Epidermal Growth Factor Induction of Apolipoprotein A-I Is Mediated by the Ras-MAP Kinase Cascade and Sp1*

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Xi-Long Zheng‡§§, Shuji Matsubara‡§§, Catherine Diao‡§§, Morley D. Hollenberg‡, and
Norman C. W. Wong‡§§**

From the Endocrine Research Group, ‡Departments of Medicine and §Biochemistry & Molecular Biology and
¶Pharmacology & Therapeutics, the Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada T2N 4N1

Insulin induces apolipoprotein A-I, apoA-I gene transcription via a membrane receptor with intrinsic tyrosine kinase activity. This finding prompted us to ask whether the gene is stimulated by epidermal growth factor (EGF), EGF a peptide hormone that binds to another member of the receptor superfamily with tyrosine kinase activity. Our data showed that like insulin, EGF increased abundance of apoA-I protein and transcription of the gene in human hepatoma, Hep G2 cells. The effects of both hormones appeared direct because their induction of apoA-I gene transcription was not affected by the protein synthesis inhibitor, cycloheximide. Although both insulin and EGF stimulate apoA-I expression, each hormone binds to a distinct membrane receptor thus suggesting differential intracellular signaling. Therefore, we used a panel of inhibitors to define the pathway(s) that mediate the actions of these hormones. Whereas, the actions of EGF required only the Ras-mitogen-activated protein, MAP kinase, those of insulin were mediated by equal participation of both the Ras-MAP kinase and protein kinase C, PKC cascades. Despite differences in signaling pathways triggered by each hormone receptor, the activation of apoA-I transcription required the participation of a single transcription factor, Sp1. Furthermore, EGF induction of transcription was attenuated by mutating the MAP kinase site at amino acid, Thr266 rendering Sp1 phosphorylation deficient. In summary, EGF stimulation of apoA-I expression is mediated solely by the Ras-MAP kinase cascade and enhanced activity of this pathway requires Sp1 with an intact phosphorylation site at Thr266. However, insulin induction of this gene is different and requires both Ras-MAP kinase and PKC pathways but their actions are also mediated by Sp1.

Apolipoprotein A-I (apoA-I) is a major protein component of HDL, the serum high-density lipoprotein (HDL) particles (1, 2). The anti-atherogenic properties of apoA-I alone or as part of HDL underlie their inverse correlation with the incidence of ischemic cardiovascular disease, the number 1 cause of premature death in modern societies (3, 4). The cardioprotective actions of apoA-I or HDL arises from their participation in a normal physiologic process, so called “reverse cholesterol transport” (5, 6). ApoA-I acts as a cofactor to facilitate an interaction between HDL particles and the cell membrane. This interaction enables the efflux of intracellular cholesterol to HDL particles, which in turn shuts the sterol to the liver for further metabolism and excretion (5, 7). Enhanced reverse cholesterol transport lowers total body cholesterol, as demonstrated clearly following the infusion of apoA-I protein into humans (8). The reduction in cholesterol lowers the risk of arteriosclerosis (9), a major cause of ischemic cardiovascular disease. In support of this idea, transgenic mice that overexpress human apoA-I protein had significant reductions of atherosclerotic lesions in vessel walls (10). Therefore, understanding the mechanisms that enhance apoA-I expression will lead us to better ways to enhance its expression and thus lower the risk of ischemic cardiovascular disease (11).

We showed recently that insulin induces rat apoA-I gene transcription and this induction is mediated by an insulin responsive core element (IRCE) a motif recognized by Sp1 (12, 13). Insulin action is initiated by its binding to a membrane receptor with intrinsic tyrosine kinase activity, prompting us to wonder whether this mechanism is unique to the peptide hormone. The actions of insulin can be categorized into immediate responses: such as glucose transport or delayed responses; including cell differentiation and proliferation (14, 15). Several intracellular pathways are activated by insulin action and may include the Ras-MAP kinase, PI 3-kinase, and phospholipase Cγ cascades (14). These pathways are not exclusive to the insulin receptor and may be used by other tyrosine kinase receptors, including that for EGF (16). Therefore, we tested the ability of another peptide hormone, epidermal growth factor, EGF on apoA-I expression. If apoA-I is inducible by EGF, it offers a new avenue to augment expression of the gene. But equally important is that this model provides an opportunity to compare or contrast the signaling mechanism(s) activated by EGF and insulin. The results summarized here show that like insulin, EGF also enhances apoA-I expression. However, whereas the actions of EGF are mediated solely by the actions of a single pathway, that of insulin requires the participation of at least two cascades.

