GLOBAL GENE EXPRESSION ANALYSIS OF ERK5 AND ERK1/2 SIGNALING REVEALS A ROLE FOR HIF-1 IN ERK5-MEDIATED RESPONSES

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Running Title: Gene Expression Analysis of ERK5 and ERK1/2 Signaling

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Extracellular signal regulated kinase 5 (ERK5) is a recently characterized MAP kinase, which is most similar to the well studied ERK1/2 subfamily, but uses distinct mechanisms to elicit responses. To understand the specificity of signaling through ERK5 vs ERK1/2, we examined global gene expression changes in response to each pathway. Microarray measurements in retinal pigment epithelial (RPE) cells revealed 36 genes regulated by ERK5, all of which were novel targets for this pathway. 39 genes were regulated by ERK1/2, which included 11 known. Of these genes, 19 were regulated by both pathways. Inspection of the 17 genes uniquely regulated by ERK5 revealed that 14 (82%) were previously associated with hypoxia, via regulation by HIF-1. In contrast, 16 (84%) genes regulated by either ERK5 or ERK1/2 were implicated in hypoxia, most through mechanisms independent of HIF-1. Of the 20 genes regulated by ERK1/2, only 9 were implicated in hypoxia, and were not well characterized hypoxia targets. Thus, unlike ERK5, a mechanistic link between ERK1/2 and HIF-1/HRE could not be established on the basis of gene regulation. Activation of both pathways enhanced transcription from an HRE and increased HIF-1α protein expression. In contrast, ERK5 but not ERK1/2 elevated transcription through GAL4-HIF-1. Interestingly, ERK5 is not significantly activated by hypoxia in RPE cells, indicating that ERK5 regulation of these genes is relevant in normoxia rather than hypoxia. Thus, ERK5 and ERK1/2 differ in their mechanisms of gene regulation, and indicate that ERK5 may control hypoxia responsive genes by a mechanism independent of HIF-1α expression control.

Mitogen activated protein (MAP) kinase signaling pathways regulate cell proliferation, transformation, differentiation, apoptosis, and responses to stress. Well characterized mammalian MAP kinases include extracellular signal-regulated kinases 1/2 (ERK1/2), c-jun N terminal kinases (JNK), and p38 MAPK pathways, which are regulated by three-tiered kinase cascades involving MKKK \( \rightarrow \) MKK \( \rightarrow \) MAPK (1,2). The ERK1/2 pathway is stimulated in response to growth factors and mitogens, via sequential activation of p21Ras, Raf-1, and MKK1/2, which in turn phosphorylates and activates ERK1/2. Phosphorylation of ERK1/2 promotes its translocation from cytoplasm to nucleus, and subsequent regulation of gene expression by phosphorylation and activation of nuclear transcription factors. Known transcription factor substrates for ERK1/2 are numerous and include Elk-1/p62 TCF, Ets-1, AP1, GATA, Myc, and NFkB. ERK1/2 signaling commonly regulates gene transcription.
through individual transcription factor binding sites, such as CREB, as well as composite elements (e.g. Ets-1/AP1 and SRF-Elk). Constitutive activation of the ERK1/2 pathway has been shown to promote cellular transformation and genomic instability and defects in this pathway have been shown to arrest growth and development.

ERK5/BMK1 is a newer member of the MAP kinase family which shows greatest similarity to ERK1/2 among the various MAPKs (3-5). ERK1/2 and ERK5 share 66% sequence identity in their kinase domains, as well as a Thr-Glu-Tyr motif in the activation lip containing sites for dual phosphorylation. Despite this similarity, ERK5 is selectively phosphorylated by MKK5, and is not recognized by the ERK1/2 activators, MKK1/2 (4). ERK5 is also stimulated in response to cell stress as well as growth factors (6,7), and activation of the ERK5 pathway upstream of MKK5 has been shown to involve MEKK2/3 as well as p21Ras and Src, and exclude Raf kinase catalytic activity (4,8-11). Like ERK1/2, ERK5 recognizes phosphorylation sites containing Pro at positions -2 and +1 to the phosphorylation site. Substrates shared by ERK5 and ERK1/2 include CREB and Ets-1 (12,13). Whereas those specific to ERK5 include members of the myocyte enhancer factor 2 (MEF2) family (2A, 2C, 2D), Sap1α, MyoD, and Bad (11,14-17). Unique to ERK5 is a large C-terminal transcriptional activation domain (18) which physically interacts with MEF2C, further increasing transcriptional activity beyond that achieved by phosphorylation alone (15). The ERK5 C-terminal domain also functions as a site of serine phosphorylation whose subsequent interaction with 14-3-3 interferes with binding, phosphorylation, and activation of ERK5 by MKK5 (19).

Regulatory mechanisms and cellular functions of ERK5 and ERK1/2 overlap in some aspects, and show distinct features in others. ERK1/2 and ERK5 regulate similar processes within the cell, including chemotaxis, cytoskeletal organization, transcription, and proliferation (7,13,20-22) and have been shown to cooperate in NIH3T3 cell transformation via convergence of p90RSK and NFκB signaling (21). However, mechanisms used by each pathway to elicit specific responses may be distinct and may involve differential localization, substrate preferences, or signal strength (12,13,20,22). For example, although CREB is a substrate for both ERK1/2 and ERK5, the cellular location of ERK5 activation dictates a preferential role for this pathway in mediating CREB-dependent neuronal survival (12). At this point, only a few gene products whose expression is controlled by ERK5 have been identified, and single readout studies examining transcriptional targets for ERK1/2 and ERK5 have shown little overlap. Selective functions of different ERK forms are also revealed in genetic studies, where mice lacking the ERK1 gene are viable but defective in thymocyte maturation (23) and mice lacking ERK2 are embryonic lethal at day E11.5 due to defects in placental development (24), whereas mice lacking ERK5 instead acquire cardiovascular defects, leading to lethality at E9.5-10.5 (25). The cardiovascular phenotype of ERK5-/- mice is strikingly similar to that of mice lacking either MEKK3 or MEF2C (26,27), supporting a physiological signaling mechanism involving ERK5, MEKK3, and MEF2C. It is clear that MEF2C is necessary for ERK5-mediated survival in cardiovascular cells (28), although overexpression of MEF2C is only partially sufficient to rescue the ERK5-/- phenotype, suggesting that additional downstream targets of ERK5 are relevant but remain unidentified (28).

