Inhibition of Phorbol Ester-Induced Monocytic Differentiation and c-fms Gene Expression by Dexamethasone: Potential Involvement of Arachidonic Acid Metabolites

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The treatment of human U-937 leukemia cells with 12-0-tetradecanoylphorbol-13-acetate (TPA) is associated with induction of monocytic differentiation. However, the signaling pathways responsible for induction of the differentiated monocytic phenotype remain unclear. The present studies demonstrate that dexamethasone blocks TPA-induced U-937 cell growth inhibition, adherence, and alpha-naphthyl acetate esterase staining. The results also demonstrate that dexamethasone inhibits the appearance of c-fms transcripts associated with TPA treatment. Run-on transcription assays demonstrated that the c-fms gene is transcriptionally active in uninduced U-937 cells and that the rate of transcription is unchanged after dexamethasone and/or TPA treatment. These findings indicated that TPA increases c-fms expression by a dexamethasone-sensitive posttranscriptional mechanism. Treatment of U-937 cells with TPA was also associated with stimulation of arachidonic acid metabolism. Furthermore, dexamethasone, an inhibitor of phospholipase A₂ activity, blocked TPA-induced increases in arachidonic acid release. These findings suggested that TPA may regulate certain features of monocytic differentiation, such as c-fms gene expression, through the formation of arachidonic acid metabolites. Indomethacin, an inhibitor of cyclooxygenase, had no detectable effect on c-fms gene expression. However, the cyclooxygenase metabolite, prostaglandin E₂, inhibited the TPA-induced increases in c-fms mRNA levels. Taken together, the results indicate that TPA regulates c-fms gene expression by a dexamethasone-sensitive mechanism and that c-fms mRNA levels are controlled by metabolites of the arachidonic acid pathway.

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TPA (12-0-tetradecanoylphorbol-13-acetate) and other tumor-promoting phorbol diesters induce human myeloid leukemia cell lines to differentiate along the monocytic lineage. This induction of a monocytic phenotype includes inhibition of growth, adherence, an increase in monocytic surface markers, and induction of alpha-naphthyl acetate esterase (NSE) staining. Furthermore, in addition to the down-regulation of c-myc, TPA-induced monocytic differentiation of HL-60 promyelocytic leukemia cells is associated with the appearance of c-fos, c-fms, colony-stimulating factor-1 (CSF-1), platelet-derived growth factor-1 (PDGF-1), platelet-derived growth factor-2 (PDGF-2), and TNF transcripts. The TNF, CSF-1, and c-fms (CSF-1 receptor) genes code for proteins involved in monocyte proliferation and activation. However, the signal transduction pathways responsible for these changes in gene expression during induction of monocytic differentiation by TPA remain unclear.

TPA-induced activation of the calcium- and phospholipid-dependent protein kinase C is presumed to represent a critical step in the induction of monocytic differentiation by phorbol diesters. For example, other structurally unrelated agents, such as bryostatin and teleocidin, also activate protein kinase C and induce monocytic differentiation. Moreover, sphingoid bases and palmitoyl carnitine inhibit activation of protein kinase C as well as phorbol ester-induced monocytic differentiation. However, it is not clear whether the multiple events associated with TPA-induced monocytic differentiation of myeloid leukemia cell lines are a direct consequence of protein kinase C activation. In this regard, TPA may mediate additional biochemical events. Furthermore, agents that activate protein kinase C are capable of stimulating arachidonic acid metabolism via the activation of phospholipase A₂. Indeed, recent findings have suggested that TPA induces TNF gene expression through the arachidonic acid cascade and that TNF mRNA levels are controlled by metabolites of this pathway.

CSF-1 is required for the proliferation and differentiation of cells along the monocytic lineage. Previous work has demonstrated that both the CSF-1 and c-fms genes are expressed during monocytic differentiation. The c-fms gene shares homology with other tyrosine kinase-encoding protooncogenes. Moreover, the c-fms product is identical to the CSF-1 receptor. These findings have suggested that CSF-1 is capable of regulating monocyte functions by an autocrine mechanism. The c-fms transcripts become detectable at 12 to 24 hours during TPA-induced monocytic differentiation, while the CSF-1 and TNF genes are expressed as earlier events. Recent studies have demonstrated that c-fms gene expression is regulated at least in part by a labile protein that stabilizes the c-fms transcript.

