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Cyclic AMP Inhibition of Tumor Necrosis Factor α Production Induced by Amyloidogenic C-Terminal Peptide of Alzheimer's Amyloid Precursor Protein in Macrophages: Involvement of Multiple Intracellular Pathways and Cyclic AMP Response Element Binding Protein

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ABSTRACT

In the present study, we focused on the molecular events involved in tumor necrosis factor-α (TNF-α) production in response to the amyloidogenic 105-amino acid carboxyl-terminal fragment (CT105) of amyloid precursor protein, a candidate alternative toxic element in Alzheimer's disease pathology, and the mechanisms by which cyclic AMP regulates the relating inflammatory signal cascades. CT105 at nanomolar concentrations strongly activated multiple signaling pathways involving tyrosine kinase-dependent extracellular signal-regulated kinase and p38 mitogen-activated protein kinases. Moreover, phosphatidylinositol 3-kinase/Akt signal was required for excess TNF-α production in human macrophages derived from THP-1 cells. Interferon-γ significantly potentiated the induction of the CT105-mediated signal cascade. These multiple signaling pathways in turn converged, at least in part, at the nuclear transcription factor known as cAMP response element binding protein (CREB), which acts on the TNF-α gene promoter through the cAMP response element. The cell-permeable cAMP analog dibutyryl cAMP partially and almost simultaneously suppressed all of these CT105-induced signaling pathways through excessive CREB phosphorylation, which led to decreased CREB DNA binding activity and reduced TNF-α expression. Furthermore, dibutyryl cAMP decreased the interaction of the p65 nuclear factor-κB with CREB binding protein, thus further inhibiting CT105-mediated TNF-α expression. Collectively, the detailed molecular mechanisms of amyloidogenic CT-induced TNF-α production as negatively regulated by cAMP may advance the possibility of targeted treatment in Alzheimer's disease.

Alzheimer's disease (AD) is the most common cause of progressive dementia and is characterized by neuropathologies, including intracellular neurofibrillary tangles and extracellular neuritic plaques composed principally of β-amyloid (Aβ) (Selkoe, 2001). The pathological role of Aβ-bearing carboxyl terminal peptide (CT), a β-secretase product of amyloid precursor protein (APP), in AD pathology has received renewed attention because of its nuclear localization and its liberation of bioactive CT57/59, a γ-secretase product, which might mediate the genomic events required to lower the cellular apoptotic threshold (DeGiorgio et al., 2000; Cupers et al., 2001; Kinoshita et al., 2002). Importantly, the in vivo expression or the in situ injection of the amyloidogenic CT effectively reconstituted the pathologic characteristics of AD, such as the neuropathological changes, memory deficits and the disruption of synaptic plasticity (Nalbantoglu et al., 1997; Berger-Sweeney et al., 1999; Kim et al., 2001). Furthermore,

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ABBREVIATIONS: AD, Alzheimer's disease; Aβ, β amyloid; CT, carboxyl-terminal peptide; APP, amyloid precursor protein; CT105, the 105 amino acid carboxy-terminal fragment; IFN-γ, interferon-γ; CRE, cAMP response element; CREB, cAMP response element binding protein; TNF-α, tumor necrosis factor-α; MAPK, mitogen-activated protein kinase; PI3-K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; IRF-1, interferon regulatory factor 1; p38, protein kinase of 38 kDa; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase; PD98059, 2′-amino-3′-methoxyflavone; SB202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole HCl; EMSA, electrophoretic mobility shift assay; ELISA, enzyme-linked immunosorbent assay; CBP, cAMP response element binding protein binding protein; dbcAMP, dibutyryl cAMP; TK, tyrosine kinase; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; H89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; NF-κB, nuclear factor-κB.

an increased accumulation of potentially amyloidogenic and neurotoxic CTs was detected in human AD brains, and a robust increase (almost 10-fold) was observed in the steady state level of these amyloidogenic CTs in transgenic mice carrying the APP Swedish mutation versus human AD brains (Cupers et al., 2001; Bodendorf et al., 2002). These findings, together with those of recent studies that reported the failure of Aβ vaccine and upon the protective role of Aβ in defensive compensation (Kontush et al., 2001; Check, 2002), strongly suggest that Aβ-bearing CT is a promising candidate causative molecule in AD pathology.

The contribution to AD pathology made by inflammatory events centered upon the chronic activation of microglial and astrocytic cells is strongly supported by multiple epidemiological studies with nonsteroidal anti-inflammatory drugs, which decrease the probability of AD development (McGeer et al., 1996; Akiyama et al., 2000). Indeed, the serum and the cerebrospinal fluid of patients with AD indicated an acute phase type reaction, and blood macrophages were chronically activated. Increased production and release of IFN-γ from the immune cells of patients with AD and increased IFN-γ in the sera of DS patients were also found (Torre et al., 1995; Solerte et al., 2000). Furthermore, the synergistic effect of IFN-γ on neurotoxic microglia activation in response to Aβ (Meda et al., 1995; Yan et al., 1996) suggests its potential role in the exacerbation of AD pathology. Moreover, the severity and duration of dementia in AD are known to correlate significantly with reduced cAMP and CREB levels (O'Neill et al., 1994; Yamamoto-Sasaki et al., 1999; Yamamoto et al., 2000). These combined findings strongly suggest the importance of IFN-γ and cAMP in the complex modulation of the immunological mechanisms associated with cognitive dysfunction in AD.

