Expression of the Transcription Factor Egr-1 in Pancreatic Acinar Cells Following Stimulation of Cholecystokinin or Gαq-Coupled Designer Receptors

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Abstract
Background/Aims: The injection of cerulein, an analogue of the pancreatic secretagogue cholecystokinin (CCK), induces acute pancreatitis in mice that is accompanied by the synthesis of the transcription factor Egr-1. The signaling cascade that connects cerulein stimulation with enhanced Egr-1 biosynthesis was analyzed. Methods: AR42J rat pancreatic acinar cells were used as a model system to measure cerulein-induced Egr-1 biosynthesis. For comparison, the signaling cascade induced by activation of Gαq-coupled designer receptors with the designer drug clozapine-N-oxide (CNO) was investigated. Results: Stimulation of AR42J cells with cerulein induced a robust and transient biosynthesis of Egr-1. The signaling cascade connecting cerulein stimulation with Egr-1 gene expression required elevated levels of cytosolic Ca2+ and the activation of the protein kinases PKC, Raf and ERK, while expression of MKP-1 prevented Egr-1 biosynthesis in cerulein-stimulated AR42J cells. In addition, ternary complex factors are required to connect cerulein stimulation with enhanced transcription of the Egr-1 gene. Egr-1 biosynthesis induced in CNO-stimulated AR42J pancreatic acinar cells expressing Gαq-coupled designer receptors required identical signaling molecules, although subtle differences were observed in comparison to cerulein/CCK receptor signaling. Conclusion: We propose that overstimulation of the canonical Gαq-induced signaling pathway may be crucial for inducing acute pancreatitis.
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**Introduction**

Cerulein, a ten amino acid oligopeptide derived from the skin of the Australian green tree frog (Litoria caerulea), is a cholecystokinin (CCK) analogue that activates both CCK1 and CCK2 receptors [1]. Injection of a supraphysiological concentration of cerulein is used in an animal model of acute pancreatitis, that is characterized by massive tissue damage of pancreatic acinar cells, a local pancreatic inflammation, and an infiltration of inflammatory cells into the pancreas. A premature activation of trypsinogen within pancreatic acinar cells is generally believed to be the initiating event for the tissue-damage of the pancreas [2].

This local inflammation of the pancreas is accompanied by the synthesis and release of proinflammatory chemokines and cytokines, including tumor necrosis factor-α (TNFα), macrophage chemotactic protein (MCP-1), macrophage inflammatory protein-1 (MIP-1), and interleukin 1β [3-6]. In addition, an upregulation of the intracellular adhesion molecule ICAM-1 has been reported [4]. Interestingly, the genes encoding TNFα, MCP-1, MIP-1, and ICAM-1 have been described as target genes for the zinc finger transcription factor Egr-1 [7-14].

Egr-1 is regulated via its biosynthesis. In unstimulated cells, only low levels of Egr-1 are detectable. Upon stimulation by extracellular signaling molecules including growth factors, hormones, and neurotransmitters, the biosynthesis of Egr-1 is induced [15, 16]. Egr-1 couples extracellular signals with long-term responses by altering the gene expression pattern of Egr-1 target genes. The fact that Egr-1 regulates expression of genes encoding proinflammatory chemokines and cytokines is the basis for the hypothesis that Egr-1 functions as a master switch during inflammation. The analysis of Egr-1-deficient mice revealed that inactivation of the Egr-1 gene diminished expression of mediators for vascular injury in an animal model of lung ischemia/reperfusion [9]. Egr-1 is synthesized in pancreatic acinar cells within 1-4 hours following induction of acute pancreatitis by cerulein injection [11, 14, 17]. Moreover, expression of inflammation-related gene products, including MCP-1, MIP-1 and ICAM-1, and lung inflammation were reduced in cerulein-treated Egr-1-deficient mice, linking Egr-1 activity with the severity of cerulein-induced acute pancreatitis [11, 14, 17].