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** Recipient of scientist awards from the Canadian Institute of Health Research and Alberta Heritage Foundation for Medical Research. To whom correspondence should be addressed: Dept. of Medicine and Biochemistry & Molecular Biology, Faculty of Medicine, University of Calgary, Health Sciences Center, 3330 Hospital Dr. NW, Calgary, Alberta T2N 4N1, Tel.: 403-220-5212; Fax: 403-270-0979; E-mail: nccwong@ucalgary.ca.
† The abbreviations used are: apoA-I, apolipoprotein A-I; HDL, high density lipoprotein; IRCE, insulin responsive core element; MAP, mitogen-activated protein; PI 3-kinase, phosphatidylinositol 3-kinase; RT-PCR, reverse transcriptase-polymerase chain reaction; EGF, epidermal growth factor; CAT, chloramphenicol acetyltransferase; PKC, protein kinase C; PDBu, phorbol 12,13-dibutyrate.

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MATERIALS AND METHODS

**Plasmid Constructs**—Construction of the reporter, pAI.474-CAT was described previously (17). The deletion constructs; pAI.425-, pAI.375-, pAI.325-, and pAI.235-CAT containing rat apoA-I DNA spanning −425, −375, −325, and −235 to −7 were synthesized using the parent pAI.474-CAT as a template in separate PCR (17). Transverse mutation of the IRCE (−411 to −404) from GAGCGCGG to TCTTATTT was accomplished using a mutant primer in a PCR (12). The RsaI+−17 retrovector and Sp1 expression plasmid were gifts from Drs. J. Stone (University of Alberta, Edmonton, Alberta, Canada) and Dr. R. Tjian (University of California, Berkeley, CA), respectively (19).

**Transient and Stable Transfection**—Human hepatoma Hep G2 cells were transiently transfected with plasmid DNA of interest using LipofectAMINE (Life Technologies, Inc.) as per the instructions recommended by the manufacturer. The efficiency of DNA uptake was monitored by co-transfecting 1 μg of the plasmid, RSV-β-galactosidase (20). Stably transfected Hep G2 cells were created by co-transfecting pAI.474-CAT (17) and the plasmid, pRF/CMV2 (Invitrogen) that carried neomycin resistance as described (13).

**Cell Culture and CAT Activity Assay**—Hep G2 cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% bovine calf serum (Life Technologies, Inc.) and penicillin/streptomycin at 37 °C. Cells were cultured overnight in serum-free medium prior to the addition of the agent(s) of interest, followed by measuring CAT activity (12, 23).

**SDS-Polyacrylamide Gel Electrophoresis and Western Immunoblotting**—Whole cell extract from control or cells treated with various factors were harvested and lysed in buffer containing orthovanadate 2 mM, Triton X-100 1%, SDS 0.1%, leupeptin and aprotonin 5 μg/ml each, benzamidine and bacitracin 1 mg/ml each, dithiothreitol 600 mM, Tris 20 mM (pH 7.4), NaCl 300 mM, EDTA 5 mM, NaF 50 mM, sodium pyrophosphate 40 mM, KH₃PO₄ 50 mM, and Na molybdate 10 mM. An aliquot of equal numbers of control or treated hepatoma cells or culture medium (10 μg of total protein) containing secreted apoA-I protein was separated by electrophoresis in a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. The blot was probed using a monoclonal antibody (Calbiochem) as described (13). To investigate the transfection efficiency, the blot was probed using a phospho-specific antibody (Cell Signaling) and β-actin. The blot was then stained with Nickel Enhanced Western Lightning Chemiluminescent Substrate (Perkin Elmer). The autoradiographs were scanned using a flatbed scanner and analyzed using the NIH Image software (U. S. National Institutes of Health). The densitometric analysis was performed using the NIH Image software (U. S. National Institutes of Health). The band intensities were expressed as an arbitrary unit (AU).

**RNA Preparation and RT-PCR**—Total RNA from cells was extracted from cells using TRIzol (Molecular Research Center, Cincinnati, OH) (25). The RNA was reverse-transcribed with a first strand cDNA synthesis kit using pd(N)₆ primer (Amersham Pharmacia Biotech) according to manufacturer's protocol. 3 μl of this solution was ampliy using PCR primed with a forward primer 5'-GGGAGCTTATCGTCT-3' and reverse primer 5'-AAGGCTCAGAGGGA-3' homologous to the CAT gene. The RT-PCR signal from CAT mRNA transcripts was normalized with the signal obtained from β-actin using the primer pair (forward: 5'-GGGTGAGCGCCCTAAGGAC-3'; reverse: 5'-TTGGCTCATAGGTCGAGG-3') as described previously (26).