The results reported here suggest that arachidonic acid metabolites may be involved in TPA-induced signaling events that result in the appearance of a differentiated monocytic phenotype and the regulation of c-fms gene expression.
MATERIALS AND METHODS

Cell culture. U-937 cells were grown in RPMI 1640 medium (Hazelton Laboratories, Vienna, VA) containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine in 10% heat-inactivated fetal bovine serum (Sigma Chemical Co, St Louis, MO) at a density of 2 × 10⁶/mL in a 5% CO₂ humidified atmosphere at 37°C. Viable cells were determined by trypan blue exclusion. TPA was dissolved in 1% acetone at a stock concentration of 3.3 μmol/L. Dexamethasone, nordihydroguaiaretic acid (NDGA), esculetin (BiOMOL Research Laboratories, Plymouth Meeting, PA), ketoconazole, caffeic acid, 5(S)-hydroxyperoxyecosatetraenoic acid (5-HPETE) (Biomol), indomethacin, and prostaglandin E₂ (PGE₂) were dissolved in 100% ethanol. The final concentration of ethanol did not exceed 0.1%. All chemicals were obtained from Sigma, unless otherwise noted.

Induction of monocytic differentiation. Cell adhesion was determined by calculating the percentage of nonadherent cells compared with total cells (after adherent cells were scraped from plastic culture flasks) after 1 day of treatment. Cytocentrifuge smears of cells were examined for NSE staining after 2 days of treatment (culture flasks) after 1 day of treatment. Autoradiograms were scanned by laser densitometry.

RNA extraction and hybridization. Total cellular RNA was purified by the guanidine isothiocyanate-cesium chloride method, analyzed by gel electrophoresis through 1% agarose-formaldehyde gels and transferred to nitrocellulose filters. Hybridization conditions were as described. Autoradiograms were scanned by laser densitometry.

Probes included the 1.6-kilobase (Kb) ClaI/EcoRI fragment of the human c-myc 3′ exon purified from the pM C41-3 RC plasmid, the 1.8-Kb PstI fragment of the chicken β-actin gene purified from the pAI plasmid, the 4.0-Kb EcoRI fragment of the c-fms gene isolated from the pc-fms 102 plasmid, and the 1.1-Kb PstI fragment of the pE4 plasmid containing tumor necrosis factor (TNF) cDNA.

Run-on transcription assay. Labeled nuclear RNA was prepared and hybridized to plasmid DNA (equivalent to 2 μg of insert) that had been run in a 1% agarose gel and transferred to a nitrocellulose filter. The prehybridization and hybridization conditions were as previously described except that the hybridizations were performed for 72 hours at 55°C and the concentration of RNase in the wash was 500 ng/mL. The 32P-labeled RNA was hybridized to PstI-digested chicken β-actin pAl plasmid, resulting in a 1.8-Kb insert, and EcoRI-digested pc-fms 102 plasmid, resulting in a 4.0-Kb fragment containing the human c-fms cDNA.

Measurement of arachidonic acid release. Arachidonic acid release was monitored as described. Cells in logarithmic growth phase were incubated for 16 hours in 0.1 μCi/mL 5,6,8,9,11,12,14,15-3H [arachidonic acid (87 Ci/mmol; Amersham Corp, Arlington Heights, IL). The cells were then washed in phosphate-buffered saline, resuspended in medium with or without dexamethasone for 30 minutes, then exposed to 8 nmol/L TPA. Release of tritium into cell-free supernatants was then determined by scintillation counting.