Details about the mechanisms used by ERK5 vs ERK1/2 pathways to elicit distinct responses remain incomplete. To examine this problem, we took an unbiased approach by studying global gene expression changes in
cells, altered in response to each pathway. Groups of genes regulated in common by ERK1/2 and ERK5 as well as genes regulated uniquely by each pathway were examined for common mechanisms of promoter regulation or gene function. The results revealed a majority of targets unique to ERK5 that were also known to be induced in response to hypoxia. Further analysis confirmed that ERK5 induces transcription through an HRE, through mechanisms involving induction of HIF-1 protein expression and transactivation. Thus, ERK5 appears to control a majority of its transcriptional targets via a novel mechanism involving enhanced transcription through HIF-1/HRE.

**EXPERIMENTAL PROCEDURES**

**Cell Culture.** Human retinal pigment epithelial cells immortalized with telomerase (hTERT-RPE1, Clontech Laboratories, Inc., Palo Alto, CA) were grown at 37° C in 5% CO2 in Dulbecco’s modified Eagle’s/Ham’s F12 medium (Sigma, St. Louis, MO), supplemented with 10% Tet-system approved fetal bovine serum (BD Biosciences, Palo Alto, CA), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), and 0.348% sodium bicarbonate (HyClone Laboratories, Logan, UT). Cells were passaged at P1-P10 and transfected at 75-80% confluency.

**Plasmid constructs.** Vectors for mammalian expression of constitutively active rat MKK5-β (Ser222Asp, Thr226Asp, pCMV5MKK5β DDHA3), wild-type human ERK5-FLAG and GAL4-MEF2C-luciferase were kindly provided by Dr. Melanie Cobb (U. Texas Southwestern Medical Center, Dallas TX). Plasmids encoding constitutively active human MKK1-ΔN3/S218E/S222D (pMM9MKK1R4F-HA) and wild-type rat ERK2 (pCMV5ERK2) were described previously (29,30). pGAL-HIF-1α (531-826) was kindly provided by Dr. Greg Semenza through the Johns Hopkins Special Collection of ATCC (#MBA-9) (31). pGLHif-luciferase containing three copies of the 3’ EPO-HRE was generously provided by Dr. Max Gassmann (the Institute of Veterinary Physiology, University of Zurich, Zurich, Switzerland) (32). Other plasmids included pCDNA3.1HisB (Invitrogen) and pRL-null Renilla (Promega, Madison, WI). Human MKK5 MGC clone #14094 was purchased from Invitrogen. The open reading frame (ORF) of splice variant MKKα was PCR amplified using primers 5’-CACCAGAAGGCTGAAGATGTAATGAC-3’ and 5’-CGGGGGCCCTGCTGGCTGCT-3’, and the splice variant MKK5β ORF was isolated using primers 5’-CACCAGAAGGCTGAAGATGTAATGAC-3’ and 5’-CGGGGGCCCTGCTGGCTGCT-3’. The MKKα and β ORFs were cloned into TOPO-vectors (Invitrogen) to generate pCDNA3.2D/V5-MKK5α and -MKK5β. MKK5α and β activating mutations (CA-MKK5) were generated by incorporating Ser311Asp and Thr315Asp or Ser222Asp and Thr226Asp mutations, respectively, using the QuikChange Mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by DNA sequencing (U. Colorado-Boulder DNA Sequencing Facility).

**Transient Transfections and Transcription Assays.** RPE cells (2 x 10⁴) were transfected with 1.8 μg DNA in 12-well plates using FuGene6 (Roche Diagnostics, Indianapolis, IN) at a FuGene:DNA ratio of 6:2, according to manufacturer’s instructions. FuGene-DNA complexes were incubated in OptiMEM (Invitrogen) for 30 min followed by drop wise addition to cells. RPE cells were harvested at 24 h post-transfection in passive lysis buffer (PLB) (Promega) and cell lysates were analyzed using the dual luciferase reporter assay system (Promega) according to
manufacturer's instructions. Luciferase activity was measured using a MicroLumat LB96P luminometer (EG&G Berthold). Firefly luciferase activity was normalized by Renilla luciferase activity to control for transfection efficiency. Transfections were performed in duplicate and assayed in duplicate. Where indicated, cells were treated with 20 ng/ml recombinant human epidermal growth factor (Gibco, Grand Island, NY) for 10 min to activate ERK1/2 and ERK5.

Hypoxia Treatment. For time course experiments, RPE cells (8 x 10⁵) were plated 16 h prior to incubation in a hypoxia chamber (3% O₂). At the indicated times, cells were harvested in EB (described below). Whole cell extracts (50 μg for ERK5 blots and 100 μg for HIF-1α blots) were subjected to Western blot analysis as described below. For transcription assays, RPE cells were plated and transfected as above for luciferase assays and treated with hypoxia 5 h after transfection. Cells were harvested 24 h post-transfection in 1X PLB and assayed for Firefly and Renilla luciferase as described above.

Western Blot Analysis. RPE cells were harvested 24 h after transfection in extraction buffer containing 1% Triton X-100, 10 mM Tris, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 0.1% bovine serum albumin, 20 μg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 2 mM Na₃VO₄. Whole cell extracts (30 μg) were separated on 7.7% low-bis (7.5:0.2) SDS-PAGE and transferred to PVDF membranes (PerkinElmer, Boston, MA). Membranes were probed with primary antibodies to ERK5 or ppERK5 (rabbit polyclonal, Sigma), ERK2 (rabbit polyclonal, C14, Santa Cruz, Santa Cruz, CA), HA.11 (mouse monoclonal, Covance, Princeton, NJ), FLAG tag (mouse monoclonal, Stratagene), ppERK1/2 (mouse monoclonal, Sigma), HIF-1α (mouse monoclonal, BD Transduction Laboratories, Mountain View, CA) in blocking buffer [5% nonfat dry milk in 20 mM Tris pH 7.4, 138 mM NaCl, 0.1% Tween (TBST)] overnight at 4⁰C. Membranes were washed with TBST and incubated with secondary goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated antibody (Jackson Immunoresearch, West Grove, PA) for 1 h at room temperature. Immunoreactivity was visualized by enhanced chemiluminescence detection (ECL, PerkinElmer).