**Retroviral Infection of HepG2 Cells**—The pBabe-Puro retroviral expression vector (27) with and without pRep3CMV-17 insert was transfected into AmphotPCa2-293 cell (CLONTECH), using LipofectAMINE as per the manufacturer's instructions. The supernatants containing the virus were harvested 48 h after infection and used to infect stable Hep G2 cells that harbored the pAI.474-CAT reporter gene in the presence of 8 μg/ml Polybrene. After elimination of uninfected cells by adding 2.5 μg/ml puromycin to culture medium, the cells were treated for 17 nM EGF or 100 microunits/ml insulin. The inset shows a typical autoradiograph of CAT activity in the presence of EGF. Panel B shows an ethidium bromide-stained gel of RT-PCR signals reflecting CAT mRNA in the same cells treated with 1 μg/ml actinomycin D (ACTD) or 10 μg/ml cycloheximide (CHX) following exposure to EGF or insulin (upper). RT-PCR signals from β-actin mRNA served as control (lower). The various treatments are denoted below each lane. Panel C shows a Western blot analysis of apoA-I protein in the lysates and corresponding spent medium from Hep G2 cells exposed to EGF, insulin, or bpV(phen), as indicated above each lane.

**RESULTS**

**EGF and Insulin Increase ApoA-I Gene Expression**—We recently described the creation of a stable Hep G2 cell line harboring the reporter, pAI.474-CAT. This plasmid is comprised of the −474 to −7 fragment of the rat apoA-I promoter fused to the reporter gene, CAT (23). In these cells, CAT activity (Fig. 1A) increased following treatment with 17 nM EGF. Induction appeared to be rapid with detectable increases at 3 h following exposure to the hormone. The extrapolation of the data points to time 0 suggested an almost instantaneous induction by EGF. Similarly, 100 microunits/ml insulin also stimulated CAT activity with comparable kinetics. The only difference was that 1 μg/ml actinomycin D (ACTD) or 10 μg/ml cycloheximide (CHX) following exposure to EGF or insulin (upper). RT-PCR signals from β-actin mRNA served as control (lower). The various treatments are denoted below each lane. Panel C shows a Western blot analysis of apoA-I protein in the lysates and corresponding spent medium from Hep G2 cells exposed to EGF, insulin, or bpV(phen), as indicated above each lane.

**FIG. 1.** EGF and insulin induction of apoA-I. Panel A, shows the time course detailing induction of the apoA-I promoter in stable Hep G2 cells that harbor pAI.474-CAT following exposure to 17 nM EGF or 100 microunits/ml insulin. The inset shows a typical autoradiograph of CAT activity in the presence of EGF. Panel B shows an ethidium bromide-stained gel of RT-PCR signals reflecting CAT mRNA in the same cells treated with 1 μg/ml actinomycin D (ACTD) or 10 μg/ml cycloheximide (CHX) following exposure to EGF or insulin (upper). RT-PCR signals from β-actin mRNA served as control (lower). The various treatments are denoted below each lane. Panel C shows a Western blot analysis of apoA-I protein in the lysates and corresponding spent medium from Hep G2 cells exposed to EGF, insulin, or bpV(phen), as indicated above each lane.

The results show that EGF and insulin enhance the induction of apoA-I gene expression in Hep G2 cells. The induction is rapid, with detectable increases at 3 h following exposure to the hormone. The extrapolation of the data points to time 0 suggests an almost instantaneous induction by EGF. Similarly, 100 microunits/ml insulin also stimulated CAT activity with comparable kinetics. The only difference was that 1 μg/ml actinomycin D or 10 μg/ml cycloheximide following exposure to EGF or insulin (upper). RT-PCR signals from β-actin mRNA served as control (lower). The various treatments are denoted below each lane. Panel C shows a Western blot analysis of apoA-I protein in the lysates and corresponding spent medium from Hep G2 cells exposed to EGF, insulin, or bpV(phen), as indicated above each lane.
these cells and assayed for abundance of CAT mRNA using RT-PCR. Results showed that whereas, cycloheximide did not inhibit EGF or insulin induction of CAT mRNA expression (Fig. 1B), as expected actinomycin D blocked transcription of the CAT gene. These findings suggest that both hormones had a direct effect on apoA-I promoter activity and did not require de novo synthesis of other proteins.

