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The Proinflammatory Cytokines Tumor Necrosis Factor-α and Interleukin-1 Stimulate Neuropeptide Gene Transcription and Secretion in Adrenochromaffin Cells via Activation of Extracellularly Regulated Kinase 1/2 and p38 Protein Kinases, and Activator Protein-1 Transcription Factors

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Immune-autonomic interactions are known to occur at the level of the adrenal medulla, and to be important in immune and stress responses, but the molecular signaling pathways through which cytokines actually affect adrenal chromaffin cell function are unknown. Here, we studied the effects of the proinflammatory cytokines, TNF-α and IL-1, on gene transcription and secretion of bioactive neuropeptides, in primary bovine adrenochromaffin cells. TNF-α and IL-1 induced a time- and dose-dependent increase in galanin, vasoactive intestinal polypeptide, and secretogranin II mRNA levels. The two cytokines also stimulated the basal as well as depolarization-provoked release of enkephalin and secretoneurin from chromaffin cells. Stimulatory effects of TNF-α on neuropeptide gene expression and release appeared to be mediated through the type 2 TNF-α receptor, and required activation of ERK 1/2 and p38, but not Janus kinase, MAPKs. In addition, TNF-α increased the binding activity of activator protein-1 (AP-1) and stimulated transcription of a reporter gene containing AP-1-responsive elements in chromaffin cells. The AP-1-responsive reporter gene could also be activated through the ERK pathway. These results suggest that neuropeptide biosynthesis in chromaffin cells is regulated by TNF-α via an ERK-dependent activation of AP-1-responsive gene elements. Either locally produced or systemic cytokines might regulate biosynthesis and release of neuropeptides in chromaffin cells, integrating the adrenal medulla in the physiological response to inflammation. This study describes, for the first time, a signal transduction pathway activated by TNF-α in a major class of neuroendocrine cells that, unlike TNF-α signaling in lymphoid cells, employs ERK and p38 rather than Janus kinase and p38 to transmit gene-regulatory signals to the cell nucleus. (*Molecular Endocrinology* 18: 1721–1739, 2004)

DURING INFLAMMATION, RECIPROCAL interactions occur between the immune and the neuroendocrine systems, leading to activation of integrated physiological circuits. Various signaling messengers including cytokines, hormones, and neuropeptides are used to elicit this coordinated organismic response (1). IL-1 and TNF-α are multifunctional cytokines produced principally by antigen-activated macrophages but also by various other cell types, including keratinocytes, fibroblasts, and cells of the nervous and endocrine systems, in response to inflammation, infection, injury, and other environmental

Abbreviations: AP-1, Activator protein-1; ATF, activating transcription factors; C/EBP, CCAAT enhancer-binding protein; CgA, chromogranin A; CRE, cAMP-responsive element; CREB, CRE-binding protein; ENK, met-enkephalin; GAL, galanin; JNK, c-jun N-terminal kinase; K^+, potassium; mCRE, mutant CRE; mTRE, mutant TRE; NF-κB, nuclear factor κB; NO, nitric oxide; PACAP, pituitary adenylate cyclase-activating polypeptide; PG, prostaglandin; Q-RT-PCR, quantitative RT-PCR; SgII, secretogranin II; SN, secretoneurin; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TNF-R, TNF receptor; TRE, TPA-responsive element; VIP, vasoactive intestinal polypeptide.

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insults (2–5). IL-1 and TNF-α are released from inflammatory foci and reach various peripheral and central targets, both locally and via the bloodstream. These cytokines then initiate a complex cascade of cell-specific reactions that ultimately modulate the inflammatory response and serve to maintain physiological homeostasis (6, 7). IL-1 and TNF-α have similar and broad ranges of physiological actions in regulating local and systemic immune responses. The two cytokines exert their effects through two distinct plasma membrane receptors, TNF receptor 1 and 2 (TNF-R1/TNF-R2) and IL-1 receptor 1 and 2 (1) that trigger signaling cascades involving numerous protein kinases such as the three classes of MAPKs, p42/44 ERK 1 and 2, p54 c-Jun NH_2-terminal kinase (JNK), and p38 MAPK, in a variety of cell types. The involvement of the MAPKs JNK and p38 in the effect of TNF-α on different cell types is now well established (8, 9). Recruitment of these pathways by TNF-α and IL-1 leads to the activation of transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), which interact with target genes to regulate their transcription (10). The role of the ERK 1/2 MAPKs in TNF signaling is, by contrast, much less well defined.

Cytokines released during inflammation can promote a variety of physiological neuroendocrine and behavioral responses involving the central nervous system and the hypothalamo-pituitary-adrenal axis. These, in turn, can modulate the immune system via local or systemic routes involving neuroendocrine pathways and/or the autonomic and peripheral nervous systems (11, 12). For instance, catecholamines and neuropeptides have been shown to profoundly affect immune system function (11). Mice lacking dopamine-β-hydroxylase, which cannot produce noradrenaline and adrenaline, have impaired T cell function and are more susceptible to infection when challenged with pathogens (13), indicating that catecholamines play a physiological role in the regulation of the immune response. Concurrently, several neuropeptides are present in the lymphoid microenvironment where they elicit a broad spectrum of biological actions, including both pro- or antiinflammatory modulation of immune cell function. In this regard, vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP), and enkephalin (ENK), all products of adrenochromaffin cells, have established immunosuppressive effects (14, 15), whereas peripheral CRH (16) and substance P (17) exert immunostimulatory actions.

Prominent reciprocal adrenomedullary-immune system interactions characterize the overall organismic response to inflammatory or immune challenge (18, 19). However, the actions of immunoregulatory cytokines on adrenomedullary chromaffin cell function are not well defined. The rat adrenal medulla exhibits IL-1 immunoreactivity, and levels of the IL-1 protein as well as its mRNA are increased in chromaffin cells in response to injection of lipopolysaccharides (20, 21). IL-1 and TNF-α are able to modulate VIP content in bovine chromaffin cells (22). However, up to now, the molecular mechanisms underlying the action of proinflammatory cytokines in chromaffin cells have not been addressed. Elucidating such a mechanism in primary cultures of chromaffin cells may well provide a basis for understanding the actions of immunoregulatory cytokines in other neuroendocrine cells and in central and peripheral neurons. In the present study, we analyzed the effects of TNF-α and IL-1 on the biosynthesis and release of several of the major neuropeptide/secretory proteins of the adrenal chromaffin cell, including galanin (GAL) (23), secretoneurin (SN), a novel peptide that derives from the processing of secretogranin II (SgII) (24), VIP (25), ENK (26), and chromogranin A (CgA) (27) in cultured bovine adrenochromaffin cells. Experiments were designed to determine first, whether TNF-α and IL-1 affect the quantity and quality of the secretory cocktail of bioactive peptides produced by the adrenal chromaffin cell, and second, whether cytokine-initiated signaling to the nucleus in chromaffin cells is similar to, or fundamentally different from, cytokine signaling in cells of the inflammatory and immune systems.

RESULTS

TNF-α and IL-1α/β Stimulate Neuropeptide Biosynthesis and Secretion in Adrenomedullary Chromaffin Cells

To study the effect of proinflammatory cytokines on neuropeptide gene expression, primary cultures of bovine chromaffin cells were incubated with TNF-α and IL1-α/β, and the mRNA levels of VIP, GAL, SgII, and CgA were measured by Northern blot analysis or quantitative RT-PCR (Q-RT-PCR) (Fig. 1). Both IL-1 and TNF-α (10 nM, 48 h) induced a significant stimulation, ranging from 3- to 70-fold, of SgII, VIP, and GAL mRNA levels in bovine chromaffin cells, TNF-α being in general much more potent than IL-1 (Fig. 1, A–C). In contrast, neither TNF-α nor IL-1 exerted any effect on CgA mRNA levels in bovine chromaffin cells under the same conditions (Fig. 1D). To examine whether the stimulatory effect of the proinflammatory cytokines on neuropeptide gene expression is associated with an action of these cytokines on peptide synthesis and release, we analyzed the effect of a 48-h exposure to TNF-α on SgII biosynthesis, and on SN and ENK release in chromaffin cells (Fig. 2). Western blot analysis, using an antibody directed against the EM66 peptide arising from SgII processing (28), showed that TNF-α markedly increased the concentration of the SgII protein and derived processing products in chromaffin cells (Fig. 2A). Concurrently, TNF-α significantly stimulated the release of SN and ENK peptides in the medium of chromaffin cells as measured by specific RIAs (Fig. 2, B and C). We next investigated whether a prior exposure to proinflammatory cytokines would

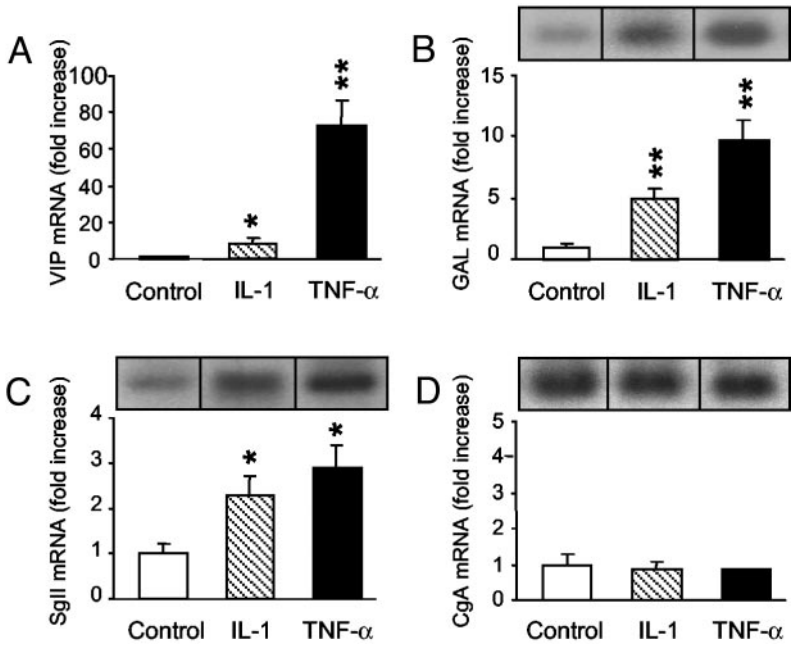

Fig. 1. TNF-α and IL-1α/β Stimulate Neuropeptide Gene Expression in Adrenochromaffin Cells

A, Cultured chromaffin cells were treated with 10 nM TNF-α or IL-1β for 48 h, and VIP mRNA levels were measured by Q-RT-PCR as described in *Materials and Methods.* B, Chromaffin cells were treated with 10 nM TNF-α or IL-1α for 48 h and harvested for Northern blot analysis as described in *Materials and Methods*. The blot was hybridized with a bovine GAL cDNA probe. C and D, The same blot was hybridized with a bovine SgII cDNA probe and with an oligonucleotide deduced from bovine CgA cDNA, respectively. Signals on Northern blots were quantified using ImageQuant 5.1 software. Values are the mean ± SEM of four separate determinations from one experiment representative of two to five experiments and are expressed as a fold increase over control untreated cells. *, $P < 0.05$; **, $P < 0.01$ *vs.* the corresponding control (Student's *t* test). Representative autoradiograms illustrating mRNA signals obtained in the different conditions are shown *above* the histograms.

influence the amount of neuropeptides that could be released from chromaffin cells upon cholinergic stimulation. For this purpose, chromaffin cells were preincubated with TNF-α before application of a high potassium (K^+) medium that depolarizes the cells and thus mimics acetylcholine stimulation. Figure 2D shows that treatment of chromaffin cells with TNF-α for 48 h resulted in a significantly higher amount of ENK released in the medium after a 1-h stimulation with elevated K^+ compared with cells that have not been treated with the cytokine (446 ± 9.8 *vs.* 271 ± 14 pg/10^6 cells, respectively), suggesting that exposure of adrenomedullary chromaffin cells to proinflammatory cytokines *in vivo* could significantly increase the amount of neuropeptide released into the circulation upon preganglionic cholinergic stimulation, which occurs during stress.