In this study, we have analyzed the signaling cascade leading to enhanced Egr-1 biosynthesis in cerulein-stimulated AR42J pancreatic acinar cells. Additionally, we have investigated the intracellular signaling cascade following stimulation of Gαq-coupled designer receptors in AR42J cells. The results show that both intracellular signaling cascades, induced by either stimulation of CCK receptors with cerulein or stimulation of Gαq-coupled designer receptors with CNO, require a rise of intracellular Ca²⁺, the activation of the protein kinases PKC, Raf, and ERK, and in the nucleus, the activation of the transcription factor Elk-1.

**Materials and Methods**

**Cell culture**

AR42J cells were kindly provided by Bernd Baumann, University of Ulm, Germany, and maintained in Dulbecco’s modified Eagles medium supplemented with 10% heat inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in 5% CO₂. Cells were cultured in medium containing 0.05 % fetal bovine serum for 24 hours prior to the stimulation. Stimulation with cerulein (10 nM, Sigma # C9026, dissolved in water) was performed for 1 hour if not stated differently. Likewise, stimulation with either clozapine-N-oxide (CNO, 1 µM, Enzo Life sciences, # NS-105-0005, dissolved in ethanol), EGF (10 nM, Promega, Mannheim, Germany, # G5021, dissolved in H₂O as a 100 µg/ml stock solution), or phorbol 12-O-tetradecanoylphorbol-13-acetate (TPA, 50 ng/ml, Calbiochem # 524400-1, dissolved in DMSO) was performed for 1 hour in medium containing 0.05 % fetal bovine serum. Cells were pre-incubated for 2 hours with either LY225910 (10 µM, Tocris # 1018, dissolved in DMSO), YM022 (10 µM, Sigma # SML0220, dissolved in DMSO), or lorglumide (10 µM, Sigma # L109, dissolved in methanol) prior to the stimulation. Cells were pre-incubated for 3 hours with either bisindolylmaleimide III (2 µM, ENZO ALX-270-051, dissolved in DMSO), BAPTA-AM (50 µM, Tocris # 2787, dissolved in DMSO), PD98059 (50 µM, Calbiochem # S513000,
dissolved in DMSO), SP600125 (5 μM, ENZO # BML-E1305-0010, dissolved in DMSO), or SB203580 (5 μM, ENZO # BML-E1286-0001, dissolved in DMSO) prior to the stimulation with either cerulein, EGF or TPA. Incubation of the cells with TPA (50 ng/ml) to reduce PKC expression was performed for twenty-four hours. 4-Hydroxytamoxifen (4-OHT, Sigma # H7904) was dissolved in ethanol and used at a concentration of 0.25 μM.

Retroviral gene transfer

Plasmid pBABEpuro3AB-Raf:ER, encoding a B-Raf/estrogen receptor fusion protein, was kindly provided by Martin McMahon, UCSF, San Francisco, USA [18]. The packaging cell line Phoenix-Ampho was obtained from Gary Nolan, Stanford University, USA. Phoenix-Ampho cells were transfected with the retroviral vector using the calcium coprecipitation procedure. Retroviral infection of AR42J cells was performed as described [19]. AR42J cells were selected with 0.25 μg puromycin/ml. Mass pools of stable transfectants were selected and used for all experiments in order to eliminate the possibility of specific clonal effects.

Lentiviral gene transfer

The lentiviral transfer vectors pFUW-Rαq, pFUW-REST/Elk-1ΔC, pFUW-MKP-1, pFUW-MKP-5, and pFUWmycDA-Raf1 have been described previously [20-23]. The viral particles were produced by triple transfection of HEK293T/17 cells with the gag-pol-rev packaging plasmid, the env plasmid encoding VSV glycoprotein, and the transfer vector [24].

Preparation of cell extracts and Western Blot analysis

Nuclear extracts and whole cell extracts were prepared as previously described [25]. 20 μg of nuclear proteins were separated by SDS-PAGE and the blots were incubated with an antibody directed against Egr-1 (Santa Cruz, Heidelberg, Germany, # sc-189). An antibody directed against HDAC1 (Santa Cruz, Heidelberg, Germany, # sc-81598) was used as a loading control as described recently [23, 26]. To detect FLAG-tagged proteins, we used the M2 monoclonal antibody directed against the FLAG epitope (Sigma-Aldrich, Steinheim, Germany, # F3165), at 1:3000 dilution in TBST. Antibodies against the myc epitope were prepared from CRL-1729 hybridomas, purchased from ATCC. Immunoreactive bands were detected via enhanced chemiluminescence as described [23, 26].