Tumor necrosis factor-α (TNF-α) has been implicated as a potent neurotoxic agent elevated in the plasma and in brain tissues containing plaques and/or in the cerebrospinal fluid of patients with AD (Fillit et al., 1991; Lanzrein et al., 1998), and a haplotype of TNF-α is associated with late-onset AD (Collins et al., 2000). In addition, a number of recent studies have shown that Aβ can activate surrounding microglia/astrocytes and that this might contribute to neurotoxicity inducing TNF-α through the activation of the various members of the mitogen-activated protein kinase (MAPK) cascades (McDonald et al., 1998; Combs et al., 2001; Smits et al., 2001). Recently, we have also reported that amyloidogenic CT peptide has a more potent capacity to activate microglia/monocytes, which in turn induce inflammatory mediators, such as TNF-α, at concentrations 2 to 3 log lower than Aβ (Chong et al., 2001; Rah et al., 2001). However, the molecular events underlying human macrophage-dependent TNF-α production in response to CT peptide in combination with IFN-γ and the mechanisms by which cAMP regulates the related inflammatory signal cascades have not been fully resolved.

To this end, in the present study, we examined more thoroughly the details of the mechanisms regulating CT105-mediated TNF-α production at both the cellular and molecular levels in human macrophages derived from THP-1 cells, a model for microglia, the so-called brain macrophages. Experiments were designed to address the following questions: 1) Do MAPKs and PI3-K/Akt cascades play a role in macrophage-dependent TNF-α production during CT105 stimulation? 2) How does IFN-γ regulate CT105-induced TNF-α expression? 3) Is CREB activation involved in CT105 signaling? 4) How does nuclear factor CREB regulate TNF-α expression? 5) How does cAMP regulate the CT105-induced signal transduction pathway? This study would establish a scientific background for the therapeutic potential of elevated cAMP levels for the delay of AD progression.

Materials and Methods

Materials. Anti-ERK1/2 and anti-phospho extracellular regulated kinase 1/2 (ERK 1/2) (Thr202/Tyr204) antibodies, anti-p38 MAPK, and anti-phospho p38 MAPK (Thr180/Tyr182) antibodies, and anti–stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) MAPK and anti-phospho SAPK/JNK (Thr183/Tyr185) MAPK antibodies were bought from New England BioLabs (Beverly, MA). Anti-CREB and anti-phospho CREB (Ser133) antibodies, anti–c-Akt and anti-phospho Akt (Ser473) antibodies, anti–ATF-2 and anti–c-Jun antibodies were also from New England BioLabs. Anti-CBP and anti–IRF-1 were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti–NF-κB p65 and anti–c-Fos antibodies were purchased from Upstate Biotechnology (Lake Placid, NY) and Oncogene (Cambridge, MA), respectively. PD098059, SB202190, sodium orthovanadate, forskolin, and H89 were obtained from Calbiochem (La Jolla, CA). Anti–β-actin antibody, IFN-γ, and other chemicals, including phorbol, were from Sigma (St. Louis. MO). [α-^{32}P]dATP and protein A-Sepharose beads were obtained from Amersham Biosciences (Buckinghamshire, UK) and DNA polymerase Klenow fragment was from Invitrogen (Carlsbad, CA), respectively.

Preparation of CT105 and Aβ peptides. Recombinant CT105 peptide was synthesized and purified as detailed previously (Chong et al., 2001). Previous protein conformational studies using an immunoblot analysis and circular dichroism experiment confirmed that CT105 peptide has the β-sheet structure that can induce self-aggregates similar to Aβ derived from AD brains (Chong, 1997; Kim et al., 2000).

Cell Culture Stimulation. The human monocytic cell line THP-1 was obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum as described previously (Chong et al., 2001). THP-1 has been widely used as a model of human monocytes/macrophages or microglia, not only because of its functional and morphological similarities, including its capacity to perform signal transduction pathways, but also because of functional differences in the distinct species (Ulvestad et al., 1994; McDonald et al., 1998). THP-1 cells (~5 × 10^5 cells/ml) seeded into 96- or 6-well culture plates and incubated with 20 nM phorbol 12-myristate 13-acetate for 48 h became adherent to the plastic culture dish and developed the morphology of differentiated macrophages, most closely resembling microglia, as described previously (Takashiba et al., 1999). After being washed, these adherent cells were incubated with serum-free RPMI media supplemented with glucose (0.5%) for 2 h at 37°C before stimulation. The cells were then stimulated by the addition of CT105 for the indicated times in the presence or absence of INF-γ as described in the legends to the figures. To determine the effects of specific inhibition of CT105 induced responses, cells were pretreated with various kinase specific inhibitors or forskolin for 30 min, or dbcAMP for 10 min before stimulation. In some experiments, cells were preincubated with H89 for 30 min before pretreatment with dbcAMP or forskolin. After stimulation with CT105 in the presence or absence of the specific agents for the indicated periods, the conditioned media were collected for quantification of TNF-α. Total cellular extracts and nuclear fractions were also prepared in parallel for Western blot analysis and electrophoretic mobility shift assay (EMSA) as described below.

Measurement of TNF-α Levels by an Enzyme-Linked Immunosorbent Assay. The concentration of TNF-α in the conditioned media was measured by a human specific sandwich ELISA according to the manufacturer's instructions (BD PharMingen, San Diego, CA). A standard curve using recombinant human TNF-α was set up for ELISA according to the manufacturer's instructions and the levels of secreted TNF-α were expressed as picograms per milliliter per 5×10^5 cells.