Nuclear Extract Preparation. RPE cells (3 x 10⁶) were transfected with 15 μg DNA using FuGene6, as described above. Cells were serum starved at the time of transfection and harvested at 24 h. Where indicated, cells were pre-treated with 10 μM U0126 (Promega) or DMSO for 1 h prior to treatment with 100 μM CoCl₂ at the time of transfection. Nuclear extracts were prepared using NE-PE Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL) which allows the stepwise separation of nuclear and cytoplasmic extracts.

Microarray Analysis. RPE cells (2 x 10⁵) were transfected with MKK5DD-HA (2.4 μg cDNA) and ERK5-flag (0.3 μg) or MKK1R4F-HA (1 μg) and ERK2 (0.3 μg), as described above, and total RNA was extracted using RNeasy mini-prep columns (Qiagen, Valencia, CA). Microarray analyses were performed in triplicate using RNA from three separate transfections. First and second strand cDNA synthesis, in vitro transcription of biotin-labeled cRNA, and fragmentation were carried out following standard protocols from the Affymetrix Expression Analysis Technical Manual (http://www.affymetrix.com). The samples were hybridized onto U133A GeneChips (Affymetrix, Santa Clara, CA) and processed at the UCHSC Cancer Center Microarray core facility (Denver, CO), following standard protocols. Data was corrected for background using MicroArray.
Suite 5.0 and normalized using robust multi-array average (RMA) quantile normalization (RMAExpress, version 0.1) (33). Differentially expressed genes were identified by two class paired analyses using software for Significance Analysis of Microarrays (SAM, Excel Add-in) (34) and RankProducts (RP software) (35). SAM Delta values were chosen which produced false discovery rates for MKK5 and MKK1 of 19% and 16%, respectively, without fold change cut-offs. False discovery rate (FDR) cut-offs of 20% were chosen for expression analysis using RP. Significant genes identified by SAM and RP were combined and further analyzed using the Spotfire DecisionSite Browser, v. 8 (Spotfire, Somerville, MA). Genes were chosen for further analysis when they were identified as significant (FDR ≤ 20%) by both SAM and RP, or when they showed FDR ≤ 5% with a low standard deviation across replicates by either SAM or RP. After passing these filters, a final filter was used which required ≥ 2-fold change in average intensity.

Real time PCR. RPE cells (2-3 x 10^6) were transfected with FuGene6 as described above and total RNA was isolated using RNeasy midi-prep columns (Qiagen). To avoid contamination by genomic DNA, on-column DNase digestion was carried out according to manufacturer’s instructions. Total RNA (6 µg) was used for first-strand cDNA synthesis using Omniscript Reverse Transcriptase (RT) (Qiagen) using oligo-(dT)20 primers (Invitrogen) according to manufacturer’s instructions. Each RT reaction was diluted 1:20 and 5 µl used in a 20 µL real time PCR reaction with 0.25 µM of each primer and 1-2 mM final MgCl2. Real time PCR reactions were carried out using LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche Diagnostics), containing Taq polymerase, SYBR green dye and nucleotides, and incorporation of SYBR Green into PCR products was detected using the LightCycler rapid thermal cycler system (Roche Diagnostics). A typical cycle included 10 min Hot Start at 95°C, 45 cycles with 10 s denaturation at 95°C, 10 s annealing at 65°C, and 8 s extension at 72°C. Fluorescence was detected at the end of each 72°C extension cycle. Melting curve analysis was carried out at the end of the run to confirm specificity of the PCR products for each primer set. PCR efficiency for each primer pair, calculation of cycle thresholds (C_T), and relative quantification of PCR products was calculated with standard curves using LightCycler Analysis software, v. 4.0 (Roche Diagnostics). PCR analysis was carried out in duplicate or triplicate, where indicated. Primers used for SYBR green PCR are shown in Suppl. Table 1.

RESULTS

Characterization of MAP kinase signaling in RPE cells

Microarray studies were undertaken using RPE cells immortalized with telomerase. These cells were chosen because they represent an immortal diploid human cell line that is not transformed, and they show functional signaling through MAP kinase pathways, where MKK1/2-ERK1/2 regulates proliferation, migration and survival (36-39) and MKK5/ERK5 gene expression is stimulated in response to hydrogen peroxide and other oxidative stresses (40). Thus, RPE cells represent an excellent system to study MAP kinase signaling under nontransformed conditions.

To understand the specificity of MKK5/ERK5 vs. MKK1/2-ERK1/2 signaling, experiments were carried out in which each pathway was activated individually and in combination. Co-transfection with constitutively active MKK5 (CA-MKK5) and wild-type ERK5 led to gel mobility retardation of ERK5 (Fig.1A, lane 2) indicating ERK5 phosphorylation and
pathway activation (8,22). The slower migrating ERK5 band is immunoreactive with an anti-ppERK5 antibody that recognizes the phosphorylated forms of the Thr218 and Tyr220 required for ERK5 activation, confirming that the slower migrating form represents the phosphorylated and active form of ERK5 (data not shown). Co-transfection with constitutively active M KK1 (CA-M KK1) + wild-type ERK2 resulted in increased anti-ppERK1/2 reactivity, indicating ERK1/2 activation at expression levels comparable to endogenous levels (Fig. 1A, lane 3). A combinatorial experiment transfecting all four constructs together showed gel mobility retardation of ERK5 as well as elevated anti-ppERK1/2 reactivity, indicating that both pathways were activated (Fig. 1A, lane 4). In all experiments, the transfection efficiency of RPE cells as determined by fluorescence of co-transfected GFP or immunostaining of HA-MKK1 or MKK5 approached 90% (data not shown). The degree of ERK5 and ERK1/2 activation in response to MKK5 and M KK1, respectively, was comparable or higher to that seen in response to epidermal growth factor (EGF) treatment of nontransfected cells (Fig. 1B, lane 2). As previously reported, we show here that the cell permeable MEK1/2 inhibitor, U0126, blocks ERK1/2 activation with IC50 below 0.3 μM (Fig. 1B and C) whereas ERK5 phosphorylation and activation is blocked with IC50 greater than 1 μM (Fig. 1B, C).