RT-PCR

Total RNA isolated from AR42J cells was purified with the Qiagen RNeasy Plus Micro Kit (Cat.No: 74034). RNA samples were reverse transcribed into cDNA with RevertAid M-MuLV RT (Fermentas) in the presence of RNase Inhibitor (Fermentas). The PCR conditions were: one cycle at 95°C for 5 min, 32 amplification cycles, each cycle consisted of denaturation at 95°C for 30 sec, primer annealing at 59°C for 30 sec, and extension at 72°C for 30 sec. RT-PCR was performed using the primer pairs 5’-GAC TCG GTA GCC CAT TAC AAT C-3’/5’-ACT TTC CCA AGT AGG TCA CGG-3’ (Egr-3) and 5’-TTG TGA TGG GTG TGA ACC AC-3’/5’-GTC TTC TGG GTG GCA GTG AT-3’ (GAPDH).

Results

Bio synthesis of Egr-1 in cerulein-treated AR42J cells

AR42J pancreatic acinar cells were serum-starved for 24 hours and then incubated with cerulein for 1 hour. Egr-1 immunoreactivity was virtually undetectable in the absence of stimulation. In contrast, cerulein treatment strikingly increased the biosynthesis of Egr-1 (Fig. 1A). The expression of HDAC1 was analyzed as a loading control. An analysis of the time frame revealed that Egr-1 biosynthesis was transient with a peak expression occurring 1 hour following stimulation (Fig. 1B). Cerulein treatment also increased the level of Egr-3 mRNA (Fig. 1C). However, we did not detect Egr-3 by Western blot analysis, indicating that the Egr-3 levels are low in AR42J cells. We therefore focused our studies on the stimulus-induced biosynthesis of Egr-1.
Fig. 1. Stimulation of AR42J pancreatic acinar cells with cerulein induces the biosynthesis of Egr-1. (A) AR42J cells were serum-starved for twenty-four hours and then treated with cerulein (10 nM) for 1 hour. Nuclear extracts were prepared and subjected to Western blot analysis. The blots were incubated with an antibody directed against Egr-1. As a control, expression of histone deacetylase-1 (HDAC1) was analyzed. (B) Serum-starved AR42J cells were treated with cerulein (10 nM) for 1, 3, 5, 8 or 24 hours as indicated and expression of Egr-1 was analyzed by immunoblotting. (C) Stimulation of AR42J pancreatic acinar cells with cerulein upregulates Egr-3 mRNA levels. AR42J cells were stimulated with cerulein for 1 hour. Total RNA was isolated, the mRNA reverse transcribed and the cDNA analyzed by PCR using primers to detect Egr-3 and GAPDH mRNAs. For quantification, the Egr-3 signal was normalized to the GAPDH signal and the fold-stimulation was calculated. (D-F) Serum-starved AR42J cells were preincubated with the CCK receptor inhibitors YM022 (10 µM) (D), LY225910 (10 µM) (E), or lorglumide (10 µM) (F) for 2 hours. Cells were then stimulated with cerulein (10 nM) for 1 hour in the presence of the inhibitors. Nuclear extracts were prepared and analyzed by immunoblotting (G). Schematic representation of the Gαq-coupled designer receptor rM3Dq. The backbone is the rat M3 muscarinic acetylcholine receptor. Two point mutations (Y148C and A238G) were introduced into the orthosteric binding pocket of the natural ligand within the third and fifth transmembrane domain to prevent binding of acetylcholine to the designer receptor. Gαq-coupled designer receptors were specifically activated by clozapine-N-oxide (CNO). (H) Induction of Egr-1 biosynthesis in CNO-stimulated AR42J cells expressing Gαq-coupled designer receptor. Cells were infected with a lentivirus encoding the Gαq-coupled designer receptor. The infected cells were cultured for twenty-four hours in medium containing 0.05% serum. Stimulation with CNO (1 µM) for 1 hour was performed with medium containing 0.05% serum. Nuclear extracts were prepared and subjected to Western blot analysis using an antibody directed against Egr-1. Each experiment illustrated here and in all subsequent figures was repeated at least twice with consistent results.