EMSA. Nuclear extracts and cytoplasmic fractions were prepared by a modified method of Sun et al. (1994). Protein concentration was determined with bicinchoninic acid using bovine serum albumin as a standard. Oligonucleotide corresponding to the CRE motif (bold) lying at $-115/-93$ relative to the transcription site in the human TNF-α promoter (5′-GTCGACCTCCAG**ATGACGTCA**TGGGT-3′) (Kuprash et al., 1999) was synthesized, annealed, end-labeled with [α-^{32}P]dATP using DNA polymerase Klenow fragment, and used as a probe for EMSA as detailed previously (Taylor et al., 1999). Binding reaction mixtures (10 μl), containing 5 μg of (4 μl) nuclear extract protein, 2 μg of poly(dI-dC), and 40,000 cpm ^{32}P-labeled probe in binding buffer (4 mM HEPES, pH 7.9, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 2% glycerol, and 20 mM NaCl), were incubated for 30 min at room temperature. For supershift assay, the nuclear extract was preincubated with 1 μg of anti-p65 antibodies or anti-CREB antibodies for 30 min. The protein-DNA complexes were separated on 5% nondenaturing polyacrylamide gels in 1 $\times$ Tris-borate/EDTA buffer and were autoradiographed. Autoradiographic signals for activated NF-κB were quantitated by densitometric scanning to determine the intensity of each band.

Western Blotting. Total cellular extracts or nuclear fractions containing equal amounts of protein (~20 μg) were subjected to reducing SDS-PAGE. After electrophoresis and electroblotting, the blots were blocked by incubation with 5% nonfat dry milk in Tris-buffered saline containing 0.15% Tween 20 for 2 h. The blots were then probed at 4°C overnight with primary antibodies, followed by incubation for 1 h with specific secondary antibodies conjugated with horseradish peroxidase. The proteins were visualized using an enhanced chemiluminescence Western blotting detection system (Amersham Biosciences).

Immunoprecipitation. Interaction of CBP with p65 was assessed by immunoprecipitation of cell extracts (200 μg) with 1 to 2 μg of anti-p65 antibody, followed by treatment with 25 μl of protein A-Sepharose beads. After extensive washing and boiling in 1$\times$ SDS sample buffer, the complexes were subjected to Western blotting with anti-CBP antibody.

Data Analysis. Data are expressed as the mean $\pm$ S.E.M. values and analyzed by two-tailed Student's t test for unpaired observations or analysis of variance to study the relationship between the different variables. Values of p are indicated in the figure legends.

Results

CT105 Induces Multiple Signaling Pathways That Are Negatively Regulated by cAMP. To determine more thoroughly the molecular signal transduction mechanisms responsible for excess TNF-α production in response to CT105 in human macrophages derived from THP-1 cells, the potency of CT105 to phosphorylate/activate three MAPKs was initially measured at different time points by Western blotting using phosphorylation-specific antibodies. At sublethal concentrations, CT105 synergistically with IFN-γ significantly induced ERK and the stress pathway kinase, p38 activities, which became detectable at 0.5 h, and the activity status of these kinases showed a similar kinetic, reaching maximal levels at 2 h and gradually declining over the next 6 h without affecting total protein levels (Fig. 1, A and B). ERK2 (p42) and p38 were significantly activated, although the magnitude of p38 phosphorylation was not as great as that observed in ERK, whereas Jun NH2-terminal kinase (JNK) was very faintly activated (Fig. 1C). No significant phosphorylation of ERK1 (p44) could be detected in the CT105-stimulated cells using antibody directed against a peptide representing the diphosphorylated forms of both ERK1 and ERK2. Under the same experimental conditions, the extent of PI3-K/Akt activation was also determined. Basal activity of Akt, also known as protein kinase B, was detected and further increased after a 0.5-h treatment and sustained at least for 6 h of incubation (Fig. 1D). On the other hand, a strong induction of IFN-regulatory factor 1 (IRF-1) was detectable at 2 h and remained at 6 h incubation, whereas β-actin levels were not altered (Fig. 1E). We next investigated effects of cAMP on these CT105-mediated signal pathways involving ERK, p38, and PI3-K/Akt. A synthetic cell-permeable cAMP analog, dbcAMP, markedly reduced but did not abrogate ERK, p38, and PI3-K/Akt activities upon CT105 stimulation with a little effect on each total protein level (Fig. 1, compare A with E and F with J). This partial suppression elicited by dbcAMP was specific because such inhibitory effect on both JNK phosphorylation and IRF-1 induction were not seen under the same experimental conditions. Thus, these time course experiments revealed that CT105 in the presence of IFN-γ could potently activate ERK, p38, and PI3-K/Akt pathways and that cAMP had a capacity to suppress partially but almost simultaneously each of these multiple signal transduction pathways.

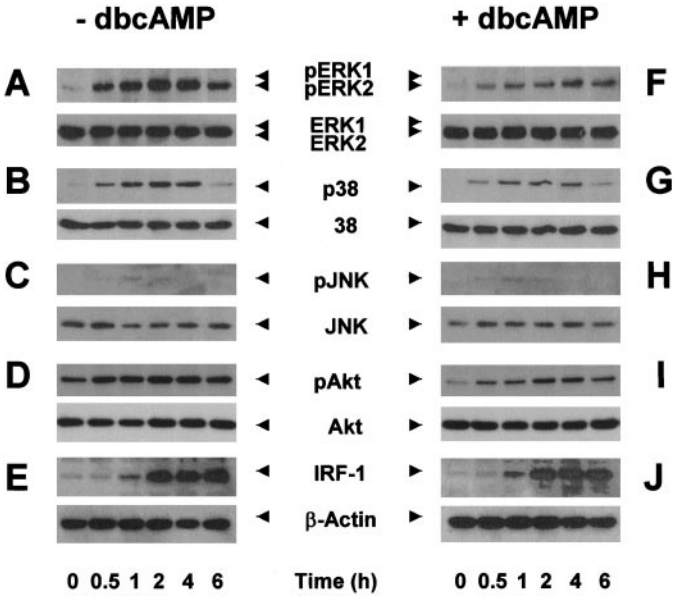

Fig. 1. The kinetics of CT105-induced activation of MAPKs and Akt that are negatively regulated by dbcAMP. Differentiated THP-1 cells were treated without (A–E) or with 0.5 mM dbcAMP (F–J) for 10 min before stimulation with CT105 (50 nM) plus IFN-γ (50 ng/ml) for indicated times in serum-free RPMI 1640 medium supplemented with glucose (0.5%). Equal amounts of total cell lysates were immunoblotted for phosphorylation of ERK1/2, p38, SAPK/JNK, and Akt (A–D, top, and F to I, top, respectively) using antibodies specific for the phosphorylated forms of each kinase as described under *Materials and Methods*. Approximately equal loading of each lane was confirmed using phosphorylation-independent antibodies of each kinase (A–D, bottom, and F to I, bottom, respectively) and also by total β-actin levels (E and J, bottom). Similarly, IRF-1 was also measured as a control for specific induction in response to CT105 plus IFN-γ (E and J, top). All of the experiments were repeated at least four times with similar results.