Whether EGF or insulin induction of apoA-I promoter correlated with increases in apoA-I protein is not known. Therefore, Western blot analysis was used to measure the abundance of the protein in both whole cell lysate and culture medium from cells treated with either EGF or insulin or 5 μM bpV(phen) for 24 h in the presence (lanes 4, 6, and 8) or absence (lanes 3, 5, and 7) of 1 μM PD153035. Panel B, shows a graph of the relative CAT activities in cells treated with conditions noted at the bottom of each bar (mean ± S.E., n = 4). Asterisk (*) denotes a significant difference with p < 0.01 between the groups with and without PD153035 treatment as determined by ANOVA.

**Activation of EGF Receptor Kinase Induces ApoA-I Expression**—The action(s) of EGF is initiated by its binding to a specific membrane receptor with intrinsic tyrosine kinase activity. To determine whether EGF induction of apoA-I is mediated by its receptor, stably transfected Hep G2 cells were exposed to 1 μM PD153035, a specific inhibitor for the EGF receptor (30), prior to the addition of hormone. The results (Fig. 2) showed that treatment with PD153035 completely blocked EGF induction of apoA-I activity. In contrast, neither insulin nor bpV(phen) induction of CAT activity in the same cells was affected by PD153035 (Fig. 2). These findings show that the activation of EGF receptor tyrosine kinase is required for apoA-I induction by the hormone. Furthermore, blockade of EGF receptor activity had no effect on the actions of insulin or bpV(phen).

**Intracellular Signaling Pathways Underlying EGF and Insulin Action**—Ligand binding to a receptor with intrinsic tyrosine kinase activity triggers this function leading to receptor autophosphorylation. These events initiate signal transduction and cellular responses. Activated tyrosine kinase receptor can initiate several intracellular pathways including; Ras-MAP kinase cascade, PI 3-kinase, PLCγ pathways (16, 31). However, the cellular responses and their signaling mechanisms are largely dependent on the cell type examined. Furthermore, the actions of a single hormone may vary from one cell type to another. Therefore, we wanted to examine the intracellular signaling pathway by which EGF and insulin induced apoA-I gene expression in Hep G2 cells.

To identify the signaling pathways underlying the actions of EGF and insulin, we used a panel of specific inhibitors known to block selected pathways. The results show that inhibitors of PI 3-kinase, 100 nM wortmannin or 10 μM LY294002 (32), did not affect the actions of EGF, insulin, or bpV(phen) (Fig. 3). Whereas, the MEK1 inhibitor PD98059 (1 μM) completely blocked EGF induction of apoA-I expression (Fig. 3), it only inhibited 50% of insulin action on apoA-I activity (Fig. 3). Although the PKC inhibitor GF109203X (2 μM) (33, 34) did not affect EGF response, it did inhibit insulin induction of apoA-I gene by 50% (Fig. 3). More importantly, the combination of PD98059 and GFX together completely blocked insulin induction of apoA-I (Fig. 3). Like insulin, apoA-I induction by bpV(phen) was also blocked in the presence of both inhibitors (data not shown).

That MAP kinase was activated during EGF, insulin, or bpV(phen) induction of the gene was assessed by treating the cells with or without the MEK1 inhibitor, PD98059. Cell lysate was assayed for p42/44 kinase using Western blot analysis (Fig. 4B, inset). The treatment of cells with PD98059 inhibited the phosphorylation of p42/44 MAP kinase (Fig. 3B, inset). The addition of these data to that above show the following: (i) the PI 3-kinase pathway does not participate in either EGF or insulin induction of apoA-I expression; (ii) the MAP kinase cascade is the sole mediator of EGF stimulation of apoA-I; and (iii) insulin or bpV(phen) action requires two independent pathways mediated by PKC and MAP kinase to stimulate the apoA-I gene.

**PKC Activation Induces ApoA-I Expression**—Since insulin or bpV(phen) induction of apoA-I is blocked by the PKC inhibitor GFX, this implies that the converse where PKC is activated should enhance apoA-I gene activity. Therefore, we tested whether the PKC activator PDBu stimulated apoA-I expression (Fig. 4) and if this induction is sensitive to the MEK inhibitor PD98059 (13). Results show treatment of cells with 25 nM PDBu increased apoA-I promoter activity and that was blocked by GFX. However, this induction was not affected by pretreatment of cells with PD98059. These findings suggest that PKC activation does not crossover to the MAP kinase cascade above the level of MEK as described in other cell systems (35, 36).