Cerulein-induced biosynthesis of Egr-1 in AR42J cells requires CCK2 receptors

AR42J cells express both CCK1 and CCK2 receptors [27]. Cerulein binds and activates both CCK receptors [1]. We used a pharmacological strategy to investigate the impact of both CCK1 and CCK2 receptors on cerulein-induced upregulation of Egr-1 expression. AR42J
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Fig. 2. Chelation of intracellular Ca\(^{2+}\) attenuates expression of Egr-1 in cerulein-stimulated AR42J pancreatic acinar cells. AR42J cells (A, C) or AR42J cells expressing Gaq-coupled designer receptors (B) were preincubated for 3 hours with the calcium chelator BAPTA-AM (50 µM). Cells were stimulated with either cerulein (10 nM) (A), CNO (1 µM) (B), or EGF (10 nM) (C) for 1 hour. Nuclear extracts were prepared and subjected to Western blot analysis. The blots were incubated with an antibody directed against Egr-1.

Cells were preincubated with the CCK2 inhibitors YM022 or LY225910, or with the CCK1 inhibitor lorglumide. Figs 1D-F show that both CCK2 inhibitors reduce expression of Egr-1 in cerulein-stimulated AR42J cells, while lorglumide did not interfere with upregulation of Egr-1 as a result of cerulein stimulation.

Bio-synthesis of Egr-1 in AR42J cells following stimulation of Gaq-coupled designer receptors with a designer drug

CCK receptors are G-protein coupled receptors that activate phospholipase C via the G protein Gaq. For comparison, we expressed a Gaq-coupled designer receptor in AR42J cells (Fig. 1G) and stimulated the cells with the designer drug clozapine-N-oxide (CNO). Fig. 1H shows that stimulation of Gaq-coupled designer receptors triggered an upregulation of Egr-1 in pancreatic AR42J cells.

Role of Ca\(^{2+}\) ions in cerulein-induced Egr-1 biosynthesis in AR42J pancreatic acinar cells

Stimulation of Gaq-coupled receptors leads to the activation of phospholipase C, the generation of IP\(_3\) and the release of Ca\(^{2+}\) ions into the cytosol via stimulation of ionotropic IP\(_3\) receptors of the endoplasmic reticulum. We assessed the importance of elevated [Ca\(^{2+}\)] for the signaling cascade connecting cerulein-induced CCK2 receptor activation with Egr-1 gene transcription. Prior to stimulation with cerulein, AR42J cells were incubated in the presence or absence of BAPTA-AM, the cell-permeable acetoxymethylester of the cytosolic Ca\(^{2+}\) chelator BAPTA. When the cerulein-induced elevation of [Ca\(^{2+}\)], was precluded by the preincubation with BAPTA-AM, the stimulus-induced upregulation of Egr-1 was reduced (Fig. 2A). Likewise, preincubation of the cells with BAPTA-AM interfered with the Egr-1 biosynthesis in CNO-stimulated AR42J cells expressing Gaq-coupled designer receptors (Fig. 2B). As a negative control, we stimulated the cells with EGF. Chelating intracellular Ca\(^{2+}\) with BAPTA-AM had no effect on the EGF-induced signaling cascade leading to Egr-1 expression (Fig. 2C).