Effects of Various Kinase Inhibitors on CT105-Stimulated MAPKs and PI3-K/Akt. To further confirm the involvement of CT105-induced ERK, p38, and PI3-K/Akt pathways as major components in mediating the inhibitory action of cAMP, cells were pretreated with specific inhibitors of these signal pathways, and the phosphorylation status of each pathway was determined by Western blot as shown in Fig. 2. The time of treatment was chosen based on our observation that maximal induction and inhibition of endogenous, ERK, p38, and Akt phosphorylation by CT105 and dbcAMP occurred at 2 h, respectively (Fig. 1). Cells stimulated with IFN-γ alone showed neither significant phosphorylation of ERK and p38 nor increase of Akt phosphorylation, whereas synergistic effect was seen with IFN-γ during CT105 stimulation. Forskolin, an adenylate cyclase activator, which should increase intracellular cAMP level, was mimicked to dbcAMP, thus leading to a partial suppression of ERK, p38, and Akt activation. Likewise, PD098059, known to selectively block the activity of MAPK kinase kinase, an activator of ERKs, and SB202190, a specific inhibitor of p38 kinase, reduced ERK and p38 phosphorylation, respectively (Fig. 2). A similar effect was obtained with LY294002, an inhibitor of PI3-K and an activator of Akt. PD098059 and LY294002 had minor effects on CT105-induced phosphorylation of PI3-K/Akt and ERK, respectively. However, SB202190 and LY294002 partially inhibited CT105-induced activation of PI3-K/Akt and p38, respectively. On the other hand, genistein, inhibitor of protein tyrosine kinase (TK), an immediate activator of ERK and p38, significantly reduced not only ERK and p38 activation as expected, but also Akt activation in response to CT105. These pharmacological agents tested had no apparent effect on IRF-1 induction. Thus, these pharmacological activation and inhibition studies, using specific inhibitors of each of the signal pathways, indicate that CT105, synergistically with IFN-γ, can potently activate TK-dependent ERK and p38 MAPKs and PI3-K/Akt and that these pathways are of major importance in mediating the inhibitory action of cAMP.

CT105-Stimulated ERK, p38, and PI3-K/Akt Activities Are Responsible for Excess TNF-α Production and Macrophage Activation. To examine the requirement of activation of each pathway in TNF-α production induced by CT105 in combination with IFN-γ, the effects of the specific inhibitors of these signaling pathways were measured by the sensitive TNF-α ELISA as shown in Fig. 3. IFN-γ in the absence of CT105 had little effect on TNF-α production, but its synergistic effects were seen when cells were exposed to CT105. Pharmacological studies revealed that PD098059 or SB202190 only partially blocked CT105-induced TNF-α production. In contrast, cAMP-elevating agents, such as dbcAMP and forskolin, led to complete inhibition. On the other hand, genistein and LY294002 also partially blocked CT105-induced TNF-α production. However, little effect was seen with H7 and H89, known inhibitors of protein kinases C and A, respectively. Thus, the extent of decreased TNF-α level largely corresponded to the inhibition pattern of each of the kinases in response to the pharmacological agents used (Figs. 1 and 2). Moreover, when exposed to CT105 plus INF-γ, cells became activated after 20 h of incubation, changing their morphology with signs of activation and developing many processes that were dramatically suppressed by dbcAMP, whereas each of the other pharmacological agents was less

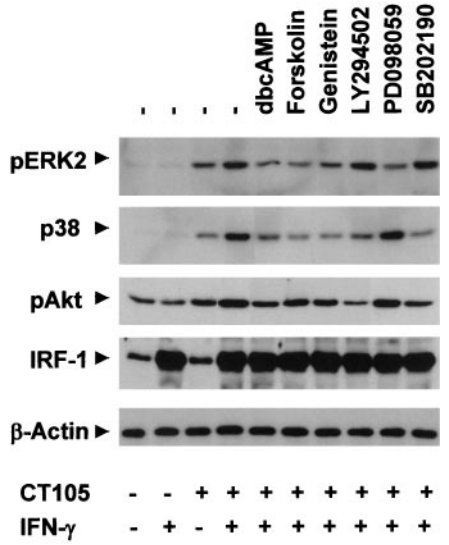

Fig. 2. Effects of various kinase inhibitors on CT105-induced ERK, p38, and PI3-K/Akt signal pathways. Differentiated THP-1 cells were incubated with 10 μM concentrations of specific kinase inhibitors [PD098059 (PD), SB202190 (SB), genistein, LY294002, H7, or H89] for 30 min, and cAMP-elevating agents [forskolin (10 μM) for 30 min or dbcAMP (0.5 mM) for 10 min] as indicated and then stimulated with CT105 (50 nM) plus IFN-γ (50 ng/ml) for 2 h. Effects of these pharmacological agents on CT105-induced activation of ERK, p38, and PI3-K/Akt pathway were determined as described in Fig. 1 and compared with those of dbcAMP and forskolin. Probing for β-actin was performed as for loading control. Shown is a representative gel of four experiments with similar results.