**Role of Ras in EGF and Insulin Induction of ApoA-I**—To determine whether Ras participates in the EGF and insulin induction of apoA-I, we infected the stable Hep G2 cells with Ras<sup>Asn-17</sup> retrovirus to express dominant negative Ras<sup>Asn-17</sup>. This mutant interrupts the Ras-dependent signaling pathway (37, 38). Results (Fig. 5) showed that expression of Ras<sup>Asn-17</sup> blocked EGF induction of the apoA-I gene. As expected, the expression of Ras<sup>Asn-17</sup> partially but significantly attenuated insulin induction of apoA-I promoter. Exposure of cells to GFX prior to Ras<sup>Asn-17</sup> retrovirus infection blocked both insulin (Fig.
5) or bpV(phen) (data not shown) induction of the gene. Additionally, the insertion of the dominant negative Ras Asn-17 in cells did not affect the stimulation of apoA-I gene induced by PDBu, a PKC activator (data not shown). These findings show that EGF stimulates apoA-I induction via a Ras-MAP kinase pathway and further solidifying the finding that insulin induction of the gene is mediated independently via both the Ras-MAP kinase and the PKC pathways.

EGF Response Requires Presence of IRCE—Next we searched for the cis-acting element(s) in the apoA-I promoter that mediated the actions of EGF. Thus serial deletion constructs of apoA-I promoter were transfected into Hep G2 cells and then treated with EGF, insulin, or bpV(phen). Results (Fig. 6A) showed that EGF stimulation of the promoter like insulin or bpV(phen) was abolished following deletion of the −425 to −376 fragment of the promoter.

Our previous studies showed that insulin induction of apoA-I gene required a motif called the IRCE (Fig. 3). Deletion analysis suggested that the same motif may also be required for response to EGF. Therefore, we tested the activity of a reporter construct containing a mutant of the IRCE. Results (Fig. 6B) showed that the reporter containing the mutant IRCE was not inducible by EGF, insulin, or bpV(phen). These finding show that the actions of all three agents require the presence of an intact IRCE.

Sp1 Augments EGF, Insulin, or bpV(phen) induction of ApoA-I—The IRCE motif is GC-rich and binds to a transcription factor, Sp1. Therefore, we speculate that Sp1 might participate in EGF, insulin, or bpV(phen) induction of the promoter. To investigate this hypothesis, we used submaximal concentrations of EGF (8.5 nM), insulin (50 microunits/ml), and

FIG. 3. Effects of PD98059, a MEK inhibitor and GFX, a PKC inhibitor, on apoA-I. Panel A shows an autoradiograph of CAT activity in stable Hep G2 cells treated either with EGF (lanes 2–7) or insulin (lanes 9–14) for 24 h in the presence (lanes 3 and 10) or absence (lanes 2 and 11) or absence (lanes 2 and 9) of 2 μM PD98059. Lines 5 and 12 indicate the pretreatment of cells with both 2 μM PD98059 and 2 μM GFX (PD98059/GFX). Other inhibitors wortmannin (lanes 6 and 13) and LY294002 (lanes 7 and 14) did not affect the actions of EGF or insulin. Panel B shows a graph of the relative CAT activities in cells treated with conditions noted at the bottom of each bar (mean ± S.E., n = 4). Asterisk (*) denotes a significant difference with p < 0.01 between the groups with and without the presence of any inhibitor as determined by ANOVA. The insert of Panel B (inset), shows a typical Western blot that reflects the phosphorylation of p42/44 MAP kinase induced by EGF, insulin or bpV(phen) in the presence and absence of PD98059 as indicated for each lane.

FIG. 4. Effect of PD98059 on PDBu induction of apoA-I. Panel A (left) shows an autoradiograph of CAT activity in stable cells treated with a PKC activator, 25 nM PDBu for 24 h in the presence or absence of the PKC inhibitor 2 μM GFX or 2 μM PD98059. Panel B shows a graph of relative CAT activities in cells treated with the conditions noted at the bottom of each bar (mean ± S.E., n = 5). Asterisk (*) denotes a significant difference with p < 0.01 between the groups treated with and without inhibitors as determined by ANOVA.

