mL of TNF-α alone for 72 hr. Results are means ± SD of three independent experiments. $P < 0.0001$ by logistic regression analysis, indicating the existence of an interaction between VPA and TNF-α.

3.5%, respectively ($P < 0.05$ for 0.1 and 1 ng/mL as compared with control). In both instances (VPA and TNF-α), PKC activity was decreased at concentrations that exhibited cytotoxicity.

To further probe the role of PKC in the modulation of cytotoxicity, we evaluated the effects of PKC modulators on VPA- or TNF-α-induced cytotoxicity. U937 cells were incubated with a 50 nM concentration of the PKC inhibitor Gö6976 for 30 min, and then were exposed to different concentrations of VPA or TNF-α for another 48 hr in the presence of the inhibitor. As expected, the percent of cytotoxicity associated with VPA at concentrations between 0.5 and 3 mM increased in the presence of Gö6976 (Fig. 2A). Similarly, in the case of TNF-α at concentrations between 0.001 and 0.1 ng/mL, cytotoxicity increased in the presence of Gö6976 (Fig. 2B). However, analysis by logistic regression indicated that there was not sufficient evidence to demonstrate an interaction between Gö6976 and VPA within the range of 0.5 to 3 mM or interaction between Gö6976 and TNF-α within the range of 0.001 to 0.1 ng/mL. The role of PKC in the cytotoxicity associated with VPA or TNF-α was evaluated further through the use of an activator of PKC, PMA. In the presence of 20 nM PMA, the cytotoxicity associated with VPA or TNF-α decreased significantly (Fig. 2, C and D). Logistic regression analysis provided evidence for the existence of an interaction for VPA as well as TNF-α. These decreases are consistent with other findings that PMA reduces apoptosis induced by TNF-α after chronic incubation with U937 cells [28, 31]. It should be noted that PMA has a dual effect on PKC activity; it produces short-term (within 30 min) activation of PKC followed by long-term reduction in intracellular PKC [32]. In the present study, PMA was introduced 30 min before the addition of VPA or TNF-α.

These results suggest that down-regulation of PKC by VPA and TNF-α is a possible mechanism for the observed synergy between VPA and TNF-α to induce cytotoxicity. Another possible mechanism of synergistic cytotoxicity for VPA and TNF-α might involve depletion of GSH cellular pools. VPA decreases cellular GSH [33], and depletion of cellular GSH in several tumor cell lines renders the cells more susceptible to TNF-mediated oxidative damage [34]. Whether a relationship exists between down-regulation of PKC and depletion of GSH by VPA remains undetermined.

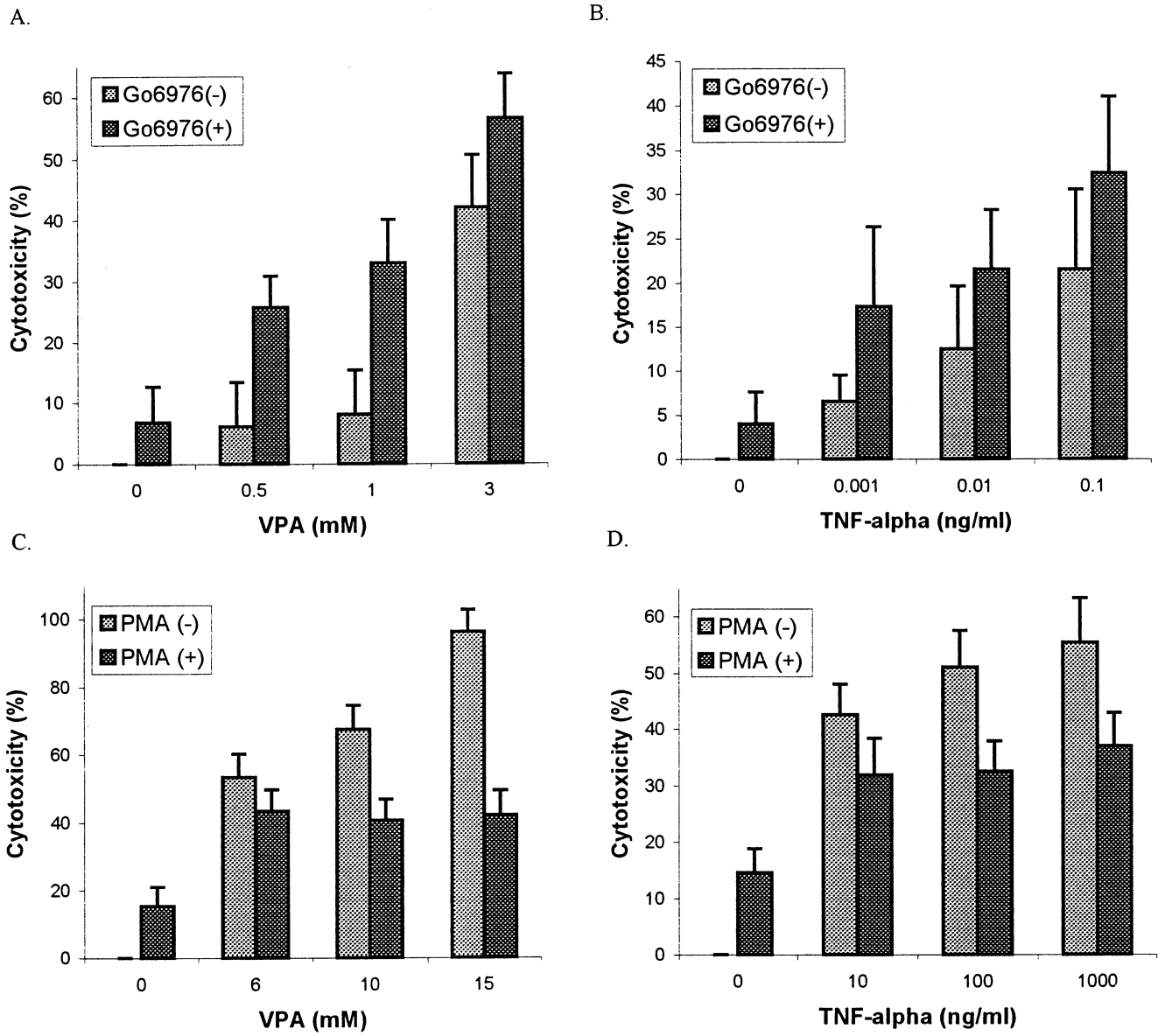

FIG. 2. Effects of PKC modulators, PKC inhibitor Gö6976 (A and B) and PKC activator PMA (C and D), on VPA- or TNF-α-induced cytotoxicity in U937 cells. Cells (1.5×10^4) were first incubated with 50 nM Gö6976 or 20 nM PMA at 37° for 30 min, and then were treated with VPA (A and C) or TNF-α (B and D) for 48 hr. Data represent means ± SD calculated from three separate experiments. Results are expressed as percent of control (without any compound treatment). $P > 0.05$ for the effect of Gö6976 on VPA or TNF-α and $P < 0.0001$ for the effect of PMA on VPA or TNF-α, using logistic regression analysis.

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