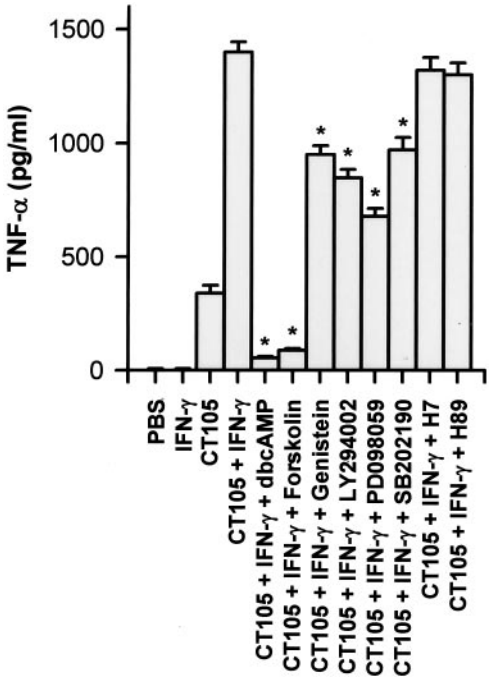

Fig. 3. Effects evoked by various kinase inhibitors on CT105-induced TNF-α production. Differentiated THP-1 cells were preincubated without or with specific kinase inhibitors and cAMP-elevating agents and then stimulated with CT105 plus IFN-γ under conditions identical to those described for Fig. 2. Conditioned media were collected at 20 h and analyzed for CT105-induced TNF-α production by TNF-α ELISA. Differential effects of these various kinase inhibitors were compared with that of dbcAMP. Data are mean ± S.E.M. ($n = 5$). *, $p < 0.001$ compared with TNF-α production in response to CT105 along with IFN-γ in the absence of inhibitor.

effective (data not shown). Thus, these findings confirm that the ERK, p38, and PI3-K/Akt activities are tightly coupled to excess TNF-α production during macrophage activation in response to CT105 plus IFN-γ and that cAMP capable of partially and almost simultaneously reducing each of these signaling pathways might be the most effective macrophage deactivator.

CT105 Induces CREB Phosphorylation and CREB DNA-Binding Activities That Are Differentially Regulated by cAMP. TNF-α gene expression is primarily regulated by the activation of NF-κB and CREB, because the NF-κB- and CREB-binding sites are found in the promoter regions of human TNF-α (Kuprash et al., 1999). Therefore, we next addressed whether CT105 synergistically activates, with IFN-γ, the transcriptional factor CREB and how cAMP influences this CREB-CRE pathway. Time course experiments under the same experimental conditions as those described in Fig. 1 revealed that CT105 in the presence of IFN-γ modestly induced CREB phosphorylation on Ser133, an event required for CREB-mediated transcriptional activation, in a manner similar to that observed with ERK and p38 (Fig. 4A). To further determine whether this CREB activation correlates with increased CREB DNA binding for TNF-α transcription, we performed EMSAs using CRE motif derived from TNF-α gene (Taylor et al., 1999). Consistent with the results of the Western blot, CT105 substantially increased CREB DNA binding activities, which reached maximal level at 2 h and gradually declined over the next 6 h (Fig. 4C). The specificity of CREB DNA binding was confirmed by supershift. The CRE complexes are supershifted by an anti-CREB antibody, whereas other antibodies specific to p65, c-Fos, c-Jun, or ATF-2 elicited little effect (Fig. 4E), implicating CREB as a major DNA binding protein on the CRE site of the TNF-α promoter for CT105-induced TNF-α expression. On the other hand, pretreatment of dbcAMP for 10 min led to more rapid, intense, and sustained increase in CREB phosphorylation than that induced by CT105 itself (Fig. 4B). This rapid and sustained CREB activation elicited by dbcAMP corresponded well with attenuation of the CT105-mediated CREB DNA-binding activities with the maximal inhibitory effect at 2 h (Fig. 4D). This inverse correlation was also confirmed dose-dependently (Fig. 5, A and B). dbcAMP-mediated CREB phosphorylation in the absence of CT105 had little effect on CREB EMSA activity. Interestingly, H89 partially reversed the pharmacological activities of dbcAMP on both CREB phosphorylation and CREB DNA-binding activities (Fig. 5, C and D). These results together suggest that the increased CREB DNA binding activity elicited by CT105 plus IFN-γ is required for up-regulation of TNF-α production and negatively regulated by cAMP via the rapid and excessive CREB phosphorylation.

Effects of Various Kinase Inhibitors on CT105-Stimulated CREB Activation and CREB DNA Binding Activities. The experiments described above indicated that CT105, with IFN-γ, synergistically activates multiple signaling pathways including TK-dependent ERK and p38 MAPK pathway and PI3-K/Akt pathway, which were tightly associated with TNF-α induction and partially diminished by cAMP in differentiated THP-1 cells. To further investigate the association of the CT105-activated signal cascades with the CREB-CRE pathway, cells were pretreated with specific inhibitors of these pathways, and the phosphorylation status of CREB and CREB-binding activities on CRE elements were determined by Western blot and EMSA as shown in Fig. 6. The results showed that suppression of CT-induced CREB