TNF-α and IL-1 Stimulation of Neuropeptide Biosynthesis and Secretion Is Concentration Dependent and Delayed

In a first set of experiments, we found by Northern blot analysis that treatment of chromaffin cells for 4 and 16 h with TNF-α and IL-1 did not significantly affect GAL and SgII mRNA levels (data not shown). We have therefore tested the effects of these cytokines at longer times of treatment (Fig. 3). At 24 h, the cytokines induced a modest increase of GAL and SgII mRNA levels (Fig. 3, A–D). At 48 h of treatment, both IL-1 and TNF-α significantly stimulated GAL and SgII gene expression (Fig. 3, A–D). These effects remained elevated after 72 h of incubation with the cytokines (Fig. 3, A–D). Because the action of TNF-α and IL-1 on neuropeptide gene expression was apparent only after approximately 48 h and was sustained thereafter, we hypothesized that these agents may exert prolonged and delayed actions on neuropeptide secretion from chromaffin cells. We have thus measured SN release after 1- to 7-d exposure to cytokines and found that addition of TNF-α or IL-1 elicits a 1.5- to 3-fold increase in SN release after 3, 5, and 7 d of treatment (Fig. 3, E and F). Together, these data indicate that proinflammatory cytokines exert long-lasting effects on neuropeptide biosynthesis and release in chromaffin cells and suggest that, under sustained cytokine action, *e.g*. in chronic inflammatory conditions, adrenomedullary cells may continuously release several neuropeptides that can exert various regulatory effects.

To show that the effects of the two cytokines on neuropeptide gene expression are dose dependent, chromaffin cells were incubated with graded concen-

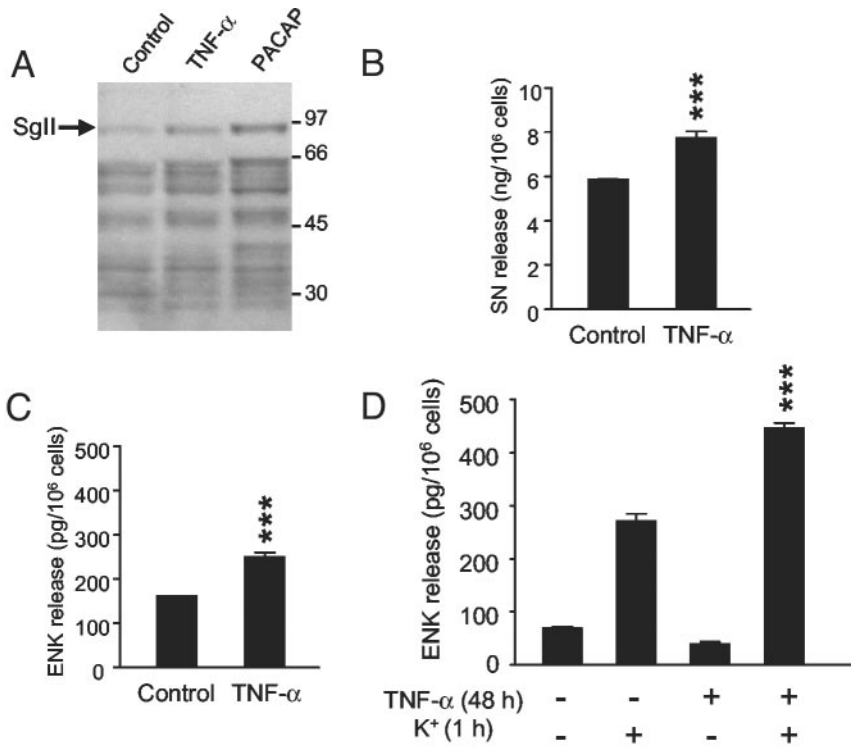

Fig. 2. Effects of TNF-α on Neuropeptide Biosynthesis and Secretion in Adrenochromaffin Cells

A, Western blot analysis of SgII concentration in chromaffin cells after 72 h of treatment with TNF-α (10 nM) or PACAP (50 nM), which was used as a positive control (24). Twenty μg of proteins were analyzed by SDS-PAGE and immunoblotted with antibodies directed against SgII (28). Both TNF-α and PACAP increased the concentration of SgII and processing products in chromaffin cells. B, SN concentration released in the culture medium was determined by RIA using a specific antibody (24). TNF-α (10 nM) induced a significant increase in SN release after 48 h of treatment. C, ENK concentration in the culture medium was determined by RIA using a specific antibody (72). TNF-α (10 nM) induced a significant increase in ENK release after 48 h of treatment. D, Effect of exposure to TNF-α (10 nM, 48 h) on K^+ (40 mM, 1 h)-stimulated release of ENK peptide. Chromaffin cells that had been treated with TNF-α exhibited a significantly higher response to cell depolarization by high K^+ than cells that had not been pretreated. Data in panels B, C, and D are expressed as the mean ± SEM of four determinations. ***, $P < 0.001$ *vs.* the corresponding control (Student's *t* test).

trations of TNF-α and IL-1 for 3 d, and mRNA or peptide levels were determined (Fig. 4). It was found that a concentration of 0.1 nM of each cytokine is sufficient to provoke a significant increase in GAL mRNA levels (Fig. 4, A and B), whereas a significant stimulation of SgII mRNA levels was observed only at a higher concentration (1 nM) of the cytokines (Fig. 4, C and D). The maximum effects of TNF-α and IL-1 on gene expression of both GAL and SgII occurred at a concentration of about 10 nM (Fig. 4, A–D). TNF-α and IL-1 stimulated the release of SN from chromaffin cells also in a dose-dependent manner; the cytokines were effective at concentrations of 0.01–0.1 nM, and the effects slightly but steadily increased at higher concentrations (Fig. 4, E and F).

Expression of TNF-R2 in Bovine Chromaffin Cells

To identify the receptors that could mediate the effects of the proinflammatory cytokines on adrenomedullary cells, we attempted to characterize the mRNAs encoding these receptors in cultured bovine chromaffin cells by RT-PCR using oligonucleotides deduced from bovine TNF-R1 and TNF-R2 sequences. Several primer pairs deduced from the bovine TNF-R1 sequence were tested, but none amplified a product from chromaffin cell RNA that was reverse transcribed using oligo(dT) or random hexamers, indicating the absence of mRNA encoding TNF-R1 in chromaffin cells. Because no positive control cell was available for RT-PCR, we used bovine genomic DNA to show that the oligonucleotides employed were suitable to amplify by PCR a TNF-R1-encoding fragment (Fig. 5). Concurrently, analysis of the RT-PCR products obtained with specific primers for TNF-R2 revealed the amplification of a DNA fragment with the expected size (Fig. 5). Cloning and sequencing of this DNA fragment confirmed its identity as the bovine TNF-R2. This finding suggests that the effects of TNF-α on chromaffin cells are mediated through TNF-R2. Because no bovine IL-1 receptor has been cloned so far, the type of receptor involved in the action of IL-1 on chromaffin cells could not be determined.

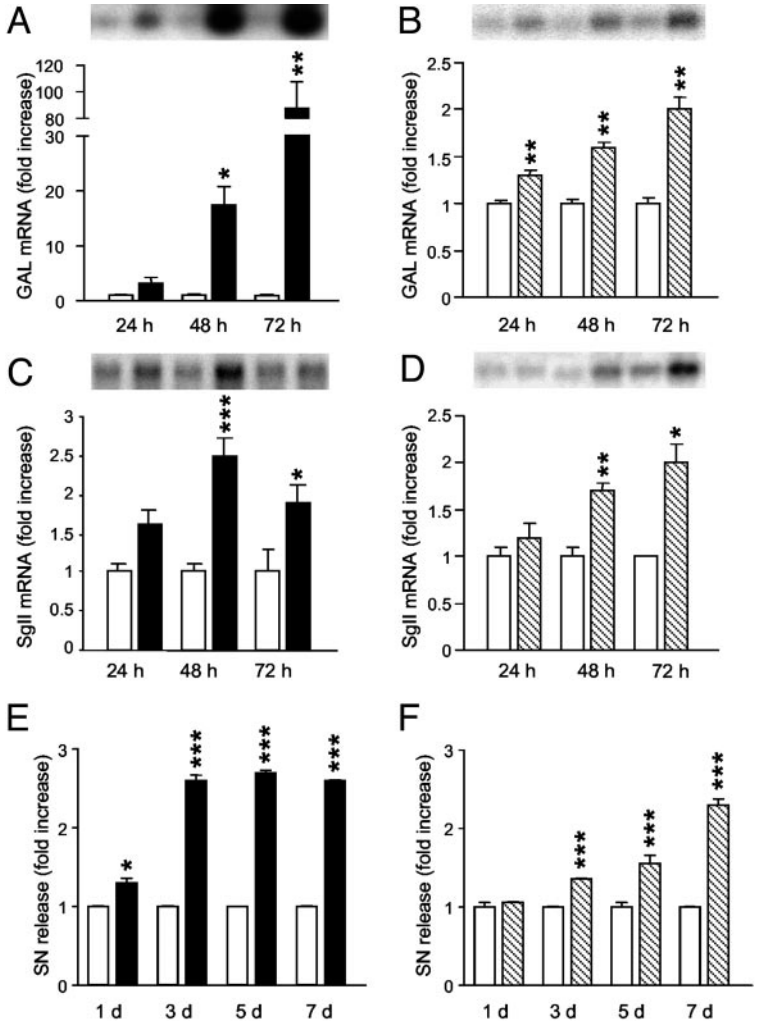

Fig. 3. Time Course of TNF-α- and IL-1α-Induced Stimulation of GAL and SgII mRNA Levels and SN Release

A, Chromaffin cells were incubated in the absence (control; *open bars*) or presence of 10 nM TNF-α (*black bars*) for various durations, and GAL mRNA levels were determined by Northern blot analysis. A representative autoradiogram illustrating GAL mRNA signals obtained at the different times is shown *above* the histogram. B, Chromaffin cells were incubated in the absence (control; *open bars*) or presence of 10 nM IL-1α (*hatched bars*) for various durations, and GAL mRNA levels were determined by Northern blot analysis. A representative autoradiogram illustrating GAL mRNA signals obtained at the different times is shown *above* the histogram. C, Chromaffin cells were incubated in the absence (control*; open bars*) or presence of 10 nM TNF-α (*black bars*) for various durations, and SgII mRNA levels were determined by Northern blot analysis. A representative autoradiogram illustrating SgII mRNA signals obtained at the different times is shown *above* the histogram. D, Chromaffin cells were incubated in the absence (control; *open bars*) or presence of 10 nM IL-1α (*hatched bars*) for various durations, and SgII mRNA levels were determined by Northern blot analysis. A representative autoradiogram illustrating SgII mRNA signals obtained at the different times is shown *above* the histogram. E, Chromaffin cells were incubated in the absence (control; *open bars*) or presence of 10 nM TNF-α (*black bars*) for various durations, and SN concentration in the culture medium was determined by RIA. F, Chromaffin cells were incubated in the absence (control; *open bars*) or presence of 10 nM IL-1α (*hatched bars*) for various durations, and SN concentration in the culture medium was determined by RIA. Results are expressed as fold increase over corresponding control values and represent means ± SEM of four determinations for each time point from one experiment representative of two or three experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* the corresponding control (Student's *t* test).