To confirm ERK5 signaling responsiveness in RPE cells, GAL4-MEF2C reporter assays were carried out. It has been established that ERK5 phosphorylates and activates GAL4-MEF2C and that MEF2 reporter assays are sensitive and reliable assays for ERK5 activation (8,22). Transcriptional activity of MEF2C is specifically enhanced by ERK5 and p38 MAPK, but not ERK1/2 (14,41). In agreement, while transfection of ERK5 alone had no effect on GAL4-MEF2C luciferase induction, expression of CA-MKK5β activated GAL4-MEF2C by 1.8-fold, while co-transfection of CA-MKK5β + WT-ERK5 activated GAL4-MEF2C by 3.8-fold (Fig. 2). As expected, expression of CA-MKK1 with or without ERK2 had no significant effect on GAL4-MEF2C-dependent luciferase induction, although activation of a c-fos SRE luciferase reporter was observed under this condition, consistent with enhanced signaling through ERK1/2 (data not shown). Interestingly, co-expression of CA-MKK1 + CA-MKK5β seemed to block GAL4-MEF2C-dependent transcription relative to CA-MKK5/ERK5 alone, suggesting that M KK1/2-ERK1/2 antagonizes responses to MKK5/ERK5 signaling (Fig. 2). The same behavior was also observed in mouse C2C12 cells (data not shown), and was therefore not unique to RPE cells.

Identification of genes induced by ERK5 vs ERK1/2

In order to distinguish between molecular changes elicited by activation of ERK5 vs. ERK1/2, RPE cells were transfected to stimulate each pathway as described in Fig. 1 and total RNA was isolated for microarray analysis using Affymetrix U133A GeneChips, which contain 22,283 probe sets corresponding to 14,500 human genes. Measurements were performed in triplicate, each microarray corresponding to an independent transfection experiment. Two calculations commonly used to assess differential expression of genes from replicate experiments involve (i) significance analysis of microarrays (SAM), which applies modified t-tests to generate corrected p-values using a permutation-based method for assessing significance of changes, and (ii) rank products (RP), which sorts genes according to the likelihood of observing them (34,35). While both methods estimate the false discovery rate (FDR) by applying non-parametric methods, SAM requires an estimate of gene-specific variance while RP
makes relatively weak assumptions regarding variance of the data, making it a potentially more sensitive method (35). We found that reliable filters were achieved by requiring probe sets to show significant changes (FDR ≤ 20%) with both SAM and RP, or more stringent changes (FDR ≤ 5% with a low standard deviation across replicates) with either SAM or RP. After passing these criteria, a final filter required ≥ 2-fold change in average intensity. By combining the three filtering criteria, we identified differentially regulated genes that by inspection revealed functionally significant classes of genes regulated by MKK5 and MKK1. These were not identifiable by applying any of the three filters alone, which individually yielded larger sets of genes that were significantly difficult to cluster.

The results showed 36 genes that were induced by ERK5 activation, of which 17 were selectively upregulated by ERK5 and not ERK1/2 (Table 1), and 19 genes were upregulated by both ERK5 and ERK1/2 (Table 3). No genes which passed our filters were observed to be repressed in response to ERK5 activation. Genes upregulated by ERK5 (Tables 1 and 3) had diverse biological roles, with known functions in cell proliferation, cell survival and apoptosis, transcription, glucose metabolism, nucleotide metabolism, and pH homeostasis. Analysis of the literature showed that none of these genes had been previously shown to be regulated by ERK5, revealing many novel gene targets under control of this pathway.

Activation of ERK1/2 led to induction of 39 genes, of which 20 were selectively upregulated by ERK1/2 (Tables 2 and 3). In contrast to ERK5, many of the genes upregulated in response to ERK1/2 in these cells were previously observed in other systems, including FOS (42), HAS2 (43), PTHLH (44), BIRC3/AIP1 (45), CCL2/MCP1 (46,47), CXCL1 (48), CXCL2 (49), IL-6 (50), IL-8 (51), PTGS2 (52), and IL1B (53). No genes were found to be repressed by ERK1/2 signaling in RPE cells.

The 19 genes regulated by both ERK5 and ERK1/2 represented approximately half of the genes significantly responsive to either pathway. This high percentage is noteworthy, given previous studies of individual gene products that showed little or no overlap between the pathways (12,14,21). Many of these genes are regulated by NFκB and AP1, nuclear factors that are both known to play necessary roles in ERK5- or ERK1/2-mediated responses (21), and may thus provide common mechanisms for coregulation of gene expression by the two pathways. Altered expression of a subset of genes found responsive to ERK1/2 and ERK5 by microarray analysis were confirmed by RT-PCR and real time PCR (Table S2).

Further analysis focused on the 17 genes selectively induced by ERK5. Examination of these genes by careful literature searching and analysis of microarray databases showed that 14 (82%) were previously linked with cellular responses to hypoxia (Table 1). Subsequent inspection of genes upregulated by both ERK5 and ERK1/2 showed 16 of 19 (84%) also previously shown to be hypoxia-regulated (Table 3). In contrast, only 9 of 20 (45%) genes selectively regulated by ERK1/2 alone showed links to hypoxia signaling (Table 2), none of which were solidly established as hypoxia-regulated. Indeed, all genes except for FOS (54,55) and stanniocalcin-1 (55,56) were linked to hypoxia only by a single microarray study of hypoxia induced responses in human umbilical cord vein endothelial cells (55).

Regulation of HIF-1 dependent transcription by ERK5

The relevance of ERK5 vs ERK1/2 to HIF-1 dependent signaling was then examined using an HRE luciferase reporter, pGL-HIF (32). This reporter contains a concatamerized HRE containing three repeats from the EPO
The HRE has previously been characterized as selectively transactivated upon binding of dimeric HIF-1α/β. While transfection of RPE cells with the HRE-luc reporter and ERK5 led to a minimal effect on luciferase induction, co-transfection of cells with CA-MKK5α + WT-ERK5 or CA-MKK5β + WT-ERK5 activated transcription by 2.6-fold and 1.7 fold, respectively (Fig. 3). This provided a preliminary indication that MKK5/ERK5 signaling positively regulates HRE-dependent transcription.

ERK1/2 have previously been shown to regulate HRE promoter activity of various genes, including VEGF, GLUT and CA9 (57-59). Thus, it was no surprise that activation of ERK1/2 signaling by co-transfection of CA-MKK1 + ERK2 also enhanced pGL-HIF promoter activity by an amount comparable to MKK5/ERK5 (2-fold, Fig. 3). Simultaneous activation of ERK5 and ERK1/2 pathways, by cotransfection of CA-MKK5α/β, CA-MKK1, ERK5 and ERK2 resulted in a 4-fold induction of transcription from the HRE promoter (Fig. 3). The additivity seen between ERK5 and ERK1/2 suggests that these pathways promote HRE-dependent transcription through independent mechanisms.