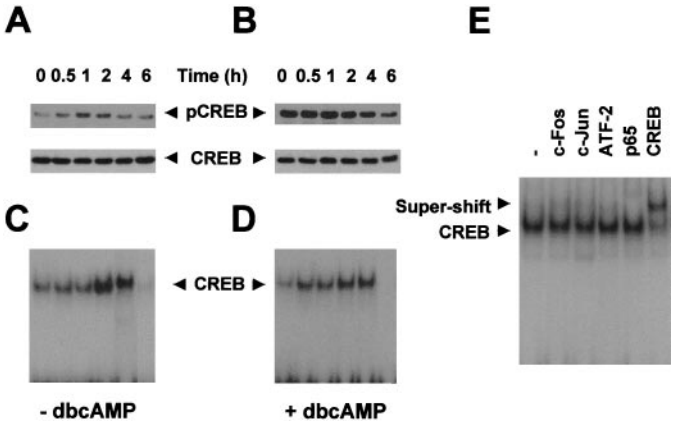

Fig. 4. The kinetics of CT105-induced CREB activation and CREB binding activities on CRE site that are differentially regulated by cAMP. Differentiated THP-1 cells were treated without (A and C) or with (B and D) 0.2 mM dbcAMP for 10 min before stimulation with CT105 (50 nM) plus IFN-γ (50 ng/ml) for indicated times under conditions identical to those described for Fig. 1. In A and B, cell lysates were immunoblotted for phospho-CREB as described in Fig. 1 (A and B, top). Approximately equal loading of each lane was confirmed by using phosphorylation-independent antibodies against CREB (A and B, bottom). In C and D, equivalent amounts of nuclear extracts were assayed for DNA binding activities of CREB with ^{32}P-labeled CREB probe using EMSA as described under *Materials and Methods*. Identification of the CREB by supershift EMSA assay was shown in E. Antibodies specific for c-Fos, c-Jun, ATF-2 p65 of NF-κB, and CREB were preincubated with the nuclear extracts for 30 min before the binding reaction with the CREB specific probe. All of the experiments were repeated at least four times with similar results.

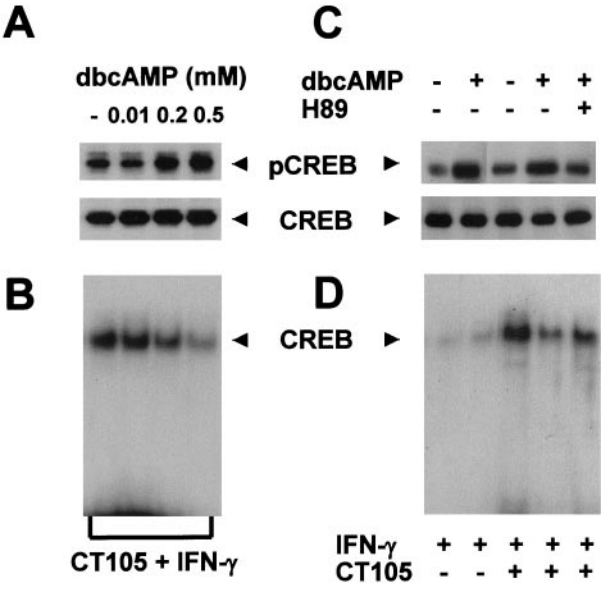

Fig. 5. Dose response of dbcAMP on CREB activation and CREB DNA-binding activities and effect of the PKA inhibitor H89 on cAMP activities. Dose-dependent effect of cAMP on CREB phosphorylation (A) and CREB DNA-binding activities (B) were examined by preincubation for 10 min with increasing concentrations of dbcAMP as indicated before stimulation with CT105 (50 nM) plus IFN-γ for 2 h as described above. THP-1 cells were incubated with H89 (5 μM) for 30 min, followed by treatment with dbcAMP (0.5 mM), and then stimulated with CT105 (50 nM) plus IFN-γ (50 ng/ml) for 2 h. Partial reversal of cAMP-mediated activities by H89 was determined by analysis of CREB phosphorylation (C) and CREB DNA binding activities (D) as described above. All of the experiments were repeated at least three times with similar results.

activation by specific inhibitors of each of CT-activated pathways involving genistein, PD098059, SB203580, or LY294002 (Fig. 6A) led to a significant reduction in CREB binding activity at CRE site as revealed in EMSAs (Fig. 6B). Forskolin mimicked dbcAMP-induced effect. IFN-γ in the absence of CT105 had little effect on both CREB phosphorylation and CREB EMSA activity, but its synergistic effects were seen when cells were exposed to CT105. These results together confirm that CT105 synergistically with IFN-γ can potently activate multiple signal pathways, including TK-dependent ERK, p38, and PI3-K/Akt, which in turn converged into, at least in part, CREB-CRE pathway for initiation of TNF-α expression. In addition, either decreased or excess phosphorylation of CREB by the relating kinase inhibitors or by cAMP-elevating agents could negatively regulate CREB-DNA binding activities required for TNF-α production in response to CT105.

CT105-Induced p65/CBP Interaction was Inhibited by dbcAMP Treatment. Recent studies have shown that cAMP increases the activation of CREB, which then competes with p65 for limiting amounts of CBP, resulting in decreased p65/CBP complexes required for NF-κB activities necessary for TNF-α expression (Falvo et al., 2000). To further define an additional regulatory role of cAMP-CREB system in NF-κB activities through coactivator CBP, cells were stimulated with CT105 in the absence or presence of dbcAMP, and total cell lysates were immunoprecipitated with antibodies to p65 and probed for the presence of CBP. CT105 stimulation along with IFN-γ resulted in the significant increase of p65·CBP complexes, and treatment with dbcAMP did decrease the interaction of p65 with CBP (Fig. 7, bottom). This inhibition pattern corresponded inversely to the level of cAMP-mediated CREB phosphorylation (Fig. 7, top). Thus, in addition to the inhibition of CREB DNA binding, cAMP, by increasing CREB phosphorylation, decreased the interaction of p65 with CBP, which is essential for optimal NF-κB transcriptional activity for TNF-α expression in response to CT105 plus IFN-γ.