PKC connects Gaq-coupled receptor activation with Egr-1 biosynthesis

The rise in intracellular [Ca\(^{2+}\)] following stimulation of Gaq-coupled receptors may activate protein kinase C (PKC), as shown in different cell types [28, 29]. Stimulation of AR42J
cells with CCK has been shown to activate PKC [27]. To assess the involvement of PKC in the signaling cascade connecting cerulein stimulation with enhanced Egr-1 expression, we incubated AR42J cells with the protein kinase C inhibitor bisindolylmaleimide III (2 μM) for 3 hours as indicated. Cells were stimulated with either cerulein (10 nM) (A), or CNO (1 μM) (B) for 1 hour. Nuclear extracts were prepared and subjected to Western blot analysis. The blots were incubated with an antibody directed against Egr-1. (C, D) AR42J cells (C) or AR42J cells expressing Gαq-coupled designer receptors (D) were preincubated with the phorbol ester TPA (50 ng/ml) for 24 hours to reduce expression of PKC. Cells were stimulated with either cerulein (10 nM) (C), or CNO (1 μM) (D) for 1 hour. Nuclear extracts were prepared and subjected to Western blot analysis. The blots were incubated with an antibody directed against Egr-1.

**Fig. 3.** Cerulein-regulated expression of Egr-1 requires the activation of PKC. AR42J cells (A) or AR42J cells expressing Gαq-coupled designer receptors (B) were preincubated with either vehicle or the PKC inhibitor bisindolylmaleimide III (2 μM) for 3 hours as indicated. Cells were stimulated with either cerulein (10 nM) (A), or CNO (1 μM) (B) for 1 hour. Nuclear extracts were prepared and subjected to Western blot analysis. The blots were incubated with an antibody directed against Egr-1. (C, D) AR42J cells (C) or AR42J cells expressing Gαq-coupled designer receptors (D) were preincubated with the phorbol ester TPA (50 ng/ml) for 24 hours to reduce expression of PKC. Cells were stimulated with either cerulein (10 nM) (C), or CNO (1 μM) (D) for 1 hour. Nuclear extracts were prepared and subjected to Western blot analysis. The blots were incubated with an antibody directed against Egr-1.

**Activation of Raf is essential for the induction of Egr-1 biosynthesis in AR42J cells stimulated with cerulein**

Elevated intracellular Ca²⁺ concentrations as a result of stimulation of Gαq-coupled receptors trigger an activation of ERK, a crucial factor for the induction of Egr-1 biosynthesis in many cell types [21, 30-32]. In pancreatic acinar cells, CCK stimulation activates ERK [33]. The connection between an elevated Ca²⁺ concentration and the activation of the ERK signaling pathway is accomplished by PKC, that in turn, activates the protein kinase Raf. To demonstrate the importance of Raf in activating Egr-1 biosynthesis, we generated a AR42J...
Fig. 4. Essential role of Raf in connecting cerulein stimulation with enhanced Egr-1 transcription in AR42J pancreatic acinar cells. (A) Schematic representation of the modular structure of B-Raf and ΔB-Raf:ER. The functional domains of B-Raf (CR3, CR2, and CR1) are depicted. Fusion of the catalytic CR3 domain to the hormone binding domain of the estrogen receptor generates the ΔB-Raf:ER fusion protein. (B) Activation of B-Raf signaling pathway in AR42J cells via expression of a ΔB-Raf/estrogen receptor fusion protein triggers the biosynthesis of Egr-1. AR42J-ΔB-Raf:ER cells were serum-starved for twenty-four hours. Cells were stimulated with 4-OHT (250 nM) as indicated. (C) Modular structure of A-Raf and DA-Raf1. (D) Expression of DA-Raf1 in lentiviral-infected AR42J cells was detected using an antibody against the N-terminal myc-tag. (E) AR42J cells were infected with a lentivirus encoding DA-Raf1. As a control AR42J cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). Cells were cultured for twenty-four hours in medium containing 0.05% serum. Stimulation was performed with medium containing cerulein (10 nM). Nuclear extracts were prepared and subjected to Western blot analysis. The blots were incubated with antibodies directed against either Egr-1, or HDAC1 as indicated. (F) AR42J cells were infected with a lentivirus encoding Gαq-coupled designer receptors. Cells were cultured for twenty-four hours in medium containing 0.05% serum. Stimulation was performed with medium containing CNO (1 µM). Nuclear extracts were prepared and analyzed by immunoblotting using antibodies directed against either Egr-1 or HDAC1.
phosphorylation of the MAP kinase kinase by Raf. Fig. 4C shows the modular structure of DA-Raf1, a splicing isoform of A-Raf that functions as an antagonist of the Raf/MEK-ERK1/2 signaling pathway [35]. Cellular proteins of mock-infected AR42J cells or cells infected with a myc-tagged DA-Raf1 encoding lentivirus were fractionated by SDS-PAGE. The fusion protein was identified by Western blot analysis using an antibody targeting the myc epitope (Fig. 4D). Next, the functional implication of DA-Raf1 expression was assessed. The results show that expression of DA-Raf1 significantly reduced the upregulation of Egr-1 expression in cerulein-stimulated AR42J cells (Fig. 4E). Likewise, DA-Raf1 reduced the expression of Egr-1 in CNO-stimulated AR42J cells expressing G\(\alpha\)q-coupled designer receptors (Fig. 4F). In addition, preincubation of the cells with PD98059 (50 \(\mu\)M, as suggested [36]) efficiently blocked the upregulation of Egr-1 in cerulein-stimulated AR42J cells, or in CNO-stimulated AR42J cells expressing a G\(\alpha\)q-coupled designer receptor (Figs. 5A, B). Incubation of AR42J cells with PD98059 also blocked the upregulation of Egr-1 in EGF-treated cells (Fig. 5C). In contrast, the MAP kinase inhibitors SB203580 or SP600125, described as p38 or JNK inhibitors, did not interfere with the upregulation of Egr-1 following stimulation of AR42J cells with cerulein (Figs. 5D and E).