The Effect of TNF-α on GAL and SgII Gene Expression Is Dependent on ERK 1/2 and p38, But Not JNK, Activities in Chromaffin Cells

The effects of proinflammatory cytokines are known to involve a wide variety of signal transduction pathways leading to the activation of several serine-threonine kinases (29). We examined the involvement of the MAPKs p38 and JNK, which have been described as mediators of cytokine action in various tissues (8, 9), as well as ERK 1/2, a major component of signaling initiated by other first messengers in chromaffin cells, in the stimulatory effect of TNF-α on neuropeptide gene expression (Fig. 6). Application of the p42/44

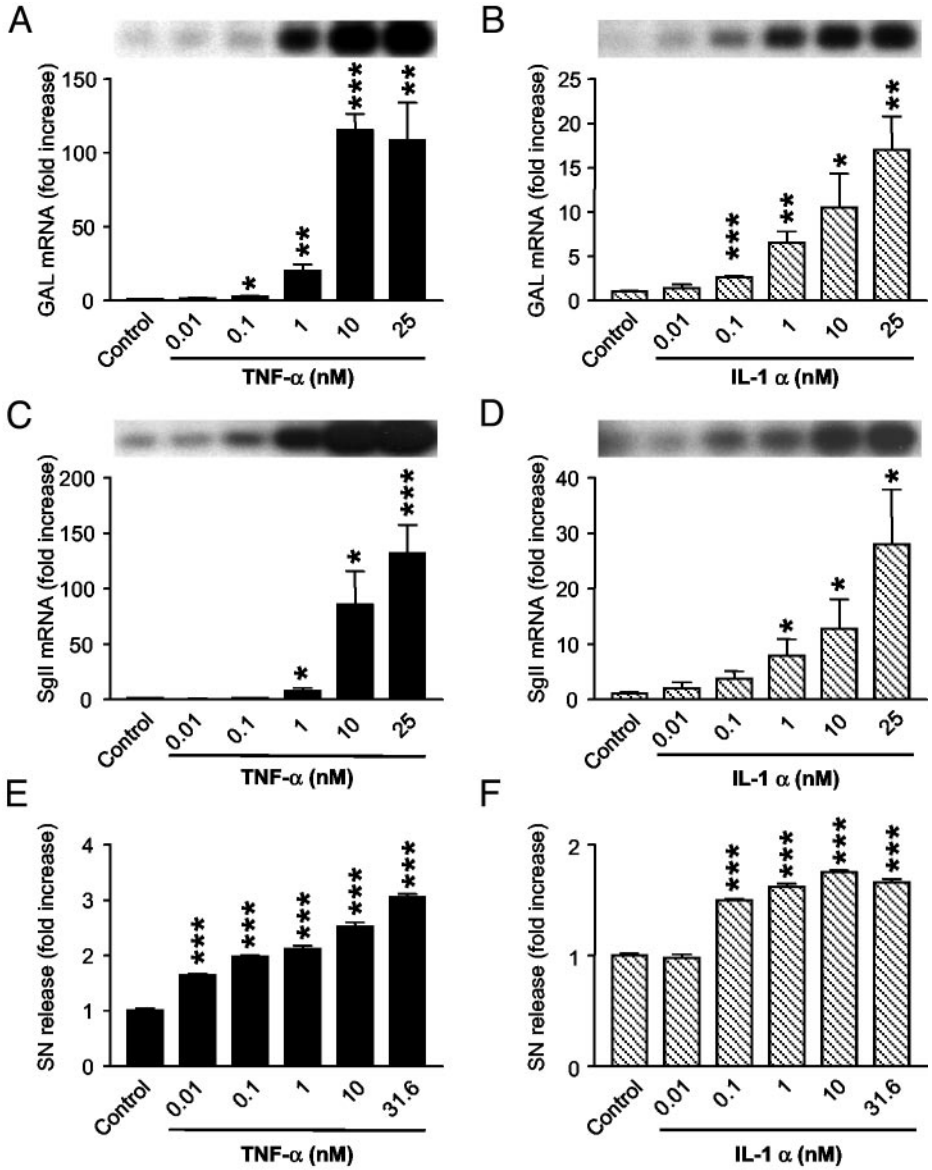

Fig. 4. Concentration-Dependent Effects of TNF-α and IL-1α on GAL and SgII mRNA Levels and SN Release

A and B, Chromaffin cells were treated for 72 h with graded concentrations of TNF-α or IL-1α, respectively, and GAL mRNA levels were determined by Northern blot analysis. Representative autoradiograms illustrating GAL mRNA signals obtained in response to the different cytokine concentrations are shown *above* the histograms. C and D, Chromaffin cells were treated for 72 h with increasing concentrations of TNF-α or IL-1α, respectively, and SgII mRNA levels were determined by Northern blot analysis. Representative autoradiograms illustrating SgII mRNA signals obtained in response to the different cytokine concentrations are shown *above* the histograms. E and F, Chromaffin cells were treated for 5 d with graded concentrations of TNF-α or IL-1α, respectively, and SN concentrations in the culture medium were determined by RIA. Results are expressed as fold increase over corresponding control values and represent means ± SEM of four determinations for each cytokine concentration from one experiment representative of two experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* the corresponding control (Student's *t* test).

ERK 1/2 MAPK inhibitor U0126 (10 μM) or the p38 inhibitor SB 203580 (10 μM) completely suppressed the effect of TNF-α on GAL and SgII mRNA levels (Fig. 6, A, B, D, and E). In contrast, exposure of chromaffin cells to the JNK inhibitor JNK II (10 μM) did not alter the stimulatory effect of TNF-α on neuropeptide gene expression (Fig. 6, C and F). These results suggest that TNF-α activates ERK 1/2 and p38 to stimulate neuropeptide gene expression in chromaffin cells. In addition, TNF-α-induced release of SN was also inhibited by U0126 and SB 203580, indicating that ERK 1/2 and p38 are also involved in the action of the cytokine on neuropeptide secretion (Fig. 6G).

Because ERK 1/2 activation could be highly relevant to a specific effect of inflammatory agents in neuroendocrine cells, we attempted to characterize the mech-

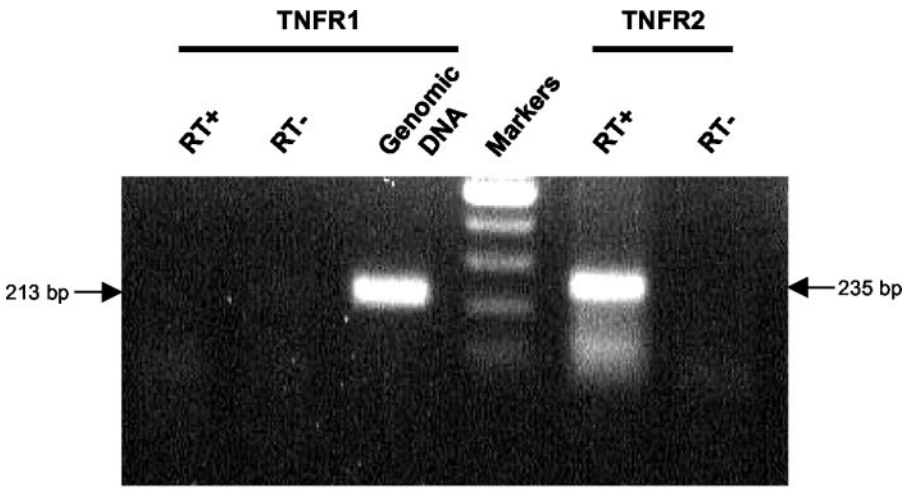

Fig. 5. Expression of TNF-R1 and -R2 in Adrenochromaffin Cells

Total RNA from chromaffin cells was reverse transcribed in the absence (RT−) or presence (RT+) of reverse transcriptase and used to amplify by PCR cDNA fragments of TNF-α receptors. Electrophoresis on agarose gel followed by ethidium bromide staining revealed that only a fragment of the TNF-R2 cDNA could be amplified from chromaffin cell-reverse transcribed RNA. Bovine genomic DNA was used as a positive control for TNF-R1 amplification. Cloning and sequencing confirmed the identity of the amplified products. Markers in base pairs are indicated.

anisms underlying its implication in neuropeptide gene regulation by TNF-α in chromaffin cells. We first analyzed the kinetics of the inhibition by U0126 of the cytokine-induced stimulation of GAL and SgII mRNA levels. We found that the inhibitor was able to block the effect of TNF-α on GAL (Fig. 7A) and SgII (Fig. 7B) mRNA levels when it was added during the first 12–24 h of exposure to the cytokine but was ineffective when added later. These data indicate that ERK 1/2 enzymes are stimulated early during TNF-α action and that the activation of these kinases probably persists for several hours to support the delayed and prolonged effect of the proinflammatory cytokine on neuropeptide gene transcription. We then examined the effect of TNF-α on ERK 1/2 phosphorylation in chromaffin cells at short and long times of treatment. Western blot analysis using antibodies directed against ERK 1/2 phosphorylated on threonine-183/tyrosine-185 showed that ERK 1/2 phosphorylation was strongly increased after a 5-min incubation of chromaffin cells with TNF-α and returned to normal values after 24 h of treatment (Fig. 8A). The total level of ERK 1/2 was not modified by TNF-α treatment (Fig. 8B). These data are in line with the observations made in the kinetic studies.