Measurement of protein kinase C activity. U-937 cells were suspended in serum-free RPMI 1640 medium and treated with 8 nmol/L TPA for 5 minutes at 37°C. In certain experiments, the cells were pretreated with 1 μmol/L dexamethasone or 50 μmol/L NDGA for 30 minutes. After pelleting and washing in phosphate-buffered saline (Ca++ and Mg++-free), cells were lysed by 10 passages through a 25-gauge needle and reconstituted in buffer A (20 mmol/L Tris-HCl, pH 7.5, 100 μg/mL aprotinin, 0.25 mmol/L leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride). The cytosolic fraction was obtained after microcentrifugation. The particulate fraction was solubilized with buffer A containing 1% Triton X-100 for 15 minutes on ice. Protein kinase C was partially purified by passage through 0.5 mL diethylaminoethyl-cellulose columns and eluted with 80 mmol/L NaCl. Aliquots of column eluate were assayed in the presence of 10 mmol/L MgCl₂, 20 μg of histone H1, 10⁴ cpm [γ-32P]adenosine triphosphate (3,000 Ci/mmol, Amersham; final specific activity, approximately 400 cpm/pmol), and 11.7 mmol/L TPA with or without 8 μg/mL phasitidylserine and 1 mmol/L CaCl₂. After incubation for 10 minutes at 30°C, bound radioactivity was determined by precipitation with 25% trichloroacetic acid and collection onto filters under vacuum. Protein kinase C activity was determined by subtracting the amount of 32P (per milligram protein) incorporation into histone noted in the absence of added phospholipids and calcium from the amount of 32P incorporation in their presence.

RESULTS

The treatment of U-937 cells with 8 nmol/L TPA resulted in the appearance of a differentiated monocytic phenotype. For example, U-937 cell proliferation was completely inhibited by TPA exposure (Fig 1). In contrast, while dexamethasone alone had no detectable effect on proliferation of U-937 cells, this agent partially blocked the growth inhibitory effects of TPA (Fig 1). Dexamethasone also inhibited the effects of TPA on other phenotypic markers. In this regard, 56% of the U-937 cells were adherent after a 24-hour exposure to TPA (Table 1). However, only 9% of these cells adhered to the flask following treatment with both dexamethasone and TPA. Dexamethasone also blocked TPA-induced increases in NSE staining (Table 1). These findings suggested that dexamethasone inhibits multiple phenotypic changes associated with the induction of monocytic differentiation.

Further studies were performed to determine whether dexamethasone also blocks changes in gene expression during TPA-induced monocytic differentiation. We previously demonstrated that c-fms transcripts are induced in HL-60

![Figure 1](https://www.bloodjournal.org) Effects of TPA and dexamethasone (DEX) on U-937 cell growth. Viable cell counts were determined by trypan blue exclusion for untreated U-937 cells (●), cells treated with 8 nmol/L TPA (●), 1 μmol/L dexamethasone (●), or 1 μmol/L dexamethasone for 30 minutes before adding 8 nmol/L TPA (○). Values represent the mean ± SE for three separate experiments.
Table 1. Effects of TPA and Dexamethasone on Monocytic Differentiation in U-937 Cells

<table>
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<tr>
<th></th>
<th>% Adherence*</th>
<th>% NSE Positive†</th>
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<tr>
<td>U-937 cells (untreated)</td>
<td>0</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>TPA (8 nmol/L)</td>
<td>56 ± 11</td>
<td>29 ± 8</td>
</tr>
<tr>
<td>Dexamethasone (1 µmol/L)</td>
<td>12 ± 1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>Dexamethasone (1 µmol/L, 30 min prior to TPA (8 nmol/L))</td>
<td>9 ± 8</td>
<td>11 ± 6</td>
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In each case, data was obtained from three separate experiments.

*Mean ± SE percent adherent cells after 1 day of treatment.
†Mean ± SE percent of strongly positive cells after 2 days of treatment.

cells by 24 hours of TPA exposure. Similar findings were obtained in the present study (Fig 2). In contrast, TNF mRNA was detectable at 3 to 6 hours and was down-regulated by 24 hours of TPA treatment (Fig 2). The level of c-myc transcripts was also down-regulated by 24 hours of exposure to this agent (Fig 2). The effects of dexamethasone on changes in c-fms and c-myc expression were therefore monitored at 24 hours of TPA treatment, while similar studies were performed at 3 hours for changes in TNF mRNA levels. Dexamethasone alone had no detectable effect on c-fms expression (Fig 3). However, this agent completely inhibited the induction of c-fms transcripts by TPA (Fig 3).