Overexpression of MKP-1 prevents cerulein-induced Egr-1 expression

MKP-1, the enzyme that dephosphorylates ERK in the nucleus, is synthesized in different cell types following ERK activation, suggesting that MKP-1 is part of a negative feedback loop that inactivates ERK [22, 29, 37, 38]. Fig. 6A shows that the biosynthesis of Egr-1 was impaired in cerulein-stimulated AR42J cells that had been infected with a MKP-1 encoding lentivirus. These data indicate that active ERK in the nucleus was required within
the signaling cascade. Likewise, Egr-1 biosynthesis was attenuated in CNO-stimulated AR42J cells expressing Gq-coupled designer receptors (Fig. 6B). In contrast, expression of MKP-5, the phosphatase that dephosphorylates and inactivates p38 and c-Jun N-terminal protein kinases in the nucleus, did not significantly interfere with the upregulation of Egr-1 following stimulation of CCK2 receptors with cerulein or Gq-coupled designer receptors. Cells were cultured for twenty-four hours in medium containing 0.05% serum. Stimulation was performed with medium containing CNO (1 µM). Nuclear extracts were prepared and analyzed by immunoblotting using antibodies directed against either Egr-1 or HDAC1.

**Suppression of ternary complex factor activity impairs Egr-1 biosynthesis in cerulein-stimulated AR42J pancreatic acinar cells**

The serum response elements (SRE) within the Egr-1 promoter function as binding sites for the serum response factor (SRF) and ternary complex factors (TCF). TCFs are proteins that contact DNA and also bind to SRF. We assessed the impact of ternary complex factor activation on the regulation of Egr-1 biosynthesis in cerulein-stimulated AR42J cells. To overcome the problem associated with redundancy of functions between ternary complex factors [39], we expressed a dominant-negative mutant of the ternary complex factor Elk-1, termed REST/Elk-1ΔC. The mutant retains the DNA-binding and SRF interaction domain, but lacks the C-terminal activation domain of Elk-1 (Fig. 7A). REST/Elk-1ΔC additionally contains the N-terminal repression domain of the transcriptional repressor REST [40], an immunological tag used for detection of the protein (FLAG epitope) and a nuclear localization
signal (NLS). Nuclear proteins of mock-infected AR42J cells or cells infected with a REST/Elk-1ΔC encoding lentivirus were fractionated by SDS-PAGE. The fusion protein was identified by Western blot analysis using antibodies targeting the FLAG epitope. Fig. 7B shows that the REST/Elk-1ΔC fusion protein was synthesized as expected. Next, the functional implications of REST/Elk-1ΔC expression were assessed. The results show that REST/Elk-1ΔC blocked the biosynthesis of Egr-1 in cerulein-stimulated AR42J cells (C). Likewise, stimulation of Gaq-coupled designer receptors did not induce an upregulation of Egr-1 in AR42J cells expressing the dominant-negative mutant of Elk-1, REST/Elk-1ΔC (D). Thus, TCF activity is essential for upregulating Egr-1 gene transcription as a result of CCK receptor or Gaq-coupled designer receptor stimulation.