FIG. 5. Effect of dominant negative RasAsn-17 on EGF and insulin induction of apoA-I. This graph represents the relative CAT activities in stable Hep G2 cells infected with either pBabe-Puro retrovirus (CV, control virus) or the virus expressing RasAsn-17 as indicated. Puromycin-selected Hep G2 cells were treated with the conditions as noted at the bottom of each bar (mean ± S.E., n = 4). Asterisk (*) denotes a significant difference with p < 0.01 between the groups with and without the expression of RasAsn-17 in response to each agent as determined by ANOVA.
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Mutation of Thr266 in Sp1 Attenuated EGF Induction of ApoA-I—Next we postulated that EGF stimulation of apoA-I was mediated by the MAP kinase pathway involving phosphorylation of Sp1. To test this idea, we inspected Sp1 protein for potential sites of MAP kinase phosphorylation according to published algorithms. Six potential sites for MAP kinase were identified (Fig. 8) (40, 41). The prediction scores ranged from 0 to 1.000 with values that exceeded 0.5 reflecting potential phosphorylation sites. Three of the sites with the highest scores were Thr266 (0.767), Thr414 (0.664), and Thr325 (0.726). Thr266 was especially interesting because it was located toward the C-terminal region of the second glutamine-rich domain, one of the known trans-activation domains of Sp1 (19). Since the amino acids surrounding Thr266 appeared most homologous against a consensus MAP kinase site, we mutated the threonine residue by replacing it with an alanine to create Sp1-T266A. The use of Sp1-T266A in transfection studies revealed the following results. Although transfection of Sp1-T266A alone into stable Hep G2 cells augmented activity of pAI.474-CAT, the activity was not significantly different from wild-type Sp1. When the transfected cells were exposed to EGF, apoA-I induction was attenuated by 54% in cells containing the mutant Sp1 compared with the wild-type. However, PDBu induction of apoA-I expression mediated by PKC was the same in the presence of the mutant or wild-type Sp1. These data suggest that amino acid Thr266 in Sp1 is required for full EGF induction of apoA-I.

DISCUSSION

ApoA-I is an essential component of HDL. The protein alone or in the form of HDL mediate a normal physiologic process called reverse cholesterol transport, which lowers total body cholesterol (5, 7) thereby reducing the risk of IHD. This function of apoA-I makes it an important target for therapies to enhance expression of the protein. To achieve this goal, our studies have helped define the mechanism(s) by which hormones regulate gene activity (11, 42). This avenue of research is attractive because simple manipulation of the hormones, mimetics, or synthetic analogues will help us control activity of the gene.

Our initial studies showed that insulin induction of apoA-I transcription in Hep G2 cells was mediated by a cis-acting element, the IRCE in the promoter (12). More recently, we found that signaling pathways involving protein kinase A, PKA, or PKC also stimulated gene activity and this induction required the transcription factor, Sp1 (13). Sp1 binds specifically to the IRCE (12, 13). Together these findings form the basis of an incomplete model depicting the hierarchy of events in the regulation of apoA-I transcription. However, we have yet to determine whether the two separate sets of results are connected. To relate the findings arising from these studies, we have defined the pathway(s) by which insulin induce apoA-I
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Fig. 8. Site-directed mutagenesis of Thr266 in Sp1 attenuates EGF induction of apoA-I. Panel A shows the amino acid sequence of Sp1. Six motifs (XXTP, bold) were identified as potential phosphorylation sites for MAP kinase. The underlined sequences (not bold) show the two glutamine-rich activation domains. The bold and underlined sequences indicate three zinc fingers. The table shows the positions of potential MAP kinase phosphorylation sites and the prediction scores. T indicates the potential phosphorylation site with a score above 0.5. Panel B demonstrates the effect of Thr266 mutant Sp1 (Thr266.Sp1) on EGF induction of apoA-I compared with wild-type Sp1 (Sp1). The apoA-I-474-CAT plasmid DNA were co-transfected with 1 µg each of either wild-type Sp1 or Thr266 mutant Sp1 or empty vector (EV). After 24 h, cells were treated with and without 8 nM EGF or 25 nM PDBu, followed by assaying for CAT activity (mean ± S.E., n = 4). Asterisk (*) denotes a significant difference with p < 0.01 between the groups with wild-type and Thr266 mutant Sp1 in response to each agent as determined by ANOVA.

and extended this line of thinking to examine the potential action(s) of another peptide hormone, EGF, that also acts via a receptor tyrosine kinase.