Phosphorylated ERKs have been shown to translocate to the nucleus where they activate transcription factors in response to certain stimuli. We applied confocal laser scanning microscopy to assess the localization of activated phospho-ERK in chromaffin cells. The effect of TNF-α on the translocation of the dually phosphorylated ERKs was compared with those of PACAP and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) that have previously been shown to stimulate phospho-ERK accumulation in chromaffin cells (24). In basal conditions, only weak staining for active phospho-ERK was detected (Fig. 9). Incubation with 10 nM TNF-α for 5 min increased phospho-ERK staining in the cytoplasm of many chromaffin cells, but no translocation of activated phospho-ERK to the nucleus could be observed. Incubation with 50 nM PACAP for 5 min increased phospho-ERK immunostaining in the cytoplasm of many chromaffin cells and also in the nucleus of a few cells. Finally, treatment with 100 nM TPA for 5 min caused the appearance of activated phospho-ERK in the nucleus of many chromaffin cells (Fig. 9). These data show that different stimuli may lead to various patterns of activated phospho-ERK localization and that, despite activation of ERK, TNF-α does not cause phospho-ERK translocation to the nucleus after 5 min of treatment, indicating the possibility that TNF-α signaling to the nucleus proceeds via ERK phosphorylation of a downstream cytoplasmic transduction component, which, in turn, translocates to the nucleus to allow the ultimate transactivation of the neuropeptide target genes examined here.

The Effect of TNF-α on GAL and SgII Gene Expression Is Cycloheximide Sensitive

Because the effect of the proinflammatory cytokines on neuropeptide mRNA levels occurs only several hours after initiation of treatment, we hypothesized that *de novo* protein synthesis was necessary. To test this hypothesis, we assessed the effect of the protein synthesis inhibitor cycloheximide on TNF-α-induced GAL and SgII gene expression. Incubation of chromaffin cells in the presence of cycloheximide (0.5 μg/ml) completely abolished the stimulation of GAL and SgII mRNA levels elicited by TNF-α (Fig. 10, A and B), indicating that the cytokine induces the biosynthesis of factors that are required for the stimulation of neu-

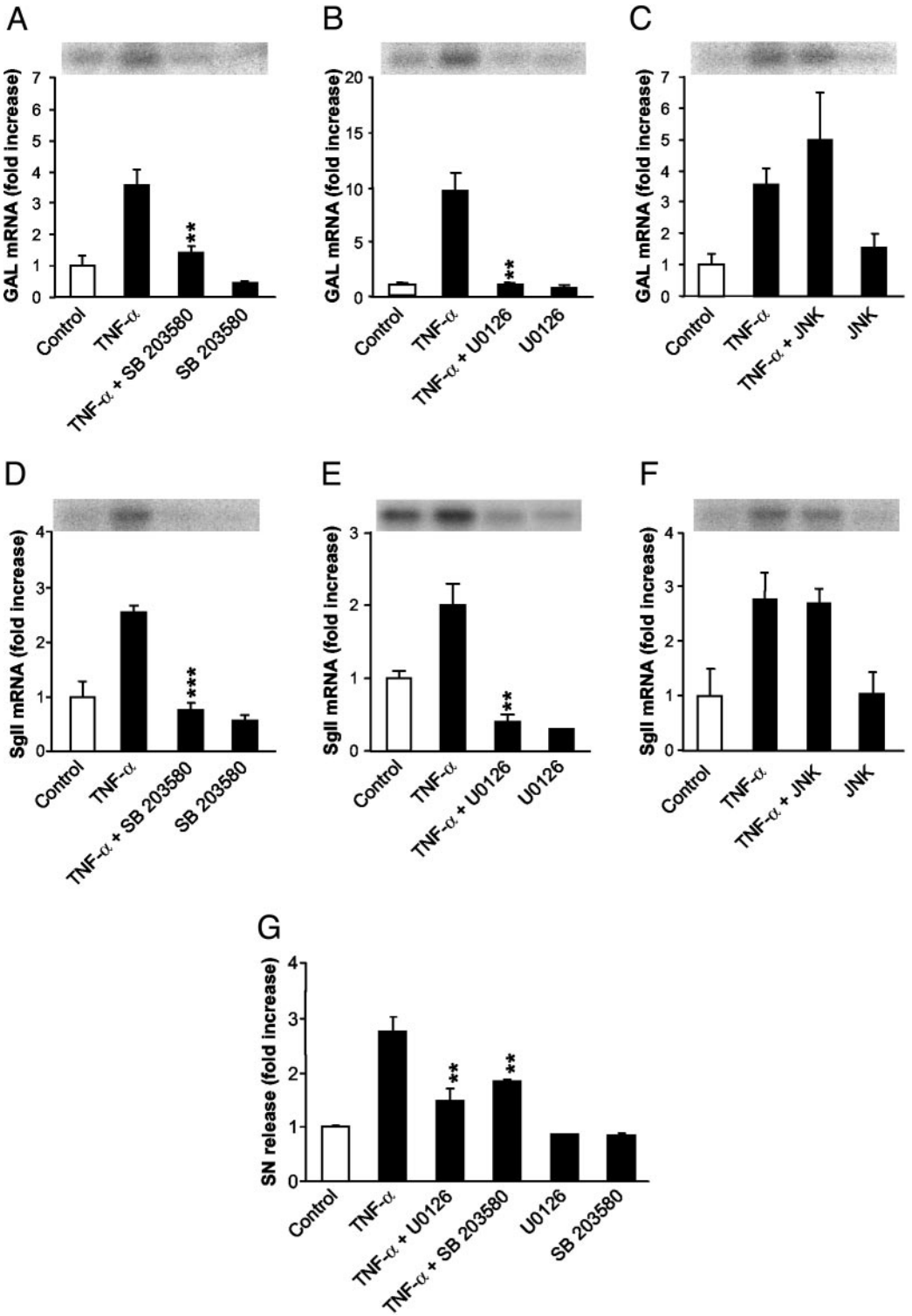

Fig. 6. Effect of Inhibitors of the MAPKs ERK 1/2, p38, and JNK on TNF-α-Induced GAL and SgII mRNA Levels and SN Release in Adrenochromaffin Cells

Chromaffin cells were incubated for 48 h in control conditions or with10 nM TNF-α, in the absence or presence of 10 μM U0126 (A and D), 10 μM SB 203580 (B and E), and 10 μM JNK II (C and F) as described in *Materials and Methods*. GAL (A–C) and SgII (D–F) mRNA levels, determined by Northern blot analysis, are expressed as fold increase over corresponding control values and represent means ± SEM of four determinations for each condition from one experiment representative of two or three experiments. Representative autoradiograms illustrating mRNA signals obtained in the different conditions are shown *above* the histograms. G, SN concentrations in the culture medium were determined by RIA and the results are expressed as described above. **, $P < 0.01$; ***, $P < 0.001$ *vs.* TNF-α alone (Student's *t* test).

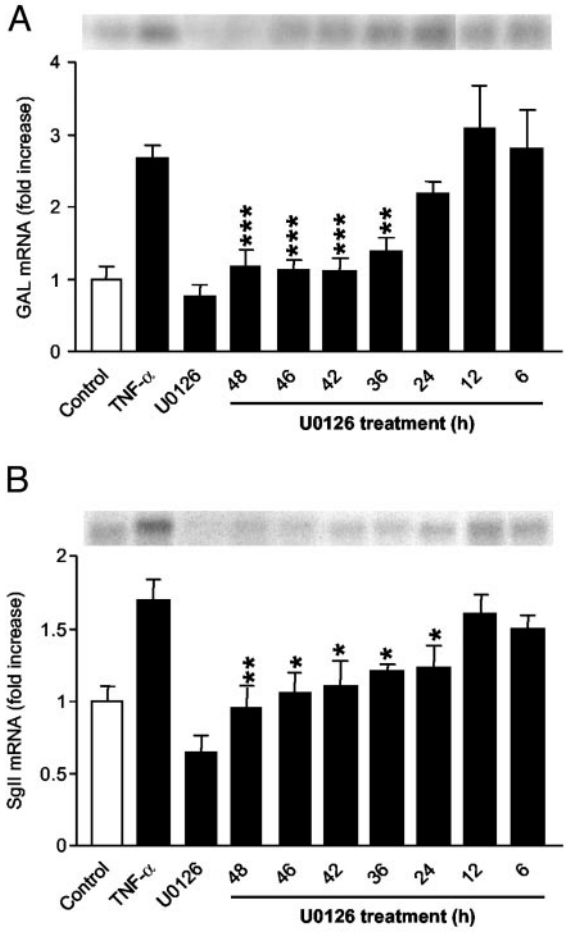

Fig. 7. Kinetics of the Effect of U0126 on TNF-α-Induced GAL and SgII mRNA Levels in Adrenochromaffin Cells

Chromaffin cells were incubated for 48 h in control conditions or with10 nM TNF-α, in the absence or presence of 10 μM U0126, which was added for the times indicated, after the initiation of the TNF-α treatment. GAL (A) and SgII (B) mRNA levels, determined by Northern blot analysis, are expressed as fold increase over corresponding control values and represent means ± SEM of four determinations for each condition. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* TNF-α alone (Student's *t* test). Representative autoradiograms illustrating mRNA signals obtained in the different conditions are shown *above* the histograms.

ropeptide gene expression in chromaffin cells. In addition, application of cycloheximide at various times revealed that *de novo* protein synthesis is required at least during the first 12–24 h of TNF-α treatment (Fig. 10, A and B).

TNF-α Induces the Binding Activity of AP-1 Transcription Factors by an ERK 1/2-Dependent Mechanism in Chromaffin Cells

Binding of TNF-α to its receptors is known to cause activation of two major transcription factors, AP-1 and NF-κB (30). To determine whether TNF-α affects the activity of these *trans*-acting factors in chromaffin

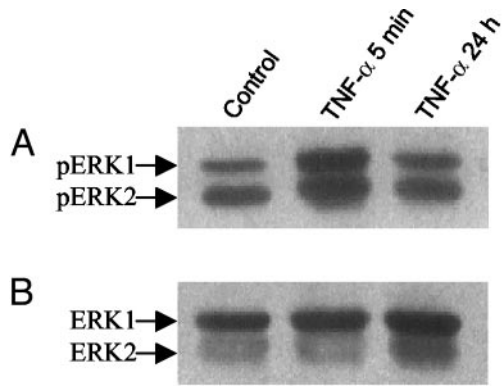

Fig. 8. Effect of TNF-α on the Phosphorylation of ERK 1/2 in Adrenochromaffin Cells

Chromaffin cells were incubated for 5 min or 24 h at 37 C in the absence or presence of TNF-α, and protein extracts were analyzed by Western blotting as described in *Materials and Methods*. A, Phosphorylation of ERK 1/2 was probed with the anti-ACTIVE MAPK pAb. B, The total amount of ERK 1/2 was determined by probing the same blot with anti-ERK 1/2 pAb. Two experiments were performed with similar results.