To confirm the link between ERK5 and the regulation of HRE through HIF-1, we tested transactivation using a GAL4-HIF-1α fusion [GAL4-HIF1(531-826)], cotransfected with a GAL4-luciferase reporter (31). HIF-1α contains an N-terminal transactivation domain (TAD-N, residues 531-575) which is constitutively active, and a C-terminal transactivation domain, (TAD-C, residues 786-826) (31), which is activated by hypoxia (60). The stability of GAL4-HIF-1α protein is not affected by oxygen tension (31), thus this construct provides an excellent system to test HIF-1α transactivation independent of protein stability or expression. Expression of ERK5 induced transcription through GAL4-HIF-1α by 1.4-fold, whereas co-expression of CA-MKK5α + ERK5 or CA-MKK5β + ERK5 enhanced transcription activity by 3-fold (Fig. 4).

Notably, CA-MKK1 + ERK2 had no effect on transcription through the GAL4-driven reporter (Fig. 4). Co-transfection with CA-MKK5α/β + CA-MKK1 along with ERK5 + ERK2 resulted in transcriptional activity similar to that observed for CA-MKK5 + ERK5 (3.6-fold, Fig. 4). Western blots confirmed that ppERK1/2 was indeed elevated in these extracts (data not shown). Thus, unlike pGL-HIF, the GAL4-HIF-1α system was selectively regulated by ERK5 and unaffected by ERK1/2 signaling. Taken together, these results indicate that ERK5 is a key signaling pathway for the regulation of HIF-1 transactivation, and involves a mechanism distinct from that of ERK1/2.

One mechanism by which cells respond transcriptionally to hypoxia involves posttranslational modifications that elevate HIF-1α protein by blocking its proteolytic turnover. In order to determine the effect of ERK5 and ERK1/2 signaling on HIF-1α expression, Western blots were carried out on nuclear extracts prepared from serum-starved RPE cells transfected with CA-MKK5α/β+ERK5 or CA-MKK1+ERK2. Under non-hypoxic conditions, HIF-1α was almost undetectable in cells transfected with control vector (pCDNA3) (Fig. 5, lane 1), and induced above background in cells co-transfected with CA-MKK5α + ERK5 or CA-MKK5β + ERK5 (Fig. 5, lanes 2 and 3). HIF-1α protein levels were also induced in the presence of CA-MKK1 + ERK2 (Fig. 5, lane 4), as well as the combination of CA-MKK5, CA-MKK1, ERK2 and ERK5 (Fig. 5, lane 5). As previously reported (61), the hypoxia mimic, CoCl2 induced HIF-1α to very high levels (Fig. 5, lane 6), and was inhibited by pretreatment of cells with the cell permeable compound, U0126 (Fig. 5, lane 6), which inhibits both MKK1/2 and MKK5 at higher concentrations (61). Regulation of protein
expression appeared to be controlled by post-transcriptional mechanisms, because HIF-1α mRNA levels were unaffected by activation of either ERK5 or ERK1/2 in the microarray datasets (data not shown).

Regulation of ERK5 by Hypoxia

To determine the significance of ERK5 signaling under hypoxic conditions, RPE cells were treated with or without hypoxia (3% O₂), monitoring the phosphorylation of ERK5 and ERK1/2 by Western blotting. Figure 6 shows that while ERK5 and ERK1/2 were activated by EGF in a U0126-dependent manner (lanes 1-3, upper panels); ERK5 and ERK1/2 were not significantly activated by hypoxia treatment for 1, 5 or 24 hours (lanes 4-9 vs. 10-15, upper panels). As a positive control to ensure hypoxic conditions were achieved, HIF-1α protein levels were monitored by Western blotting, and was found to be induced at 5 and 24 h after incubation at 3% O₂ (Fig. 6, lanes 4-9 vs. 10-15). Thus, the regulation of hypoxia-responsive genes by ERK5 appears to be important under normoxic rather than hypoxic conditions in RPE cells. Likewise, the induction of HIF-1α is not affected by U0126 (Fig. 6, lanes 12-15), consistent with our findings that transcription from pGalHIF and EPO-HRE reporters was unaffected by U0126 following hypoxia (data not shown). Taken together, these data indicate that hypoxia induced transcription does not require ERK5 and ERK1/2 signaling in RPE cells.

We conclude that MKK5-ERK5 signaling induces HIF-1α protein expression and HRE transactivation to a similar extent as that induced by the MKK1/2-ERK1/2 pathway. On the other hand, MKK5-ERK5, but not MKK1/2-ERK1/2, enhances transcription from a GAL4-HIF-1α reporter system. Taken together, these studies indicate that the control of HRE gene expression via induction of HIF-1α represents a mechanism that appears to be shared with ERK1/2, whereas alternative mechanisms involving regulation of HIF-1α are controlled by ERK5 in a manner distinct from ERK1/2.

DISCUSSION

In this study we used global gene expression measurements to profile responses to activation of separate ERK pathways. Selective stimulation of ERK5 and ERK1/2 respectively increased expression of 36 and 39 genes, respectively. None of the ERK5 responses were previously identified as regulated by this pathway, in contrast to ERK1/2 responses, where 11 genes were previously identified as downstream targets. In general, the magnitude of changes responsive to ERK5 were lower compared to ERK1/2, and interestingly, no gene whose message was decreased in response to ERK5 or ERK1/2 passed our filters.

The profiling results revealed new insight into the specificity of signaling through each pathway. Of the responses observed, 47% of ERK5 targets and 51% of ERK1/2 targets were uniquely regulated by each pathway. Significantly, of the 17 genes controlled by ERK5 alone, 14 were found by literature searching to be regulated in response to hypoxia (Table 1). Most of these were previously validated by experiments demonstrating a link to transcription by HIF-1, a basic helix-loop-helix factor that promotes transcription under conditions of low oxygen tension (55,62-65). For example, of the set of 14 ERK5-responsive genes, nine were induced or blocked upon WT HIF-1α or dominant-negative HIF-1α overexpression, respectively, in cell lines (bHLHB3, PFKFB3, PFKFB4, ADM, CA9, PPP1R3C, SLC2A3, HIG2, and CCNG2, Table 1), and four were downregulated upon homologous recombination to delete expression of HIF-1α or β (CCNG2, ADM, DDIT4, SLC2A3, Table 1). In addition, six genes had promoter sequences which were verified as hypoxia- or HIF-inducible elements or which bound to HIF-1 (bHLHB3, ADM, PFKFB3, PFKFB4,
CA9, and DDIT4, Table 1), and of the remaining, one showed conserved E box elements containing potential HREs with HIF-1 consensus sequences (5'-RCGTG-3': CALR).