That EGF stimulated apoA-I expression comes from two sets of complementary data. First, 17 ng EGF induced apoA-I promoter 7-fold in stable Hep G2 cells that harbored a DNA fragment (−474 to −7) in the pA1.474-CAT reporter (Fig. 1). Second, this induction of apoA-I trancription underlies the increase in abundance of the protein in both cell lysate and culture medium (Fig. 1C). The mechanism by which EGF induces apoA-I expression appeared to be transcriptionally mediated because hormone action was not affected by cycloheximide inhibition of protein synthesis. This finding implies that EGF trans-activation of this gene is direct and did not require de novo protein synthesis. The direct effect of EGF is further supported by the rapid response of promoter activity, noted in the kinetics of induction (Fig. 1A). Although EGF is a known hepatocyte mitogen (44, 45) and its activity may induce gene expression in the liver, this is the first demonstration that the hormone up-regulates apoA-I. The preceding studies add EGF to the list of hormones that modulate apoA-I including, thyroid hormone, steroid hormones, and insulin (42, 46, 47). EGF is the second and insulin the first peptide hormone shown to regulate apoA-I expression. Thus it is of interest to compare and contrast the actions of these hormones that act via comparable intracellular signaling pathways.

Activation of the EGF receptor may trigger signaling pathways including those mediated by Ras-MAP kinase, PI 3-kinase, or PLCγ (16, 31). Despite the variety of choices, EGF activation of apoA-I appears to be channeled solely through the Ras-MAP kinase cascade, a pathway commonly used by the hormone to regulate intracellular events (40, 50). This explanation was supported by finding that the specific EGF receptor inhibitor PD153035 blocked hormonal induction of the gene. In addition, EGF activation of the Ras-MAP kinase cascade appeared distinct from that of insulin or bpV(phen) because the same inhibitor had no effect on the actions of the latter two agents. Additional support that EGF induction of apoA-I was mediated by a single pathway arose from the finding that hormone action was inhibited completely by the MEK inhibitor PD98059 at a low concentration of 2 µM (51). This inhibitor also abolished phosphorylation of p42/44 MAP kinase induced by EGF (Fig. 3B, inset). Infection of the cells with a retrovirus expressing a dominant negative Ras, RasAsn-17 (38), blocked EGF induction of apoA-I and confirmed the participation of Ras in this process. Additional evidence for the single pathway mechanism came from data showing that the EGF action was not affected by GFX or wortmannin, inhibitors for PKC and PI 3-kinase, respectively. These data show that EGF induction of apoA-I is mediated by the Ras-dependent MAP kinase, a pathway known to be present and active in Hep G2 cells (52–55). For example, recent data show that hepatopoietin stimulates MAP kinase through EGF receptor activation in these cells (55).

Although the preceding observations showed the Ras-MAP kinase cascade to mediate EGF induction of apoA-I transcription, this finding only details the cytosolic portion of the mechanism. Activation of transcription requires conversion of the cytosolic signal to nuclear event(s). Many transcription factors are known to mediate the actions of activated MAP kinase (56). However, the specific one(s) required for EGF induction of apoA-I is not known. To examine this question, we used deletion and mutational analysis to locate a motif, the IRCE (−411 to −404) within the promoter that mediated the actions of EGF. Our recent studies showed that the IRCE was bound specifically by the transcription factor, Sp1 but not Sp2 nor Sp3 and this binding enhanced apoA-I gene transcription (13). Overexpression of Sp1 in cells augmented EGF induction of apoA-I, thus showing the participation of Sp1 in this process (Fig. 7).

The finding that the Ras-MAP kinase cascade mediates EGF activity and this pathway eventually requires Sp1 to activate transcription provides a catalogue of the components of the induction. Further insight into this mechanism requires the connection between the kinase cascade and Sp1. Therefore, we postulated that Sp1 may serve as a substrate for MAP kinase. To examine this hypothesis we searched for and found many potential kinase sites in Sp1. However, amino acid Thr266 was targeted for mutagenesis because this motif was most homologous to the consensus MAP kinase site. Although Thr266 is located in the second trans-activation domain of Sp1, the mutation of this amino acid did not affect significantly the basal activity of the transcription factor. However, exposure of the transfected cells to EGF showed a clear difference resulting in a 54% lower level of induction in cells with mutant Sp1 versus the wild type (Fig. 8). This finding is consistent with the idea that Thr266 serves as a functional phosphorylation site for the actions of EGF.
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In another model, EGF induction of gastrin gene expression in epithelial cells was mediated by the MAP kinase cascade and required phosphorylation of the transcription factor, Sp1 (57). Thus our finding that MAP kinase regulates Sp1 adds to the list of kinases including; Sp1 kinase (57) or DNA-dependent kinase (58), PKA (13, 18, 21), casein kinase II (43), and PKC (13, 48) known to modulate Sp1 activity, which in turn, leads to changes in gene expression. Although phosphorylation of Sp1 by various kinases is possible and in some cases demonstrated, the specific sites that are modified remain undefined. Therefore, the observation that mutation of Thr266 attenuated EGF induction of apoA-I is consistent with the idea that MAP kinase modulates Sp1 activity by phosphorylating Thr266. Since EGF induction was not completely abrogated, this suggests that additional phosphorylation sites are likely involved.