cells, we carried out gel shift assays using double-stranded DNA fragments containing consensus binding sites for these proteins, and untreated and cytokine-treated chromaffin cell nuclear extracts. Binding to a labeled TPA-responsive element (TRE), a *cis*-active element that interacts with AP-1 transcription factors in a wide variety of gene promoters, was markedly increased by a 2 h-treatment of chromaffin cells by TNF-α (10 nM) in comparison to untreated cells (Fig. 11A). The increase in TRE-bound proteins was still apparent after 24 h of exposure to TNF-α, indicating that this cytokine exerts a sustained effect on the binding to the TRE. Enhanced binding of the TRE to chromaffin cell nuclear extracts was markedly reduced by the addition of the ERK 1/2 inhibitor U0126 (10 μM), demonstrating that TNF-α-induced elevation of AP-1 activity in the nucleus is ERK dependent (Fig. 11A). To confirm that the major protein of the complex was indeed AP-1, a supershift assay was performed using antibodies directed against Fos and Jun proteins or cAMP response element (CRE)-binding protein (CREB)/activating transcription factors (ATFs) (Fig. 11B). Incubation with an anti-Fos antibody completely abolished the binding to the TRE, confirming that, indeed, the bound complex comprises mainly AP-1-like proteins (Fig. 11B). Anti-Jun antibodies also supershifted the TRE-bound complex whereas the anti-CREB/ATF had no effect (Fig. 11B). Taken together, these results show that TNF-α increases the binding activity of AP-1-like proteins in chromaffin cells through a mechanism requiring activation of ERK 1/2. We next investigated the effect of TNF-α on the binding of the CRE *cis*-active sequence, which is also present in the promoters of a variety of genes expressed in chromaffin cells (31, 32). Consensus CRE elements are able to interact with CREB/ATF as well as

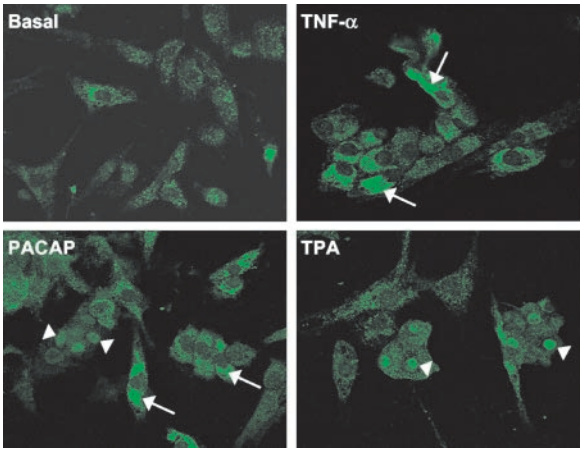

Fig. 9. Immunocytochemical Localization of Activated Phospho-ERK in Adrenochromaffin Cells

Chromaffin cells were exposed to 10 nM TNF-α, 50 nM PACAP, or 100 nM TPA for 5 min at 37 C, and then fixed and processed for immunofluorescence. Active ERK was visualized by using antibodies against dually phosphorylated ERK and Alexa-488-conjugated goat antirabbit Igs. Cytoplasmic staining is indicated by *arrows*, and nuclear staining is indicated by *arrowheads*. Representative fields of cells are shown.

with AP-1 transcription factors in chromaffin cells (33). Binding of nuclear extract proteins of chromaffin cells to this element was also increased after a 2-h exposure to TNF-α, and this effect, like the effect exerted on TRE binding, was also observed at 24 h of treatment (Fig. 11C). Moreover, the effect of TNF-α on the CRE binding was also inhibited by U0126. Supershift experiments showed that a large portion of the CRE-bound proteins in TNF-α-treated chromaffin cells are displaced by anti-Fos or anti-Jun antibodies, whereas anti-CREB antibodies interfered only with a minor fraction of the complexes bound to this element (Fig. 11D). Thus, it appears that the proinflammatory mediator TNF-α induces a persistent elevation in the amount of AP-1 proteins that interact with both TRE and CRE DNA sequences in adrenochromaffin cells.

TNF-α and ERK Activation Increase AP-1-Mediated Gene Transcription in Chromaffin Cells

To determine whether the transcriptional effect of TNF-α on neuropeptide genes is mediated by AP-1 proteins through enhanced binding to TRE/CRE elements, we transfected an AP-1 *cis*-reporting system containing a TRE sequence, or control plasmids, in chromaffin cells before treatment with the cytokine. We found that TNF-α increases the transcriptional activity of a promoter bearing AP-1 binding sites in chromaffin cells (Fig. 12A), indicating that this proinflammatory cytokine is able to induce gene transcription through the increased binding of AP-1 to the TRE that was observed by EMSA experiments. In contrast,

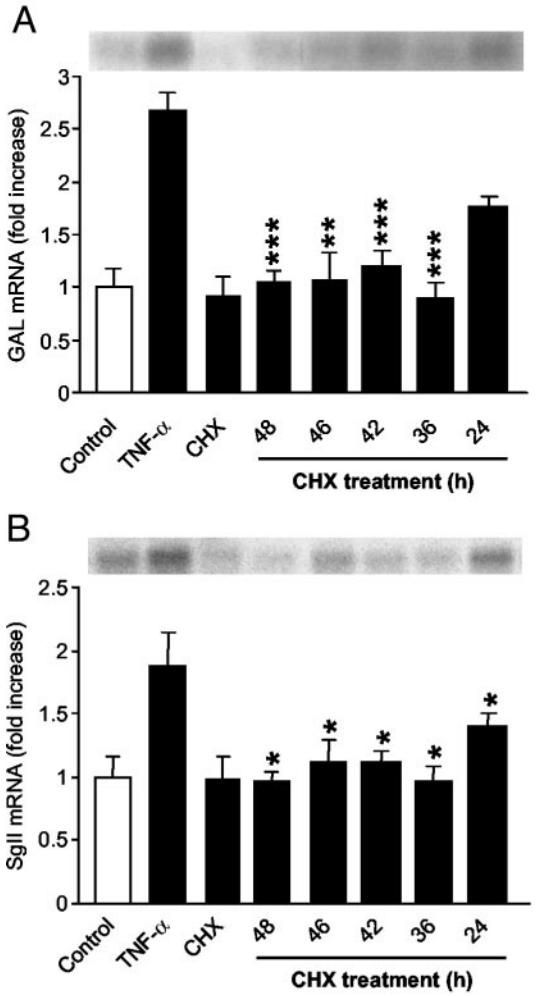

Fig. 10. Effect of Cycloheximide on TNF-α-Induced GAL and SgII Gene Expression in Adrenochromaffin Cells

Chromaffin cells were incubated in the absence (control) or presence of TNF-α for 48 h with or without 5 μg/ml cycloheximide (CHX), which was added for the times indicated, after the initiation of TNF-α treatment. GAL (A) or SgII (B) mRNA levels were determined by using Northern blot analysis and ImageQuant 5.1 software. Results are expressed as fold increase over corresponding control values and represent means ± SEM of four determinations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* TNF-α alone (Student's *t* test). Representative autoradiograms illustrating mRNA signals obtained in the different conditions are shown *above* the histograms.

TNF-α had no effect on a promoterless vector or on a vector in which the luciferase reporter gene is under the control of a promoter sequence, *i.e*. repeats of a CCAAT enhancer binding protein (C/EBP) element, that is different from the TRE sequence (Fig. 12A).

To demonstrate the existence of a link between ERK activation and stimulation of AP-1-mediated gene transcription, we cotransfected a constitutively active form of MAPK kinase kinase and the AP-1 *cis*-reporting vector in chromaffin cells. These experiments revealed that overexpression of the constitutive kinase

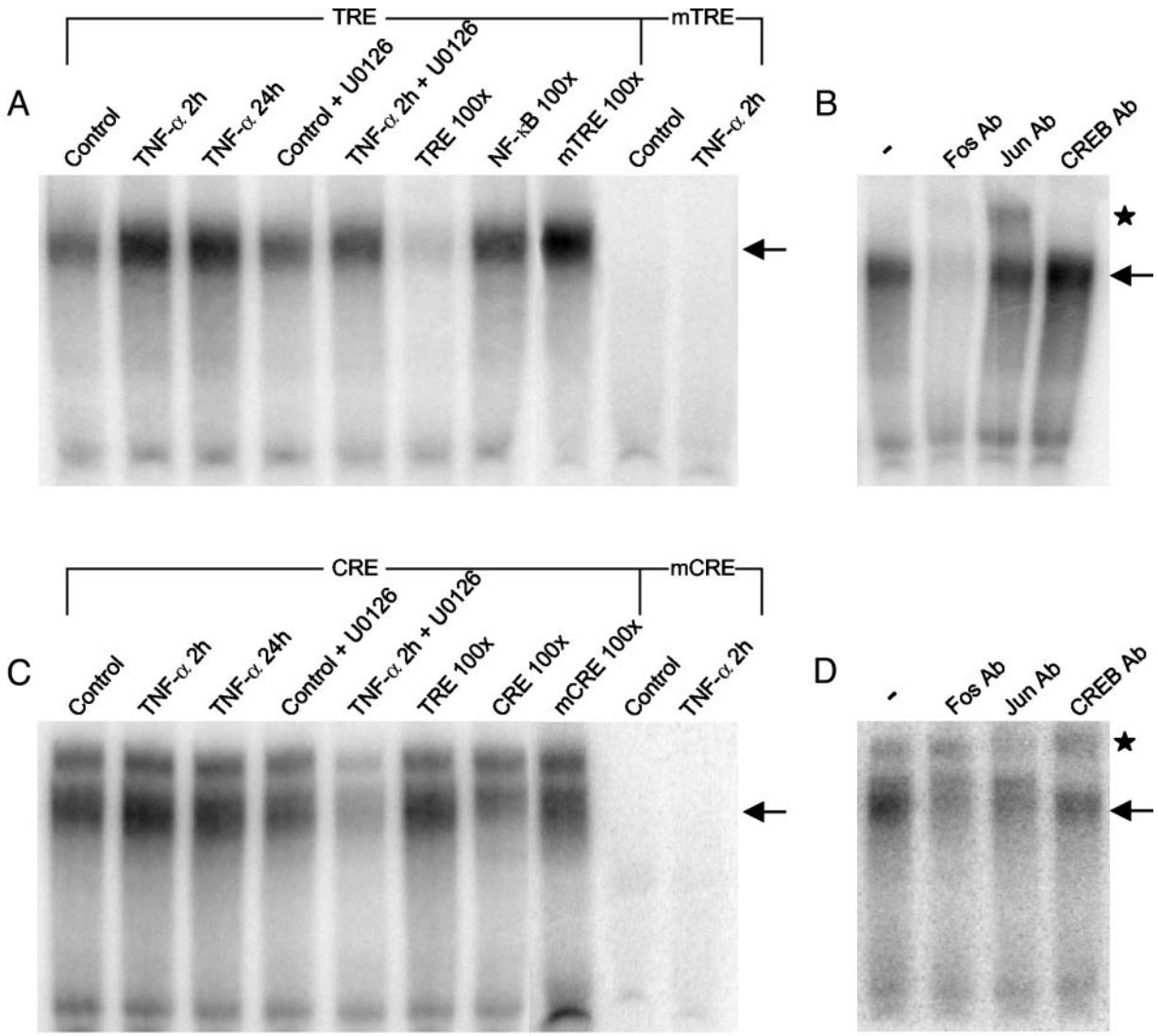

Fig. 11. TNF-α Increases the Binding Activity of TRE and CRE Sequences to AP-1-Like Transcription Factors through an ERK 1/2-Dependent Mechanism