Discussion

Acute pancreatitis is an inflammatory disease that is life-threatening in its severe form. The disease is accompanied by an activation of pancreatic enzymes in the pancreas and a subsequent exaggerated inflammatory response that is not limited to the pancreas but
includes other tissues, most importantly the lung. The precise molecular events leading to the induction of acute pancreatitis have not yet been elucidated in detail. There are several animal models of experimental pancreatitis. One of the best-characterized and frequently used model is based on the injection of the CCK analogue cerulein into mice. This treatment results in a pancreatitis phenotype including trypsin activation in the pancreas, cytoplasmic vacuolization, edema formation, and infiltration of inflammatory cells into the pancreas. The fact that expression of the inflammatory transcription factor Egr-1 has been causally connected with acute pancreatitis [11, 14, 17] provided the stimulus for investigating the signal transduction following cerulein stimulation of pancreatic acinar cells. The objective of this study was the elucidation of the cerulein-induced signaling cascade in AR42J pancreatic acinar cells leading to enhanced expression of Egr-1.

Cerulein binds to both CCK1 and CCK2 receptors that both are Gq-coupled receptors [41]. However, G protein-coupled receptors may couple to more than one G protein with the result that distinct signaling pathways are activated. In cardiomyocytes, for instance, the β2 adrenergic receptor couples to both Gαs and Gαi [42]. The GnRH receptor couples to Gq in αT3-1 gonadotrophs, while an interaction with Gαi has been observed in prostate cancer cells [43], indicating that the G protein specificity of a particular receptor may be cell type-dependent. Likewise, coupling of CCK2 receptors to pertussis-toxin sensitive Gi/o has been observed in parvalbumin-positive GABAergic basket cells of the hippocampus, while in pyramidal cells the canonical Gq-pathway is induced by CCK via activation of CCK2 receptors [44]. Therefore, we expressed for comparison a Gq-coupled designer receptor exclusively activated by designer drug (DREADD) in AR42J pancreatic acinar cells. These designer G protein-coupled receptors have been developed to specifically activate a particular G protein induced signaling cascades. The backbone for the Gq-coupled designer receptor is the rat M3 muscarinic acetylcholine receptor. Binding of acetylcholine to the designer receptor is prevented by introducing two point mutations (Y148C and A238G) into the orthosteric binding pocket of the natural ligand within the third and fifth transmembrane domain of the receptor. This receptor lacks constitutive activity, is unresponsive to the endogenous ligand acetylcholine, but can be activated by the otherwise pharmacologically inert compound clozapine-N-oxide (CNO) [45, 46]. The results of this study show that cerulein signals via the CCK2 receptor in AR42J cells to induce Egr-1 expression. Moreover, the data reveal that cerulein-stimulated CCK2 receptor signaling and CNO-induced Gq-coupled designer receptor signaling activated identical intracellular signaling molecules, indicating that cerulein-induced pancreatitis relies on the overstimulation of the canonical Gq-characterized signaling cascade.

Hallmarks of Gq-induced signaling are the rise in intracellular Ca²⁺ and the subsequent activation of PKC. Pharmacological experiments confirm that both Ca²⁺ ions and phorbol ester responsive PKC isoforms are crucial for the signaling connecting CCK2 receptor activation with enhanced Egr-1 gene transcription. These data support the view that CCK2 receptor stimulation leads to an activation of the Gq-signaling pathway. In fact, activation of PKCε has been demonstrated to be crucial for CCK-mediated activation of ERK [27]. A comprehensive analysis of Gq-coupled designer receptor signaling revealed that stimulation of this receptor type activates the protein kinases Raf and extracellular signal-regulated protein kinase, as shown in studies analyzing AT₁ angiotensin II receptors, M3 muscarinic acetylcholine receptors, gonadotropin releasing hormone receptors, calcium-sensing receptors, and Gq-coupled designer receptors [20, 21, 31, 47]. The data presented here show that both Raf and ERK are required to connect cerulein stimulation with Egr-1 gene transcription in AR42J pancreatic acinar cells.