Dexamethasone also blocked TPA-induced TNF expression (data not shown), as previously shown in HL-60 cells, but had no effect on the down-regulation of c-myc expression (Fig 3). Moreover, dexamethasone alone or in combination with TPA had no detectable effect on actin mRNA levels (Fig 3). These findings indicated that dexamethasone inhibits pathways involved in the induction of c-fms and TNF expression, but not in the down-regulation of c-myc expression.

To further analyze the effects of dexamethasone on c-fms expression, we measured the rate of c-fms gene transcription in isolated nuclei by the run-on transcription assay procedure. Although c-fms transcripts were undetectable by Northern analysis (Fig 2), the c-fms gene was transcriptionally active in uninduced U-937 cells (Fig 4). Furthermore, there was no detectable increase in c-fms gene transcription associated with the appearance of c-fms transcripts after 24 hours of TPA treatment. These findings suggested that c-fms expression is controlled posttranscriptionally in these cells. Treatment with dexamethasone alone or in combination with TPA also had no effect on rates of c-fms transcription (Fig 4). These results further implied that dexamethasone inhibits the posttranscriptional regulation of c-fms expression by TPA.

The finding that dexamethasone, an inhibitor of phospholipid...
pase A1 activity,27 blocked certain features associated with TPA-induced monocytic differentiation prompted further studies monitoring the effects of this agent on arachidonic acid release. In this context, phorbol esters that activate protein kinase C also stimulate arachidonic acid metabolism.10,28 Similar findings were obtained in TPA-treated U-937 cells. For example, TPA increased tritium release from [3H]arachidonic acid-labeled cells by approximately 40% over that in uninduced cells (Fig 5A). In contrast, while dexamethasone alone had no detectable effect on arachidonic acid release, this agent blocked the increases induced by TPA (Fig 5B). These findings suggested that the effects of dexamethasone may be, at least in part, related to inhibition of arachidonic acid release.

After its release from membrane-bound phospholipids, arachidonic acid is further metabolized to leukotrienes by 5-lipoxygenase and to prostaglandins by cyclooxygenase.29 Consequently, further experiments were performed in the presence of NDGA, an inhibitor of 5-lipoxygenase.30 NDGA (50 μmol/L) alone had no detectable effect on levels of c-fms mRNA (Fig 6). However, treatment with this 5-lipoxygenase inhibitor and TPA resulted in nearly complete inhibition of TPA-induced c-fms expression (Fig 6). NDGA also blocked the induction of TNF expression by TPA, but had no detectable effect on actin mRNA levels (Fig 6). However, given the potential of this concentration of NDGA to affect other pathways,31 additional inhibitors of 5-lipoxygenase were tested.32–34 Neither 10 to 100 μmol/L caffeic acid, 20 to
DEXAMETHASONE INHIBITS c-fms INDUCTION

100 μmol/L ketoconazole, nor 5 to 50 μmol/L esculetin inhibited the induction of c-fms expression observed after 24 hours of TPA treatment (data not shown). Furthermore, treatment of U-937 cells for 24 hours with 10 or 100 nmol/L 5-HPETE, the primary metabolite of arachidonic acid via the 5-lipoxygenase pathway, failed to induce c-fms mRNA (data not shown). These findings suggested that the formation of eicosanoids other than those responsible for the activity of 5-lipoxygenase may be involved in the regulation of c-fms gene expression.

Studies were also performed with indomethacin, an inhibitor of cyclooxygenase, \(^{13}\) and PGE, a prostaglandin metabolite of the cyclooxygenase pathway. Treatment of U-937 cells with indomethacin or PGE, alone had no detectable effect on c-fms mRNA levels (Fig 7). Furthermore, indomethacin had no effect on the induction of c-fms expression by TPA (Fig 7). However, exposure of U-937 cells to PGE, was associated with a 53% decrease in levels of c-fms transcripts induced during TPA treatment (Fig 7). These agents had similar effects on TNF expression, although PGE, completely blocked the induction of TNF transcripts by TPA (Fig 7). In contrast, indomethacin and PGE, had little, if any, effect on changes in c-myc expression or actin mRNA levels (Fig 7).