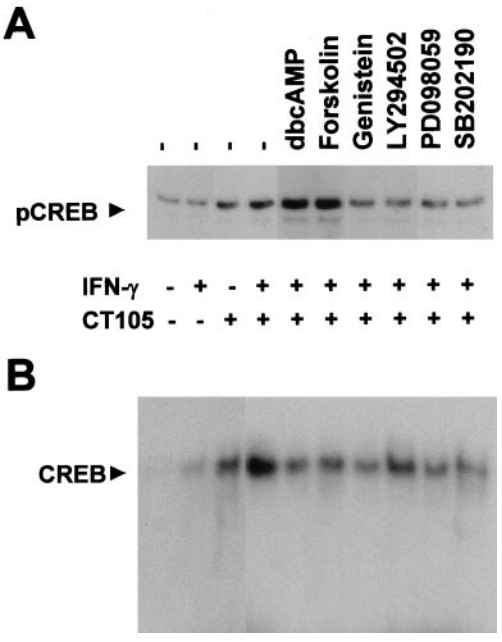

Fig. 6. Effects of various kinase inhibitors on CT105-induced activation of CREB and CREB-DNA binding activities. Under conditions identical to those described for Fig. 2, effects of various kinase inhibitors (10 μM) on CT105-induced activation of CREB (A) and CREB-DNA binding activities (B) were determined and compared with those of dbcAMP (0.5 mM) and forskolin (10 μM) as described in Fig. 5. Shown is a representative gel of triplicate experiments with similar results.

Discussion

The results of our previous studies suggest that the neurotoxic inflammatory response to amyloidogenic CTs may be a mechanism that leads to the chronic neurodegeneration associated with AD (Chong, 1997; Chong et al., 2001; Rah et al., 2001). The present study demonstrates that amyloidogenic CT105 at nanomolar concentrations may act as a potent stimulator of human macrophages, thus inducing multiple signaling pathways involving ERK, p38, and PI3-K/Akt signals, which result in excessive TNF-α production. Second, the underlying mechanism of the synergistic effect of INF-γ involves the enhancement of these multiple signaling pathways. Third, these CT105-mediated multiple signaling pathways in turn converge, at least in part, at the nuclear transcription factor CREB that acts on the TNF-α gene promoter through CRE in human macrophages. Fourth, that cAMP partially and almost simultaneously suppresses all of these CT105-induced signaling pathways through excessive CREB phosphorylation, which leads to a reduction in the CREB DNA binding required for TNF-α production. Finally, cAMP decreased the interaction of the p65 NF-κB with CBP, thus further inhibiting CT105-induced TNF-α expression.

Our most striking finding is that CT105-induced TNF-α production was dependent not on the activation of a single signaling pathway but on multiple pathways, which included TK-dependent ERK and p38, and PI3-K/Akt pathways in human macrophages derived from THP-1 cells. Time-course experiments and pharmacological studies with specific inhibitors of each of the related signaling pathways demonstrated the involvement of two parallel TK-dependent pathways, involving ERK and p38, the two major members of the MAPK family (Whitmarsh et al., 1997), but not JNK, as major components in this CT105-induced inflammatory process. This finding is in line with recent studies, which showed the

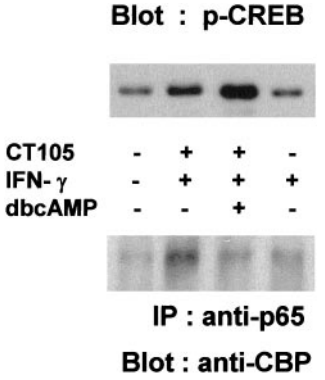

Fig. 7. Inhibitory effect of dbcAMP on CT105-induced p65/CBP interaction. Differentiated THP-1 cells were treated with CT105 (50 nM) along with IFN-γ (50 ng/ml) in the absence or presence of dbcAMP (0.5 mM) for 2 h. Cell extracts were subjected to immunoprecipitation (IP) with anti-p65 antibodies and analyzed by Western blot with anti-CBP (bottom). In parallel, activation level of CREB was shown for comparison (top). Equal protein loading was demonstrated by using an anti-CREB antibody (not shown). All of the experiments were repeated at least three times with similar results.

importance of these MAPK pathways in CT105- or Aβ-mediated inflammatory responses in human macrophages/monocytes (Chong et al., 2001; Smits et al., 2001). Recent studies demonstrated that amyloid fibrils can activate ERK and p38 in microglia in vitro and, further, that activated p38 can be immunolocalized to microglia that are associated with amyloid plaques in AD brains; this supports the in vivo relevance of our study (McDonald et al., 1998; Hensley et al., 1999). Moreover, the present study is the first to report the involvement of the PI3-K/Akt pathway in human macrophage-dependent TNF-α production in response to CT105. In contrast to the function of PI3-K/Akt upstream of the p38 pathway, as recently described (Laprise et al., 2002), our inhibition studies further indicate that there is, in part, cross-talk between PI3-K/Akt and p38, so that blocking PI3-K/Akt inhibits p38 and vice versa, whereas PI3-K/Akt and ERK seem to function independently of each other, as do ERK and p38. Genistein, an inhibitor of TK, an immediate activator of ERK and p38, further substantiated this possible connection because it reduced not only ERK and p38 activation but also PI3-K/Akt activation in response to CT105.

The second novel point of interest is that the CREB and the CRE site are involved in signal transduction cascades of macrophage dependent TNF-α expression elicited by CT105 in the presence of IFN-γ. To our knowledge, the present study provides the first evidence for a modest activation of nuclear transcriptional factor CREB, which transduces TK-dependent ERK and p38, and PI3-K/Akt signals for CT105-mediated TNF-α expression. This evidence includes the observation that CT105 was found to activate CREB and CREB binding at the CRE element. Moreover, when CREB binding activity was induced, TNF-α production increased. Decreases in both CREB activation and binding activity by specific inhibitors of the relating multiple signaling pathways led to a reduction in CT105-mediated TNF-α production. This finding is in part consistent with an earlier study, which showed that Aβ could activate CREB in both ERK- and p38-dependent manners in microglia/monocyte although no direct association between this Aβ-mediated signal cascade and TNF-α production was observed (McDonald et al., 1998). Thus, this study and our recent report (Chong et al., 2002) strongly support the hypothesis that both the NF-κB site and the CRE are required for maximal TNF-α transcription in response to CT105.