A, Chromaffin cells were incubated with TNF-α for 2 or 24 h in the absence or presence of 10 μM U0126, and nuclear extracts were prepared and assayed for their binding to a ^{32}P-labeled TRE or a mTRE. The binding specificity of the TRE to TNF-α-treated nuclear extracts was verified by adding a 100-fold excess of unlabeled TRE, mTRE, or NF-κB. Unlabeled TRE totally displaced the binding, in contrast to an excess of mTRE or NF-κB binding site, which had no effect. B, Supershift assays of the TRE-bound complex obtained in the presence of TNF-α-treated nuclear extracts, using antibodies raised against all members of Fos (Fos Ab), Jun (Jun Ab), and ATF/CREB (CREB Ab) families of transcription factors. The symbol (−) denotes control supershift reactions with no antibody. C, A ^{32}P-labeled CRE or a mCRE probe was used in gel shift assays in the same conditions as those described in panel A. The binding specificity was verified by adding a 100-fold excess of the CRE, the mCRE, or the TRE sequences. D, Supershift assays of the CRE-bound complexes obtained in the presence of TNF-α-treated nuclear extracts, using the antibodies described in panel B. The shifted (*arrows*) and the supershifted (*stars*) complexes are indicated. Note that anti-Fos is a neutralizing antibody whereas anti-Jun and anti-CREB are supershifting antibodies. Two separate experiments were performed with similar results.

leads to a robust increase in the transcriptional activity of the reporter gene (Fig. 12B). Altogether, these data strongly suggest that TNF-α stimulates neuropeptide gene transcription through increased binding of AP-1 transcription factors to TRE/CRE sequences present in the promoters *via* activation of ERK 1/2 protein kinases.

TNF-α Induces the Binding Activity of NF-κB Transcription Factor by an ERK 1/2-Independent Mechanism in Chromaffin Cells

Incubation of a labeled double-stranded oligonucleotide containing a NF-κB binding site with chromaffin cell nuclear extracts revealed that TNF-α also induced

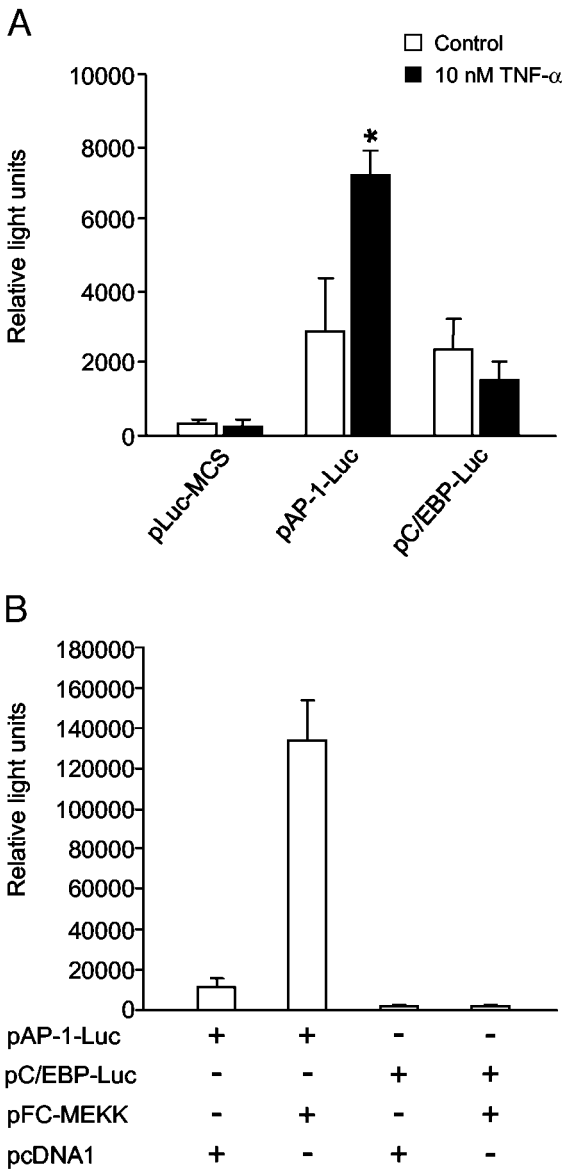

Fig. 12. TNF-α and ERK Activation Increases AP-1-Mediated Gene Transcription in Chromaffin Cells

A, Chromaffin cells were transfected with 1 μg of the plasmids pLuc-MCS, pAP-1-Luc, or pC/EBP-Luc, together with 2 μg of β-galactosidase (see *Materials and Methods*) and incubated overnight in the presence or absence of 10 nM of TNF-α. The cells were harvested, the luciferase activity was measured, and the transfection efficiency was corrected with the β-galactosidase activity in each well. The results represent means ± SEM of three independent experiments performed in triplicate. *, $P < 0.05$ *vs.* the corresponding control (Student's *t* test). B, Chromaffin cells were cotransfected with 1 μg of the plasmids pAP-1-Luc or pC/EBP-Luc and 50 ng of pFC-MEKK or pcDNA1 along with 2 μg of β-galactosidase. The cells were harvested 48 h later, the luciferase activity was measured, and the transfection efficiency was corrected with the β-galactosidase activity in each well. The results represent means ± SEM from one representative experiment performed in triplicate that was repeated once with similar results.

the formation of a DNA-protein complex that was displaced only by the homologous unlabeled probe NF-κB (Fig. 13). However, the effect exerted by TNF-α on NF-κB binding is transient because it disappeared at 24 h of treatment, and is independent of ERK 1/2 activation because it was not inhibited by U0126 (Fig. 13). These findings suggest that, in chromaffin cells, TNF-α activates various transduction pathways that lead to increased binding of AP-1 and NF-κB to cognate DNA sequences on neuropeptide genes in the nucleus.

DISCUSSION

There is now compelling evidence that catecholamines and neuropeptides released from the sympathetic nervous system and the adrenal medulla, in addition to the hormones of the pituitary-adrenocortical axis, play fundamental roles in the homeostatic regulation triggered by activation of the immune system (13, 14, 19, 34–36). Different studies have shown that the production of proinflammatory cytokines is altered not only in response to inflammatory/infectious challenges, but also to physical and psychological stressors, which are known to primarily activate adrenomedullary catecholamine and neuropeptide production (37–39). These observations suggest the existence of a regulatory loop between the immune and the sympathoadrenal systems under inflammatory

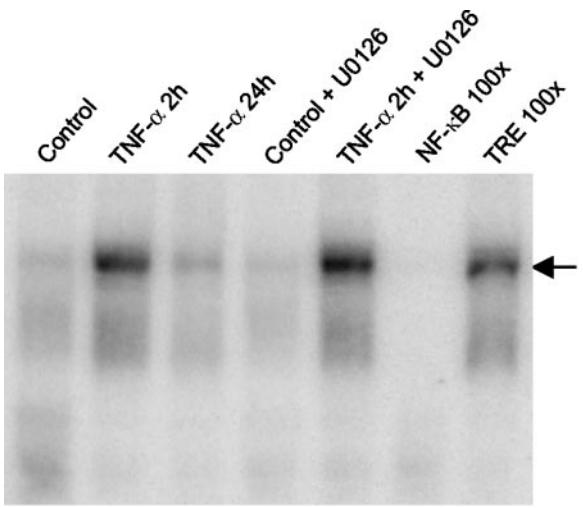

Fig. 13. TNF-α Increases the Binding Activity of an NF-κB Binding Site to Chromaffin Cell Nuclear Extracts through an ERK 1/2-Independent Mechanism

Chromaffin cells were incubated with TNF-α for 2 or 24 h in the absence or presence of 10 μM U0126, and nuclear extracts were prepared and assayed for their binding to a ^{32}P-labeled NF-κB sequence. The binding specificity of the NF-κB binding site to TNF-α-treated nuclear extracts was verified by adding a 100-fold excess of unlabeled NF-κB or TRE. Two separate experiments were performed with similar results.

and/or stressful conditions. There is substantial evidence that proinflammatory mediators may directly regulate the secretory activity of chromaffin cells as part of this regulatory loop. Factors released from human immune cells can mediate adrenaline and noradrenaline release from adrenomedullary cells (40), and IL-1 is able to stimulate catecholamine secretion (41) and VIP biosynthesis (22) in cultured bovine adrenal medullary cells.

The present study extends the findings described above in several important ways. We describe here, for the first time, the molecular mechanisms underlying the effects of TNF-α and IL-1 on gene expression and release of several neuropeptides in chromaffin cells of the adrenal medulla. TNF-α and IL-1 induced a robust stimulation of VIP, GAL, and SgII mRNA levels and increased the release of SN and ENK peptides in bovine chromaffin cells, in a time- and concentration-dependent manner. Each of these neuropeptides has been previously implicated in the response to inflammation (36, 42–44). TNF-α and IL-1 had no effect on the expression of the gene encoding the secretory granule protein CgA (45), demonstrating specificity in the effect of these cytokines on chromaffin cell secretory response.

The effect of TNF-α and IL-1 on neuropeptide biosynthesis in chromaffin cells occurs only after a significant delay after initial exposure and requires *de novo* protein synthesis during the first 12–24 h of treatment. Thus, proinflammatory cytokines likely induce new protein products in chromaffin cells to stimulate neuropeptide gene expression. This protein may be either a released (autocrine) factor, or an intracellular mediator necessary for the generation of second messengers such as prostaglandins (PGs) or nitric oxide (NO), key effectors in neuroimmune communication the production of which is regulated by proinflammatory cytokines in other cell types (46, 47). If the latter hypothesis obtains, TNF-α and IL-1 may activate the production of PGs or NO by induction of cyclooxygenase 2 or neuronal NO synthase, respectively (48, 49). In agreement with this notion, several studies have shown the induction of these enzymes and the subsequent production of PGs or NO under various conditions in primary chromaffin cells and pheochromocytomas (50–53). Further study will be required to elucidate the nature of the newly synthesized factors in chromaffin cells. These may well represent important intermediates in the regulatory action of proinflammatory cytokines in neuroendocrine cells.

In addition to their effect on gene expression, TNF-α and IL-1 also promoted a delayed and long-lasting effect on the release of ENK and SN peptides in chromaffin cells. We have also found that the amount of the neuropeptide ENK that is released by a brief high K^+-depolarization of chromaffin cells is increased after a 48-h exposure to TNF-α. Thus, after exposure to cytokines *in vivo*, adrenomedullary chromaffin cells would be expected to exhibit a higher response, in terms of neuropeptide secretion and presumably also catecholamine release, to splanchnic stimulation during acute stress. In support of this hypothesis, there is evidence that the responsiveness of the sympathetic adrenomedullary system to stress is altered in subjects with chronic inflammation. In patients with atopic dermatitis, a chronic allergic inflammatory disease, there is an overreaction of the sympathetic adrenomedullary system, causing greater catecholamine release when the patients are exposed to a psychosocial stressor (54). It has also been shown that in patients with chronic fatigue syndrome, a disease characterized by a debilitating fatigue associated with immunological dysfunction, plasma concentration of adrenaline is elevated compared with control subjects (55), indicating that the response of the adrenal medulla could be modified under immune/psychological disorders. Taken together, these observations strongly suggest that the regulation of the expression and release of neuropeptides and catecholamines in adrenomedullary chromaffin cells in response to psychological and physical stress could be altered in conditions of chronic inflammation. Altered responsiveness of the adrenal medulla may represent one of the psychobiological events leading to stress-related aggravation of some inflammatory diseases.