The preferential representation of hypoxia-responsive genes in the set of ERK5 targets was striking, and strongly suggested a positive role for the ERK5 pathway in the control of HIF-1-dependent HRE transcription. This was confirmed experimentally by demonstrating elevated transcription from a promoter containing three repeats of the EPO-HRE, in response to ERK5 pathway activation. This finding provides evidence that ERK5 is able to promote transactivation from an HRE-dependent promoter, and that this regulation is relevant under normoxic conditions.

Mechanisms for regulation were then tested by examining the potential control of HIF-1 expression by this pathway. The role of the transcription factor HIF-1α/β in adaptive hypoxia-mediated signaling is well established (66,67), and known to be controlled by hypoxia at the level of protein stability. Under normoxic conditions, human HIF-1α is hydroxylated at Pro402 and Pro564 which promotes ubiquitination by the E3-ligase, von-Hippel-Lindau (VHL), and proteasome mediated degradation (68-70). HIF-1α is also hydroxylated at Asn803 within its C-terminal transactivation domain, which prevents binding of the co-activator p300/CBP (71,72). Under conditions of low oxygen tension hydroxylation is repressed, HIF-1α is stabilized, and gene expression is activated by formation of dimeric HIF-1α/β as well as derepression of p300/CBP binding (68-70).

We found that ERK5 signaling resulted in a significant increase of HIF-1α expression compared to controls, concomitant with the upregulation of HRE-dependent transcription. In addition, ERK5 activation led to stimulation of GAL4 transcriptional activity in the presence of a GAL4-HIF-1 fusion protein. Previous studies have shown levels of this fusion protein are stable and remain unchanged even under conditions of hypoxia (31). This was confirmed in our system by controls showing no effect of ERK5 on GAL4-HIF-1 protein (data not shown). The ability of ERK5 to activate GAL4-luciferase transcription indicates a mechanism independent of expression, conceivably through GAL4-HIF-1 post-translational events although at this point we find no evidence for regulated phosphorylation. Taken together, our results indicate that ERK5 may control hypoxia responsive genes by at least two mechanisms, one involving elevated protein stability or synthesis, and another that is independent of protein expression control.

In contrast to ERK5, many of the 20 genes found to be selectively regulated by ERK1/2 had been previously characterized as targets for this pathway, including genes known to control cellular events related to proliferation and tissue remodeling. Of these, nine were previously reported to be associated with hypoxia signaling (Table 2). However, few of these genes were well characterized as hypoxia targets, the majority being identified in a single microarray study which profiled gene expression changes at low oxygen (55), and none have been explicitly linked to HIF-1 regulation or shown to depend on HRE-controlled transcription. Thus, global responses to ERK1/2 differed substantively from ERK5 responses, in that a mechanistic link between ERK1/2 and HIF-1/HRE could not be established on the basis of target gene regulation in our system.

This result implies that ERK5 and ERK1/2 might differ in their respective mechanisms of gene regulation. ERK1/2 is known to promote expression of HIF-1α and has also been shown to increase its transactivation either by direct phosphorylation within its C-terminal transactivation domain (73-75), or by indirect phosphorylation of p300/CBP (76). However,
the fact that both ERK5 and ERK1/2 elevate HIF-1α protein levels to the same degree suggests that induced expression is irrelevant to the mechanism by which well characterized hypoxia genes are selectively upregulated by ERK5. Conceivably, the relevant mechanism reflects the events involved in preferential activation of GAL4-HIF-1 by ERK5, and does not involve control of HIF-1α protein. We may expect these two MAPK pathways to have very different mechanisms for activation of hypoxia-responsive genes in cells. We further note that many previous studies implicating ERK1/2 in the regulation of HIF-1α are based on repression of transcription using cell permeable inhibitors of MKK1/2, such as compounds U0126 and PD98059, which have been used at concentrations ranging from 10-100 μM (61,75,77). As demonstrated by Cohen’s laboratory in HeLa cells (78) as well as our study (Fig. 1 and data not shown), ERK5/MEK5 signaling is sensitive to inhibition by U0126 at concentrations above 10 μM. Thus, many previous studies cannot preclude the involvement of ERK5 rather than ERK1/2 in the regulation of HIF-1α, highlighting the importance of our studies supporting a role for ERK5-MKK5 in the regulation of HIF-1α transactivation.

Significant overlap was seen in the regulation of gene expression by these pathways, observed in approximately half of the genes responsive to ERK5 and ERK1/2, respectively, and representing a wide range of biological function. This indicates that the ERK5 and ERK1/2 pathways converge on more targets than had been previously recognized (12,14,21). This was intriguing, given the different signaling mechanisms used by ERK5 vs ERK1/2, which often differ even in cases where the two pathways share a common target. For example, both ERK5 and ERK1/2 phosphorylate the transcription factor, CREB, but the localization of ERK5 activation specifies its preferential involvement in CREB phosphorylation and neuronal survival (12). Additionally, in regulating CYP24 gene expression, ERK5 directly phosphorylates and activates the nuclear factor, Ets-1, whereas ERK1/2 phosphorylates and activates the retinoid X receptor to control CYP24 promoter transactivation (13). On the other hand, ERK5 and ERK1/2 sometimes target the same molecule to elicit specific responses. For example, ERK5 and ERK1/2 cooperate to transform NIH3T3 cells, possibly through synergy between p90RSK and NFκB (21). Interestingly, 14 of the 19 genes upregulated by both ERK5 and ERK1/2 were previously reported to be responsive to hypoxia (Table 3). Literature searching showed two that were regulated by HIF-1α. Thus, like ERK1/2, hypoxia responsive genes controlled by both ERK5 and ERK1/2 appear to be in a separate class than those regulated by ERK5 alone, suggesting control by independent mechanisms. Six genes in the former class are known to be transcribed in response to hypoxia via NFκB (Table 3), implicating this transcription factor in the regulation of ERK1/2 responsive genes.