The third point of interest concerns the fact that the underlying mechanism of the synergistic effect of IFN-γ on CT105-mediated TNF-α production involves the enhancement of the related multiple signaling pathways and CREB DNA binding activity. These observations thus further support the hypothesis that IFN-γ may act as an inflammatory amplifier that aggravates the neurodegenerative process by priming microglia/macrophages to secrete proinflammatory cytokines (Meda et al., 1995; Jensen et al., 2000). In fact, the immune cells of patients with AD produced IFN-γ, a classic T cell cytokine (Solerte at al., 2000), and increased numbers of T cells have been reported recently in the brains of patients with AD and other neurological disease (Togo et al., 2002). On the other hand, it was reported that the combination of TNF-α and IFN-γ increases Aβ production and inhibits the secretion of soluble APPs (Blasko et al., 1999). These findings together strongly support the in vivo relevance of our study and the complexity of the mechanisms by which inflammatory components can exacerbate the fundamental pathology of AD. Further study is required to clarify whether IFN-γ exerts its synergistic effect on CT105-mediated TNF-α production through signal transducer and activator of transcription-1 pathway, as well described for IFN-γ (Boehm et al., 1997).

The fourth novel point of interest is that contrary to IFN-γ, cAMP negatively regulates CT105-mediated TNF-α induction in human macrophages. These findings highlight the divergent mediatory effects of IFN-γ and cAMP, which act at inflammatory sites that differentially regulate the TNF-α profile for pro- or anti-inflammatory activities of macrophages/microglia. The inhibitory mechanism of cAMP includes the partial and almost simultaneous suppression of the CT105-induced signal pathways implicated via excessive CREB phosphorylation and a consequent reduction in the CREB DNA binding activities required for TNF-α production. This study thus demonstrates for the first time, to our knowledge, that cAMP suppresses CT105-induced TNF-α production by excessive and rapid CREB activation, even though CT105 itself activates CREB. This observation resembles the inhibitory effect of rapid activation of p38 MAPK on TNF-α signaling, despite the fact that TNF-α itself activates p38 (Bowie and O'Neill, 2000), or the relationship between the strong activation of ERK and Akt by IGF-1, which protects against Aβ toxicity, despite the fact that Aβ itself weakly activates ERK and Akt (Wei et al., 2002).

Importantly, our findings further indicate that the molecular mechanisms governing signals for CT105-induced CREB phosphorylation might be distinct from those for cAMP-induced CREB phosphorylation, which typically elicit a rapid and potent response, as shown by the present study and by others (Bonni et al., 1999). In support of this view, a recent study reported that CREB has the capacity to discriminate the signals resulting from cAMP and non-cAMP stimuli, such as mitogen/stress signals at the level of CBP recruitment, and consequently coordinates the inhibition or activation of target gene expression (Mayr et al., 2001). In addition, our finding that cAMP reduced CBP availability for p65, thus decreasing NF-κB/CBP complexes required for CT105-mediated TNF-α expression, provides a link between the cAMP-mediated CREB pathway and the NF-κB pathway, which further supports a recent hypothesis that competition for CBP is another mechanism for the transcriptional regulation of TNF-α (Falvo et al., 2000).

Finally, the severity and duration of dementia in AD are significantly correlated with the early loss of locus ceruleus neurons (Bondareff et al., 1987; German et al., 1992), leading to decreased levels of cortical norepinephrine, which can negatively regulate inflammatory events in brain cells although the up-regulation of intracellular second messenger cAMP, as mediated by β-adrenergic receptors. Furthermore, several studies have reported that a decreased level of adenyl cyclase causes a reduction in cellular cAMP synthesis and impaired CREB activities in the hippocampus and that these are selectively affected in brains of patients with AD (O'Neill et al., 1994; Yamamoto-Sasaki et al., 1999; Yamamoto et al., 2000). In this regard, cell-permeable cAMP analogs that can reduce CT105-induced inflammatory response without affecting cell viability may represent a promising aspect of a combined strategy for current, largely symptomatic treatments, aimed at enhancing the levels of depleted neurotrans-

mitters, particularly acetylcholine. Recent studies reporting the attenuation of Aβ-induced neurotoxicity by the elevation of intracellular cAMP levels (Parvathenani et al., 2000) and the potentiation of Aβ-induced cortical inflammation by noradrenergic depletion (Heneka et al., 2002) further support our findings.

In conclusion, this study, in combination with our recent findings (Chong et al., 2002) suggests that Aβ-bearing CT peptide in the presence of IFN-γ may strongly activate multiple signal cascades comprising TK-dependent parallel ERK and p38 MAPKs and PI3-K/Akt pathways, which in turn converge on the transcription factors CREB and NF-κB to orchestrate excessive TNF-α production and macrophage activation. The activation of these common targets seems to require input from each single pathway so that the transcription of TNF-α in response to amyloidogenic CT peptide is turned on when the overall input reaches a threshold. This explains why blockage of a single pathway did not completely abolish TNF-α expression and why cell-permeable cAMP analogs, which could elicit a partial and almost simultaneous suppression of all of the CT105-induced multiple signaling events that consequently inhibited both the CREB-CRE and the NF-κB pathways, is such a potent inhibitor of TNF-α production and macrophage activation. Overall, the findings presented here provide insights into the detailed molecular basis of TNF-α induction by amyloidogenic CT peptide and inhibitory cAMP action in human monocyte-derived macrophages. Thus, pharmacological agents that elevate cAMP levels offer promise as potential therapeutic agents for counteracting microglia/macrophages-related neuronal damage in the dementing processes of AD.

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