Cytokines mediating altered adrenomedullary responsiveness and secretory cocktail composition and quantity may be generated from the adrenal itself. IL-1α and IL-1β immunoreactivities can be detected in rat adrenal medulla (20), and ip or iv injection of lipopolysaccharides increases IL-1 mRNA expression and protein levels in chromaffin cells (21), establishing a link between inflammation and the effect of IL-1 in the adrenal medulla. The occurrence of TNF-α in the human adrenal gland has also been demonstrated. However, this cytokine is not produced by medullary cells but only by cortical cells and resident macrophages present in the transition zone between the cortex and the medulla (3), suggesting that, in inflammatory disorders, TNF-α acts on chromaffin cells in a paracrine manner. Finally, proinflammatory cytokines acting on the adrenal gland *in vivo* during inflammation may have a systemic origin. In this regard, it is worth noting that the concentrations of cytokines used in the present study are in the same range as those measured in the plasma during various inflammatory diseases or in septic shock. Normal values of plasma TNF-α and IL-1 are usually in the pM ($\sim$10 pg/ml) range but they can reach values in the low to high nM range (up to $>$100 ng/ml) in inflammatory diseases or syndromes like rheumatoid arthritis, alcoholic hepatitis, or septic shock. Such concentrations are compatible with those that were used in the present study.

A central question about how IL-1 and TNF-α act on adrenomedullary function *in vivo* concerns the presence of their receptors in the adrenal gland. The expression of the receptor of another proinflammatory cytokine, IL-6, has been reported in the adrenal medulla (56). However, direct evidence for IL-1 and TNF-α receptors in the adrenal gland is lacking. At

present, IL-1 receptor cDNAs cannot be amplified by RT-PCR from bovine chromaffin cell RNA using degenerate primers because the bovine receptors have not been cloned. We were able to amplify a fragment of the TNF-R2, whereas TNF-R1 mRNA appears to be absent from bovine adrenal chromaffin cell RNA.

Thus, TNF-α most likely stimulates neuropeptide gene expression in adrenochromaffin cells through activation of its type 2 receptor. The two TNF-α receptor subtypes, TNF-R1 or p55 TNF receptor and TNF-R2 or p80 TNF receptor, exhibit only very modest sequence homology and are generally associated with different effects of the cytokine (1). Emerging evidence, based on recent knock-out experiments, suggests that TNF-R1 is implicated in TNF-α-induced effects on cell death, whereas TNF-R2 is involved in the trophic effects of the cytokine, as shown in hippocampal (57) and retinal (58) neurons. In the periphery, several recent reports indicate the crucial role of TNF-R2 in the metabolic effects of TNF-α. For instance, it has been shown that the expression of the TNF-R2, but not TNF-R1, is markedly increased in the hypothalamus and adipose tissue of obese mice (59), consistent with the involvement of TNF-α in the induction of insulin resistance and obesity (60). It has also been reported that hypothermia, hyperglycemia, and coma due to a syndrome of severe malaria caused by a parasite infection of susceptible mice is highly dependent on the presence or absence of the TNF-R2, but not TNF-R1, further supporting the critical role of TNF-R2 in metabolic regulations (61). In addition, TNF-R2 gene polymorphism has been linked to the pathogenesis of different metabolic disorders, including hypertension (62, 63). Finally, there is increasing clinical and experimental evidence for an important, independent role of TNF-R2 signaling in the chronicity of inflammation, as found for instance in Crohn's disease (64). Results presented here suggest that the actions of TNF-α in the regulation of catecholamine/neuropeptide-producing cells may also be mediated through TNF-R2.

The transduction pathways that could be activated by TNF-R2 in adrenomedullary chromaffin cells were studied by using inhibitors of the serine-threonine protein kinases ERK 1/2, p38, and JNK, which are involved either in TNF-α action in lymphoid cells or in various intracellular signaling pathways that impact on neuropeptide genes in adrenochromaffin cells (9, 10, 24, 25). The fact that U0126 and SB 203580 abolished the stimulation of SgII and GAL mRNA levels and SN release indicates that activation of ERK 1/2 and p38 plays a critical role in the transduction mechanisms leading to increased neuropeptide gene expression in response to TNF-α. Thus, at variance with lymphoid cells, ERK 1/2, but not JNK, is an obligatory intermediate in the cascades leading to gene regulation by TNF-α in chromaffin cells. The augmentation of the concentration of phosphorylated ERK 1/2 observed in chromaffin cells in response to cytokine treatment further supports a role of the ERK 1/2 pathway in the stimulatory effect of TNF-α on neuropeptide gene expression. Immunocytochemical labeling of phospho-ERK was applied to further characterize the effect of TNF-α on ERK activation and localization in chromaffin cells, in comparison to the effect of the protein kinase C activator TPA and the protein kinase A activator PACAP, both of which also activate ERK 1/2 (24, 25). Interestingly, TPA and, to a lesser extent, PACAP induced the translocation of activated phospho-ERK to the nucleus of chromaffin cells whereas the immunostaining observed after TNF-α was restricted to the cytoplasm. Because nuclear accumulation of phospho-ERK is known to occur within a few minutes of incubation with activating factors in various cell types (65–67), as also observed in chromaffin cells incubated with PACAP and TPA, this finding suggests that TNF-α is likely to recruit an intermediary effector downstream of ERK to transduce the signal to the nucleus in chromaffin cells. Overall, the results of the present study, along with recent reports showing that TNF-α can stimulate the activity of ERK 1/2 in primary neurons (68) and in adipocytes (69), suggest that this MAPK mediates key transcriptional signaling events initiated by TNF-α in neuroendocrine cells. In addition, application of U0126 at different times after the onset of TNF-α treatment revealed that ERK 1/2 activity is required during the first 12–24 h of exposure to the cytokine, indicating that this event is necessary during a prolonged period after the exposure to TNF-α.

Activation of MAPKs by TNF-α is known to impinge on AP-1 and NF-κB transcription factors in the nucleus in order to regulate target genes (10, 30). Using specific binding sites of these *trans*-acting proteins and chromaffin cell nuclear extracts, we found that the cytokine enhances the binding of AP-1 to both TRE and CRE sequences but not to mutant congeners. We have previously shown that mutation of TRE/CRE sequences in the GAL and/or SgII promoters abrogates their transactivation by AP-1 proteins in functional assays (33, 70) and their binding to these transcription factors in EMSA studies (71). Here, we show that TNF-α is able to stimulate AP-1-mediated gene transcription in chromaffin cells through a TRE-driven promoter. It is thus reasonable to assume that TRE/CRE sequences may play an important role in the effect exerted by TNF-α on neuropeptide gene transcription, by interacting with AP-1 proteins in chromaffin cells. The cytokine also induced a marked increase in the binding of NF-κB to its cognate sequence, although the implication of this regulatory factor in neuropeptide gene transcription remains to be established. Altogether, these findings show that, in adrenomedullary cells, TNF-α is able to stimulate the binding of the major transcription factors associated with the effects of this proinflammatory cytokine and suggest that AP-1 and/or NF-κB could be involved in the regulation of neuropeptide gene transcription in these cells. The TNF-α-elicited increase in AP-1 binding activity was blocked by the ERK 1/2 inhibitor U0126, which also suppressed neuropeptide gene stimulation by TNF-α, strongly suggesting that, in bovine chromaffin cells,

TNF-α regulates neuropeptide gene transcription through a signaling pathway involving ERK 1/2 and AP-1. This notion is supported by the cotransfection experiments performed in the present study showing that expression of a constitutively active MAPK kinase kinase increases AP-1-mediated gene transcription in chromaffin cells. In contrast, the induction of NF-κB binding activity by TNF-α was not altered by inhibition of ERK 1/2, indicating that this transcription factor is not a target of the transduction pathway activated by the cytokine that involves ERK 1/2 MAPK. Whether p38 could mediate the effect of TNF-α on NF-κB binding activity in chromaffin cells remains to be determined. Finally, it should be noticed that, whereas the effect on NF-κB binding was transient, the stimulation of AP-1 binding was maintained up to 24 h, in agreement with the kinetics of the effect of TNF-α on neuropeptide gene expression.

In conclusion, we have characterized the stimulatory effect of proinflammatory cytokines on gene expression and release of several neuropeptides in adrenomedullary chromaffin cells and proposed a molecular mechanism by which the immunoregulatory agent TNF-α exerts its action in neuroendocrine cells. These data suggest that local or systemic generation of cytokines may significantly alter the kinds and quantities of neuropeptides secreted from chromaffin cells under stressful conditions, linking the adrenal medulla to the overall physiological response to inflammation.

MATERIALS AND METHODS

Materials

Human recombinant TNF-α and IL-1α/β were purchased from Pepro Tech Inc. (Rocky Hill, NJ). Bovine SN and PACAP38 were synthesized by the solid-phase methodology as described previously (24). Cycloheximide and poly-L-lysine were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France). JNK II and SB 203580 were obtained from Calbiochem (La Jolla, CA), and U0126 was obtained from Promega Corp. (Charbonnières, France).

Cell Culture and Treatments

Primary cultures of bovine adrenochromaffin cells were obtained after retrograde perfusion of bovine adrenal glands with 0.1% collagenase (Serlabo, Bonneuil-sur-Marne, France) and 30 U/ml DNAse I (Sigma-Aldrich), followed by dissociation of the digested adrenal medullae. The cells were cultured in DMEM (Sigma-Aldrich) supplemented with 5% fetal calf serum (Biowhittaker Europe, Verviers, Belgium) and 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone (Invitrogen, Cergy-Pontoise, France). Chromaffin cells were purified by differential plating as described previously (71). Cells were then plated in the same medium as above at a density of 10^6 cells/ml in poly-L-lysine-coated 24-well plates, and treated with TNF-α or IL-1α/β in the presence or absence of protein kinase inhibitor and protein synthesis inhibitor. The inhibitors were dissolved in dimethyl sulfoxide (U0126 and SB 203580) or directly in the medium (cycloheximide and JNK II) and were added 30 min before the onset of secretagog treatment. The final concentrations of dimethyl sulfoxide never exceeded 0.1%. For acute secretion experiments under high K^+ stimulation, medium was collected for analysis of secreted met-ENK 60 min after addition of 40 mM K^+ (isotonic replacement of NaCl in complete medium).