Taken together, these results suggested that the induction of c-fms expression by TPA is mediated through the arachidonic acid cascade. However, the use of dexamethasone and NDGA in these studies may have blocked the increases in c-fms expression by altering TPA-induced activation of protein kinase C. We therefore monitored the effects of these agents on the translocation of protein kinase C activity from the cytosol to membrane fractions. Treatment of U-937 cells with TPA was associated with an increase in membrane-bound protein kinase C activity and a decline in that found in the cytosol (Fig 8). Dexamethasone or NDGA alone had little, if any, effect on the distribution of protein kinase C activity in uninduced U-937 cells (data not shown). Moreover, TPA-induced translocation of protein kinase C activity was similar in the presence of these agents to that obtained with TPA alone (Fig 8). These results suggested that the effects of dexamethasone and NDGA on TPA-induced c-fms expression are not associated with inhibition of protein kinase C.

**Fig 6.** Effects of TPA and NDGA on c-fms, TNF, and actin mRNA levels. U-937 cells were treated with 8 nmol/L TPA, 50 μmol/L NDGA, or 50 μmol/L NDGA for 30 minutes before adding TPA. Total cellular RNA was isolated at 3 and 24 hours for hybridization to the 32P-labeled DNA probes.

**Fig 7.** Effects of TPA, indomethacin (INDO), and PGE, on gene expression in U-937 cells. U-937 cells were treated with 8 nmol/L TPA, 10 μmol/L indomethacin, 1 μmol/L PGE, 10 μmol/L indomethacin for 30 minutes before adding TPA, or 1 μmol/L PGE, for 30 minutes before adding TPA. Total cellular RNA (20 μg) was isolated at 3 and 24 hours for hybridization to the indicated 32P-labeled probes.
The block in differentiation associated with leukemogenesis is associated with expression of the monocytic phenotype. Phorbol esters, such as TPA, that activate protein kinase C also induce differentiation. However, the induction of monocytic differentiation is associated with changes in both phenotypic characteristics and gene expression. While these findings have demonstrated that the block in differentiation associated with leukemogenesis is reversible, little is known about the intracellular signaling mechanisms responsible for inducing the differentiated phenotype. Other studies have demonstrated that TPA-induced monocytic differentiation is associated with the down-regulation of c-myc expression and appearance of c-fos transcripts. The decrease in c-myc mRNA levels may be sufficient to induce monocytic differentiation. In contrast, the induction of c-fos expression is neither sufficient nor required for induction of the differentiated monocytic phenotype. Other studies have demonstrated that TPA-induced monocytic differentiation is associated with expression of the TNF, c-fms, CSF-1, PDGF-1, and PDGF-2 genes. These genes code for products involved in the regulation and function of cells differentiated along the monocytic lineage. While certain insights are available regarding the down-regulation of c-myc transcripts during monocytic differentiation, signaling events involved in the regulation of genes coding for monocyte membrane or secreted products remain poorly understood.

The induction of TNF transcripts is one of the earliest detectable changes in gene expression associated with TPA-induced monocytic differentiation. Treatment of certain myeloid cells with TPA is associated with increases in arachidonic acid release. Furthermore, inhibitors of phospholipase A2 block both TPA-induced increases in arachidonic acid metabolism and TNF gene expression. These findings suggested that arachidonic acid metabolites are involved in the regulation of TNF expression during monocytic differentiation.

Previous studies have examined arachidonic acid metabolism during induction of monocytic differentiation. For example, treatment of U-937 cells with TPA is associated with increases in arachidonic acid release. Furthermore, inhibitors of phospholipase A2 block both TPA-induced increases in arachidonic acid metabolism and TNF gene expression. These findings suggested that arachidonic acid metabolites are involved in the regulation of TNF expression during monocytic differentiation. Treatment of U-937 cells with TPA was associated with increases in arachidonic acid release that were comparable to that reported for HL-60 cells. Moreover, dexamethasone inhibited the stimulation of arachidonic acid metabolism by TPA. The hydrolysis of membrane phospholipids by phospholipase A2 is rate-limiting in leukotriene and prostaglandin synthesis. Furthermore, glucocorticoids, such as dexamethasone, have been shown to inhibit phospholipase A2, presumably through the induction of lipocortin. Treatment of U-937 cells with dexamethasone also blocked certain phenotypic characteristics of TPA-induced monocytic differentiation, such as growth inhibition, adherence, and NSE staining. While other work has demonstrated that dexamethasone blocks the appearance of a differentiated murine erythroleukemia cell phenotype, previous studies have not demonstrated an inhibitory effect of this agent on induction of human myeloid differentiation.