Finally, we speculate on the importance of ERK5 signaling in mediating responses to hypoxia. Two previous studies have shown that ERK5 is phosphorylated in response to low oxygen tension and also functions to promote angiogenesis (79,80). Interestingly, both reports showed that ERK5 activation inhibited hypoxia-induced VEGF expression, most likely by suppression of HIF-1α (79,80). These studies, which were carried out in bovine lung microvascular endothelial cells (BLMECs) and mouse embryonic fibroblast cells, showed behavior opposite to that of the RPE system used in our study, where ERK5 instead promotes HIF-1/HRE signaling. Such differences are most likely explained by cell function. Epithelial cells are more vulnerable to ischemia, responding by increasing cellular permeability and
breakdown of barrier function (81). RPE cells maintain the blood-retinal barrier by facilitating macromolecular transport between the choroids and the outer neural retina, and abnormal behavior of RPE cells has been shown to contribute to age-related macular degeneration and ischemic retinopathies (82,83). A reasonable hypothesis is that regulation of genes by ERK5 may be relevant to the adaptive response to hypoxia, which is instantaneous and involves Hif-1α/β. In contrast, convergent regulation of genes by ERK1/2 with or without ERK5 may preferentially regulate targets relevant to an inflammatory hypoxia response in epithelial cells which is known to involve NFκB, CREB, and API (84). Thus, the participation of different ERK pathways may provide a mechanism to respond selectively to hypoxia in RPE cells.

**FIGURE LEGENDS**

**Figure 1. Expression of CA-MKK5 and CA-MKK1 and activation of ERK5 and ERK1/2.** (A) RPE cells (2 x 10⁵) were transfected with the indicated combinations of rat CA-MKK5β, CA-MKK1, ERK5, or ERK2 and harvested 24 h after transfection. Expression of CA-MKK1 and CA-MKK5 was monitored by Western blotting with antibodies against HA (1:2000). Activation of ERK5 and ERK1/2 was monitored by probing Western blotting with antibodies against ERK5 (1:1000) and ppERK1/2 (1:2000), respectively. (B) RPE cells (2 x 10⁵) were pretreated with the indicated amounts of U0126 for 1 h prior to stimulation with 20 ng/ml EGF for 10 min. Cells were harvested and ERK5 and ERK1/2 activity was monitored as in 1A except that the gel running time was increased to maximize the ppERK5 phosphorylation shift. Similar results were seen in two additional experiments. (C) ERK5 and ERK1/2 inhibition by U0126 (Fig. 1B) was quantified from Western blots using Adobe Photoshop. ERK5 phosphorylation was quantified as the ratio of gel mobility retarded species to the faster mobility species. ERK5 and ERK1/2 stimulated by EGF in the absence of U0126 was set to 100% and the degree of basal ERK5 phosphorylation observed under control conditions (no stimulation, 5%) was subtracted from each measurement. Results presented are the mean +/- S.D. of two experiments.

**Figure 2. MKK5 and MKK1 activation of MEF2C in RPE cells.** RPE cells (2 x 10⁴) were transfected with the Gal4MEF2C-luciferase reporter along with the indicated effector or control plasmids, as in Fig. 1A, and pRLnull Renilla. Cells were harvested 24 h after transfection and firefly luciferase activity was normalized to Renilla. Results are the mean +/- standard deviation of 3 transfections done in duplicate.

**Figure 3. MKK5 and MKK1 activation of the EPO HRE.** RPE cells were transfected with the pGL(HRE)3-luciferase promoter, the indicated effector plasmids, along with pRLnull Renilla as in Fig. 2 except hMKKα/β were used. Cells were harvested and assayed for luciferase and Renilla as in Fig. 2. Results are the mean +/- standard deviation of 3 transfections done in duplicate.

**Figure 4. MKK5 and MKK1 activation of Hif-1α in RPE cells.** RPE cells were transfected with the pGal4-Hif luciferase reporter along with the indicated effector or control plasmids, and pRLnull Renilla as in Fig. 3. Cells were harvested and assayed for luciferase and Renilla as in Fig. 2. Results are the mean +/- standard deviation of 3 transfections done in duplicate.
Figure 5. Western blot analysis of Hif-1α. RPE cells (3 x 10^6) were transfected using FuGene with pCDNA3 (lane 1), human CA-MKK5β/ERK5 (lane 2), human CA-MKK5α/ERK5 (lane 3), CA-MKK1/ERK2 (lane 4), or CA-hMKK5β/CA-MKK1/ERK5/ERK2 (lane 5). At the time of transfection, cells were also pre-treated with 10 μM U0126 (lane 6) or vehicle (lane 7) for 1 h prior to treatment with 100 μM CoCl2. Cells were serum starved and harvested at 24 h for nuclear extract preparation, as described under “Experimental Procedures”. Expression and activation of Hif-1α, was monitored using antibodies recognizing Hif-1α (1:1000).

Figure 6. ERK5 is not activated under conditions of hypoxia. RPE cells (8 x 10^5) were incubated with 10 μM U0126 for 1 h prior to treatment with or without hypoxia (3% O2) for the indicated times. Expression and activation of ERK5, ERK1/2 and HIF-1α was monitored by Western blotting as described in Figs. 1 and 4. Where indicated, RPE cells were treated with 20 ng/ml EGF +/- 10 μM U0126, as described in Fig. 1, as a positive control for ERK5 and ERK1/2 activation. Statistical analysis (Student’s t test) of Western blots quantified as in Fig. 1C showed that ERK5 phosphorylation did not significantly differ between normoxic vs. hypoxic conditions.