RIAs

The SN peptide was iodinated by the chloramine-T method and separated from free iodine on a Sep-Pak C18 cartridge using a gradient of acetonitrile (20–52%) in water/0.1% trifluoroacetic acid. The concentration of SN in the culture medium was assayed using ^{125}I-labeled SN as a tracer and a rabbit antiserum against bovine SN at a final dilution of 1:60,000, as previously described (24). Met-ENK was assayed in aliquots of culture medium using [^{125}I]met-ENK as a standard and the RB4 rabbit antiserum, as previously described (72).

RNA Extraction and Northern Blot Analysis

RNA was harvested from individual cell culture wells by adding Tri-Reagent (Sigma-Aldrich) according to the manufacturer's instructions. RNA was electrophoresed on denaturing agarose gels, blotted on Hybond NX nylon membranes (Amersham Pharmacia Biotech, Les Ulis, France), and fixed by UV irradiation. The filters were hybridized with a 773-bp fragment of bovine SgII cDNA, corresponding to nucleotides 781-1554 of the original clone described by Fischer-Colbrie *et al.* (73), and a 468-bp fragment of bovine GAL cDNA, which were labeled by random priming (70), or with an antisense 48-mer oligonucleotide, 5′-TTCTGCAGCATCCTTGGAGGACACCTCTTCTGTCACCTCTTTCGGCTC-3′, corresponding to nucleotides 488–535 of the bovine CgA cDNA (GenBank accession no. X04298), which was labeled by terminal deoxynucleotidyl transferase (Promega) in the presence of [α-^{32}P]dCTP (Amersham Pharmacia Biotech). The membranes were then exposed to fluorescent screens that were subsequently scanned using a STORM PhosphorImager 840 system (Amersham Pharmacia Biotech). The mRNA signals were quantified using ImageQuant 5.1 software (Amersham Pharmacia Biotech) and were corrected for RNA loading variations by scanning the ethidium bromide-stained 18S rRNA of each sample using the DensyLab 2.0.5 software (Bioprobe Systems, Montreuil, France). Statistical analysis was performed using Student's *t* test.

Quantitative RT-PCR

Approximately 1 μg of total RNA extracted as described above was submitted to DNAse I (RNAse free; Promega) digestion and reverse transcribed using random hexamers pdN_6 (Amersham Pharmacia Biotech) and SuperScript II RNase H^- reverse transcriptase (Invitrogen). Gene-specific forward and reverse primers for VIP mRNA amplification, 5′-TTGAGTCCCTTATTGGAAAACGA-3′ and 5′-AGCATCTGAGTGGCGTTTGA-3′, respectively, were chosen using the Primer Express 2 software (Applied Biosystems, Courtaboeuf, France). Real-time PCR (Q-RT-PCR) was carried out in a premade reaction mix (Applied Biosystems, Foster City, CA) in the presence of the transcribed cDNA and 90 nM specific primers, using an ABI Prism 7000 (Applied Biosystems). Relative amounts of VIP mRNA were determined from a standard curve generated using different dilutions of the cDNA and by normalizing against a nonvariable control gene, glyceraldehyde-3-phosphate dehydrogenase, that was analyzed in parallel on the same reverse transcription.

RT-PCR, DNA Cloning, and Sequencing

Total RNA was isolated from primary cultures of bovine adrenochromaffin cells using the Tri-Reagent (Sigma-Aldrich).

Approximately 5 μg of total RNA were reverse transcribed using an oligo(deoxythymidine)$_{12-18}$ primer (Invitrogen) or random hexamers (Promega), and the SuperScript II RNAse H$^-$ reverse transcriptase (Invitrogen), in the buffer supplied with the enzyme. Bovine genomic DNA was purified from adenal cortex tissue through proteinase K digestion followed by phenol extraction. The following forward and reverse primers were used to amplify TNF-R1 and TNF-R2, respectively: 5′-TACATCTCCTGTGACCGGTC-3′; 5′-GCTGGCTTCCCACTTCTGAAC-3′, and 5′-CTCGACCAGCAGCACGGAC-3′; 5′-GCTGGCGTCTGTGTCCCTCG-3′. PCR was performed on 5 μl of the reverse transcription reaction or 200 ng of bovine genomic DNA in the presence of 1 μM of the primers, 2.5 U Taq DNA polymerase (Promega), 200 μM deoxynucleotide triphosphate, 20 mM Tris-HCl (pH 8.4), 1.5 mM $MgSO_4$, and 50 mM KCl. Amplification was achieved in a GeneAmp PCR system 9700 (Applied Biosystems) through 30 cycles of DNA denaturation at 94 C for 45 sec, primer hybridization at 50 C for 45 sec, and DNA elongation at 72 C for 1 min. PCR products were then electrophoresed on a 1.5% agarose gel, and DNA fragments with the expected size were purified using the QIAquick Gel Extraction kit (QIAGEN, Courtaboeuf, France), and ligated into the pGEMT vector (Promega). Cloned DNA fragments were sequenced using the Thermosequenase kit (Amersham Pharmacia Biotech), and the DNA sequences were acquired with the LI-COR 4000L sequencer (ScienceTec, Les Ulis, France).

Western Blot Analysis

Protein extracts were obtained by lysis of the cells with 10 mM Tris-HCl (pH 7.4), 0.05% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride followed by sonication (three times for 10 sec) of the lysate. After centrifugation (12,000 × *g*, 15 min), the supernatant was precipitated with 10% trichloroacetic acid, and the protein precipitate was washed by ethanol/ether (vol/vol) and dissolved in electrophoresis-denaturing buffer. Proteins were quantitated by the Bradford protein assay (Bio-Rad, Marnes-la-Coquette, France) and analyzed by SDS-PAGE followed by electrotransfer onto nitrocellulose membranes (Amersham Pharmacia Biotech). The expression of the SgII protein was examined using a rabbit antiserum directed against the SgII-processing product EM66 (28). This antiserum was used at a 1:4,000 dilution. The activation of ERK 1/2 was examined by using the rabbit polyclonal antibody Anti-ACTIVE MAPK pAb (Promega) at a 1:5,000 dilution. The total amount of the enzyme (active and inactive) was determined with the Anti-ERK 1/2 pAb polyclonal antibody (Promega) at a 1:5,000 dilution. The enzyme-antibody complexes were visualized by the chemiluminescence enhanced chemiluminescence Western blotting analysis system (Amersham Pharmacia Biotech).

Immunocytochemistry

Chromaffin cells were plated at a density of 5 × 10^5 cells/ml on poly-L-lysine-coated glass coverslips and incubated overnight in serum-free medium. The cells were treated with 10 nM TNF-α, 50 nM PACAP, or 100 nM TPA for 5 min at 37 C. The cells were washed twice with PBS at room temperature and fixed with 4% paraformaldehyde in PBS for 20 min. After two washes, the cells were permeabilized for 5 min in a solution of PBS containing 0.1% BSA and 0.5% Triton X-100 and then blocked with 3% normal goat serum in PBS containing 0.1% BSA during 20 min at room temperature to reduce nonspecific staining. After a wash, coverslips were incubated with the Anti-ACTIVE MAPK antibody at a 1:400 dilution in PBS containing 0.1% BSA overnight at 4 C. The cells were washed with PBS and then incubated for 1 h at room temperature with goat antirabbit Igs coupled to Alexa-488, diluted 1:100. Finally, the coverslips were washed with PBS, rinsed with distilled water, mounted in PBS-glycerol (1:1), and examined using a confocal laser scanning microscope (Leica, Heidelberg, Germany) equipped with a diaplan DMRXA2 optical system and an argon ion laser. To verify the specificity of the immunoreaction, the primary or secondary antibodies were substituted with PBS.

EMSA

Chromaffin cells were treated with 10 nM TNF-α for 2 h or 24 h, in the absence or presence of the MAPK ERK 1/2 inhibitor U0126 (10 μM), and nuclear extracts were prepared as described previously (71). Double-stranded oligonucleotides containing consensus cAMP response element (CRE), 5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′, mutant CRE (mCRE), 5′-AGAGATTGCCAAGATGTGGAGAGCTAG-3′, TPA response element (TRE), 5′-GTTGATGAGTCAGCCGGAA-3′, mutant TRE (mTRE), 5′-GTTGACAGAGACGCCGGAA-3′or NF-κB response element, 5′-AGTTGAGGGGACTTTCCCAG-3′, were labeled by fill-in reaction using [α-^{32}P]dCTP and the Klenow enzyme, and used in gel-shifting studies. Chromaffin cell nuclear extracts (2 μg) were incubated at room temperature for 10 min in a 10-μl volume reaction containing 10 mM Tris-HCl (pH 7.5), 25 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 4% Ficoll, and 0.1 μg/μl poly(dI-dC) (Amersham Pharmacia Biotech). The ^{32}P-labeled TRE, mTRE, CRE, mCRE, or NF-κB oligonucleotide (40,000 cpm per reaction) was added, and the incubation continued for 20 min. In competition studies, the nuclear extract was incubated with the unlabeled homologous probe before the labeled probe. In the supershift EMSA, specific antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were incubated with the nuclear extracts for 30 min before addition of the labeled oligonucleotides. The antibodies used (1 μl per reaction) were affinity-purified IgG fractions recognizing ATF1 p35, CREB-1 p43, and CRE modulator-1 in the case of the anti-ATF1 antibody, all known Fos homologs in the case of the anti-Fos antibody and all known Jun homologs in the case of the anti-Jun antibody, as previously reported (33). The DNA-protein complexes were analyzed on 3.75% nondenaturing polyacrylamide gels in 0.25× TBE (10 mM Tris-borate, pH 8.3; 1 mM EDTA). The gels were then dried and exposed to fluorescent screens that were subsequently scanned using a STORM Phosphor Imager 840 system.

Transient Transfection Experiments

Chromaffin cells were plated in poly-L-lysine-coated 12-well plates at a density of 10^6 cells per well in complete medium and transfected 24 h later using the ProFection mammalian transfection system (Promega) as described previously (33). The cells were transfected with 1 μg of either the promoterless plasmid pLuc-MCS (Stratagene, La Jolla, CA), the AP-1 *cis*-reporting plasmid pAP-1-Luc driven by repeats of TRE elements (Stratagene), or the control plasmid pC/EBP-Luc containing repeats of CCAAT-enhancer binding protein elements (Stratagene), together with 2 μg of a rous sarcoma virus-β-galactosidase vector (33). In ERK transactivation experiments, 50 ng of the pFC-MEKK plasmid, which encodes a constitutively active MAPK kinase kinase under the control of the cytomegalovirus promoter (Stratagene), or the empty plasmid pcDNA1 with the cytomegalovirus promoter only (Invitrogen) were cotransfected with either pAP-1-Luc or pC/EBP-Luc. After overnight transfection, the cells were washed twice with complete medium and allowed to recover for 8 h before treatment with TNF-α (10 nM) overnight. After a wash in PBS, the cells were harvested in 200 μl of reporter lysis buffer (Promega), and 20 μl of the lysate was used for the luciferase or β-galactosidase assays in a luminometer (Berthold Lumat LB 9507, Evry, France).

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