The possible role of arachidonic acid metabolites in TPA-induced monocytic differentiation of U-937 cells was examined further in terms of the regulation of c-fms gene expression. The c-fms gene codes for the CSF-1 receptor and is expressed during induction of monocytic, but not granulocytic, differentiation. While c-fms transcripts are also detectable in trophoblasts, expression of this gene appears to be specific for the monocytic lineage in hematopoietic cells. Recent studies have demonstrated that c-fms transcripts are stabilized by a labile protein during induction of monocytic differentiation. The present studies similarly demonstrate posttranscriptional regulation of c-fms mRNA levels in TPA-treated U-937 cells. Furthermore, the results indicate that dexamethasone inhibits this effect of TPA on c-fms expression. While these findings suggested that arachidonic acid metabolites are involved in the posttranscriptional regulation of this gene, dexamethasone may have blocked this effect of TPA by modulating other signaling events. However, there was no detectable inhibitory effect of dexamethasone on TPA-induced activation of protein kinase C in these cells.

Fig 8. Effects of TPA, dexamethasone (DEX), and NDGA on protein kinase C activity. U-937 cells were treated with 8 n mole/L TPA for 5 minutes. Cells were also pretreated with 1 μmole/L dexamethasone or 50 μmole/L NDGA for 30 minutes before adding the TPA. Protein kinase C activity was determined for the cytosolic (□) and membrane (■) fractions. The results are expressed as the mean ± SD for three determinations. CO, untreated cells.
inhibitor of 5-lipoxygenase and leukotriene synthesis, blocked TPA-induced c-fms gene expression. These findings suggested that 5-lipoxygenase metabolites might be involved in the regulation of this gene. However, other known inhibitors of 5-lipoxygenase had no detectable effect on the induction of c-fms transcripts by TPA. Furthermore, there was no detectable effect of adding 5-HPETE on c-fms expression. Taken together with the previous findings in TPA-treated U-937 and HL-60 cells, the present results would suggest that leukotrienes may have little, if any, involvement in regulating c-fms mRNA levels during monocytic differentiation.

The involvement of eicosanoids in the regulation of c-fms gene expression is, however, supported by the findings with PGE$_2$. In the present work, PGE$_2$ partially down-regulated TPA-induced increases in c-fms mRNA levels. Previous studies have also demonstrated that PGE$_2$ inhibits macrophage colony formation. The basis for these effects remains unclear, although PGE$_2$ has been shown to increase cyclic adenosine monophosphate (cAMP) levels in myeloid cells and, thus, certain monocyte functions may be inhibited through cAMP-dependent protein kinase activity. On the basis of the present results, indomethacin, an inhibitor of cyclooxygenase activity, might have been expected to increase levels of c-fms transcripts, unless PGE$_2$ synthesis was already partly inhibited during induction of differentiation. Indeed, the increase in PGE$_2$ secretion from TPA-treated U-937 cells was relatively low in the absence of exogeneous arachidonic acid.

In summary, the finding that dexamethasone blocks TPA-induced increases in c-fms gene expression has suggested involvement of the arachidonic acid cascade. The present results and previous studies have demonstrated that TPA treatment of U-937 cells is associated with increases in phospholipase $A_2$ activity and that this effect is sensitive to dexamethasone. While there is little evidence to support leukotrienes in the regulation of c-fms mRNA levels, the results suggest that the cyclooxygenase metabolite, PGE$_2$, down-regulates expression of this gene. Further studies are required to define which arachidonic acid metabolites play a role in the induction of c-fms expression.

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Inhibition of phorbol ester-induced monocytic differentiation and c-fms gene expression by dexamethasone: potential involvement of arachidonic acid metabolites

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