The phosphorylated and activated ERK translocates into the nucleus and may change the transcriptional program by phosphorylating transcriptional regulatory proteins. In the nucleus, MKP-1 inactivates ERK via dephosphorylation and attenuates ERK regulated gene transcription [21, 22, 29, 38, 47, 48]. Expression of MKP-1, a dual-specific phosphatase that dephosphorylates and inactivates ERK in the nucleus, blocked the upregulation of Egr-1 following stimulation of AR42J cells with cerulein, indicating that nuclear MAP kinase
activity is essential for the signaling cascade connecting cerulein-induced CCK2 receptor stimulation with enhanced transcription of Egr-1.

A major nuclear substrate for ERK is Elk-1, a member of the Ets family of transcription factors. Elk-1 is an essential component of the serum response ternary complex that binds to DNA and to the serum response factor SRF. The transcriptional activity of Elk-1 depends on its phosphorylation-status. Elk-1 is phosphorylated by several protein kinases including ERK, leading to enhanced DNA binding, ternary complex formation and SRE-mediated transcription. Phosphorylation of Elk-1 connects the ERK signaling cascade with SRE-mediated gene transcription. The human Egr-1 promoter contains five SREs encompassing the consensus sequence CC[A/T]GG, known as the CArG box, and multiple binding sites for Elk-1 and other ternary complex factors adjacent to the CArG boxes. Transcriptional activation of Egr-1 is often preceded by an activation of Elk-1, indicating that the SREs within the Egr-1 promoter mediate signal-induced activation of Egr-1 gene transcription. To assess the necessity of ternary complex factor activation within the signaling cascade connecting cerulein stimulation with enhanced Egr-1 gene transcription, we performed loss-of-function experiments. Genetic inactivation of Elk-1 or other ternary complex factors in transgenic mice revealed minimal changes of the phenotype [39, 49, 50], suggesting that functional redundancy may exist. Therefore, we have expressed a dominant negative version of Elk-1. Due to its binding to DNA and SRF, the Elk-1 mutant REST/Elk-1ΔC most likely also inhibits the activity of two other ternary complex factors, SAP-1 and SAP-2. These experiments revealed that expression of REST/Elk-1ΔC completely blocked the stimulus-induced biosynthesis of Egr-1 in cerulein-stimulated AR42J cells as well as in CNO-stimulated AR42J cells expressing Gαq-coupled designer receptors. Thus, ternary complex factor activation is a key step in connecting Gαq-stimulation with enhanced Egr-1 biosynthesis.

The induction of acute pancreatitis via injection of cerulein into mice is a frequently used model system of this disease. Several observations suggest that activation of the proinflammatory transcription factor Egr-1 is required to induce the inflammatory response following induction of acute pancreatitis. Using the AR42J cellular model, we identified the signaling molecules Ca²⁺, Raf, ERK, MKP-1, and TCF that connect cerulein stimulation with enhanced expression of Egr-1. These molecules represent new targets for pharmacological intervention in pancreatitis. The essential role of Ca²⁺ ions, and the functions of ERK and MKP-1 have been discussed in the framework of cerulein-induced pancreatitis [6, 51-54]. Raf and TCFs have not yet been the focus of studies of the cerulein-induced signaling cascade leading to an acute pancreatitis. Future experiments, involving mouse models, will clarify the role of these signaling molecules in healthy and dysfunctional pancreatic acinar cells.

Abbreviations

CCK (cholecystokinin); CNO (clozapine-N-oxide); Egr (early growth response); ERK (extracellular signal-regulated protein kinase); MKP (MAP kinase phosphatase); PKC (protein kinase C); TCF (ternary complex factor).

Disclosure Statement

The authors declare that there is no conflict of interest.

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