The identification of the mechanism underlying EGF induction of apoA-I provides an opportunity to better understand the regulation of the gene by comparing or contrasting the actions of EGF with that of insulin in the Hep G2 cells. For these studies, the first step is to define the mechanism(s) mediating insulin induction of apoA-I. Since insulin induction is weak, we also examined an insulin mimetic, bpV(phen) and tested its action on apoA-I gene expression. BpV(phen) acts by inhibiting the specific protein-tyrosine phosphatase for the insulin receptor and thus mimics many of the actions of insulin, but intra-cellular signaling mechanisms may be different from those of insulin. For example, PI 3-kinase in cultured hepatocytes is required for insulin but not for bpV(phen) to regulate expression of the insulin-like growth factor-binding protein (49). Our first studies showed that like insulin, bpV(phen) induced apoA-I transcription, except that the induction was 4–5-fold (Fig. 2) as compared with 3–3.5-fold for insulin. Both insulin and bpV(phen) induction of apoA-I was not affected by the EGF receptor kinase inhibitor, PD153035, suggesting that although exposure of cells to these agents increased apoA-I gene activity, the pathways leading to induction were likely different.

Next, the approach that we used to define the signaling pathways for EGF induction of apoA-I was applied to the actions of insulin and bpV(phen). Exposure of cells to PD98059 following infection with RasAsn-17 retrovirus blocked both the actions of insulin or bpV(phen) induction of apoA-I by 50%. The remaining induction was sensitive to the PKC inhibitor, GFX. As expected the combination of both GFX plus PD98059 or RasAsn-17 retrovirus completely abrogated insulin or bpV(phen) induction of apoA-I. Consistent with previous studies, activation of PKC by exposure to PDBu enhanced apoA-I activity, and this was blocked by prior treatment with GFX (13, 33, 34). Our previous studies, showed that PDBu induction of apoA-I was mediated by PKC activation and that this pathway required the interaction of Sp1 with an intact IRCE (13). These data suggest that the PKC and Ras-MAP kinase pathways act independently through Sp1, because there does not appear to be cross-talk between two pathways. For example, the inhibition of MEK1 with PD98059 or RasAsn-17 expression does not affect the actions of PDBu. More importantly, the Sp1-Thr266 mutant did not affect PKC induction of this gene (Fig. 8), suggesting a distinct regulatory site for PKC phosphorylation within Sp1.

Although the pathway by which EGF activates MAP kinase leading to Sp1 phosphorylation and apoA-I induction is clear, the role of PKC in mediating actions of insulin or bpV(phen) is not. Insulin or bpV(phen) induction of PKC activity may be achieved by stimulation of PLC γ, which triggers the hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol leading to PKC activation (14, 15). This speculation is based on our finding that PI 3-kinase inhibitors, wortmannin and LY294002, did not affect the induction of the gene by either insulin or bpV(phen). This finding suggests that insulin or bpV(phen) activation of PKC is unlikely via the PI-3 kinase pathway. It remains undetermined whether PKC directly phosphorylates to stimulate Sp1 because there are 14 potential PKC recognition sites in Sp1 (39). The mutagenesis approach may not be the most efficient way to tackle this problem in the search for functional Sp1 site(s) targeted by PKC.

In summary, our studies show that EGF up-regulates apoA-I gene transcription. This process requires a single signaling pathway mediated by Ras-MAP kinase. The activation of this kinase may potentially lead to phosphorylation of the transcription factor, Sp1 at Thr266. The modified Sp1 interacts with the IRCE to enhance transcription of the gene. Insulin and bpV(phen) also induce apoA-I transcription but rather than a single cascade, their actions are mediated by two parallel independent pathways that involve the activation of PKC and MAP kinase. ApoA-I induction by these two agents also require the participation of Sp1.

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