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Table 1. Genes upregulated by CA-MKK5 but not CA-MKK1

| Gene Title | Gene Symbol | Average Fold Change | Hypoxia TF involved |
|------------|-------------|---------------------|---------------------|
| **Genes previously reported to be upregulated by hypoxia** | | | |
| Adrenomedullin | ADM | 2.3 | 1.2 | HIF1-1α (55) |
| basic helix-loop-helix domain containing, class B, 3 (DEC2) | BHLHB3 | 2 | 1.1 | HIF1-1α (85) |
| chromosome 10 open reading frame 10 (DEPP) | C10orf10 | 2.2 | 1.2 | |
| carbonic anhydrase IX | CA9 | 2.2 | 1.1 | HIF-1α (62) |
| Calreticulin | CALR | 7.4 | 1.8 | |
| Cyclin G2 | CCNG2 | 2.1 | 1.2 | HIF1-α (86,87) |
| hypothetical protein DKFZp434K1210 | DKFZp434K1210 | 2.3 | 1.2 | |
| Hypoxia-inducible protein 2 | HIG2 | 5 | 1.1 | HIF1-α (88) |
| MAX interacting protein 1 | MXI1 | 2.7 | 1 | |
| 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 | PFKFB3 | 3.8 | 1.5 | HIF-1α (89) |
| 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 | PFKFB4 | 2 | 1.3 | HIF-1α (63,64) |
| Protein phosphatase 1, regulatory (inhibitor) subunit 3C | PPP1R3C | 2.4 | 1 | |
| DNA-damage inducible transcript 4/REDD/RTP801 | DDIT4 | 5.2 | 1.9 | HIF-1α (90), Sp1 (91) |
| solute carrier family 2, member 3 (GLUT3) | SLC2A3 | 2 | 1.3 | HIF-1α (90,92,93) |

| **Other genes upregulated by MKK5 but not linked to hypoxia** | | | |
| chemokine (C-X-C motif) ligand 6 | CXCL6 | 2.1 | 1.5 | |
| Insulin induced gene 2 | INSIG2 | 2.1 | 1.1 | |
| Tumor necrosis factor, alpha-induced protein 2 | TNFAIP2 | 2.4 | 1.6 | |
Table 2. Genes upregulated by CA-MKK1 but not CA-MKK5

| Gene Title                                                                 | Gene Symbol | Average Fold Change | TF Involved          |
|---------------------------------------------------------------------------|-------------|---------------------|----------------------|
| Genes previously reported to be upregulated by hypoxia                    |             |                     |                      |
| dual specificity phosphatase 6                                            | DUSP6       | 3                   | 0.8                  |
| endothelial cell-specific molecule 1                                      | ESM1        | 5.5                 | 1.2                  |
| v-fos FBJ murine osteosarcoma viral oncogene homolog                      | FOS         | 2.7                 | 1.1                  |
| hyaluronan synthase 2                                                     | HAS2        | 2                   | 0.8                  |
| parathyroid hormone-like hormone                                          | PTHLH       | 5.1                 | 1                    |
| serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2 | SERPINB2    | 14                  | 1.2                  |
| stanniocalcin 1                                                           | STC1        | 2.2                 | 1.3                  |
| tissue factor pathway inhibitor 2                                          | TFPI2       | 2.5                 | 1.5                  |
| Thrombomodulin                                                            | THBD        | 2.6                 | 1                    |
| Other genes significantly upregulated by MKK1                             |             |                     |                      |
| AFFX-r2-Hs18SrRNA-M_x_at                                                  | ---         | 2                   | 1.3                  |
| amphiregulin (schwannoma-derived growth factor)                          | AREG        | 4.2                 | 0.9                  |
| 2,3-bisphosphoglycerate mutase                                            | BPGM        | 1.6                 | 1.1                  |
| chromosome 20 open reading frame 42                                       | C20orf42    | 2.3                 | 1                    |
| dual specificity phosphatase 5                                            | DUSP5       | 2                   | 0.9                  |
| early growth response 1                                                   | EGR1        | 2.2                 | 0.9                  |
| early growth response 3                                                   | EGR3        | 2.2                 | 1.1                  |
| interleukin 11                                                            | IL11        | 2.4                 | 1.1                  |
| matrix metalloproteinase 1 (interstitial collagenase)                     | MMP1        | 16.3                | 1.2                  |
| matrix metalloproteinase 10 (stromelysin 2)                              | MMP10       | 4                   | 1                    |
| pleckstrin homology-like domain, family A, member 1                       | PHLDHA1     | 2                   | 1                    |
| Gene Title                                                                 | Gene Symbol | Fold Change | TF involved                      |
|---------------------------------------------------------------------------|-------------|-------------|----------------------------------|
| Genes previously reported to be regulated by hypoxia                      |             |             |                                  |
| baculoviral IAP repeat-containing 3                                       | BIRC3       | 3.3         | M KK5                            |
| bone morphogenetic protein 2                                              | BMP2        | 1.6         | M KK1, M KK5                     |
| chemokine (C-C motif) ligand 2                                            | CCL2        | 2.3         | M KK5                            |
| colony stimulating factor 2 (granulocyte-macrophage)                      | CSF2        | 4.3         | M KK5, M KK1, M KK1              |
| chemokine (C-X-C motif) ligand 1                                          | CXCL1       | 21.6        | M KK5, M KK1, M KK1, M KK1, M KK1 |
| chemokine (C-X-C motif) ligand 2                                          | CXCL2       | 17.2        | M KK5, M KK1, M KK1, M KK1, M KK1 |
| chemokine (C-X-C motif) ligand 3                                          | CXCL3       | 8.9         | M KK5, M KK1, M KK1, M KK1, M KK1 |
| Ephrin-A1                                                                 | EFNA1       | 5.7         | M KK5, M KK1, M KK1, M KK1, M KK1 |
| intercellular adhesion molecule 1 (CD54)                                  | ICAM1       | 3.3, 2.5    | M KK5, M KK1, M KK1, M KK1, M KK1 |
| interleukin 6 (interferon, beta 2)                                       | IL6         | 3.7         | M KK5, M KK1, M KK1, M KK1, M KK1 |
| interleukin 8                                                             | IL8         | 12.3, 31.2  | M KK5, M KK1, M KK1, M KK1, M KK1 |
| N-myc downstream regulated gene 1                                         | NDRG1       | 4.4         | M KK5, M KK1, M KK1, M KK1, M KK1 |
| nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha | NFKBIA     | 2.3         | M KK5, M KK1, M KK1, M KK1, M KK1 |
| prostaglandin-endoperoxide synthase 2                                    | PTGS2       | 2.7         | M KK5, M KK1, M KK1, M KK1, M KK1 |
| pentaxin-related gene, rapidly induced by IL-1 beta                      | PTX3        | 2.2         | M KK5, M KK1, M KK1, M KK1, M KK1 |
| superoxide dismutase 2, mitochondrial                                    | SOD2        | 2.6         | M KK5, M KK1, M KK1, M KK1, M KK1 |
| Other genes significantly upregulated                                    |             |             |                                  |
| hypothetical protein FLJ23231                                            | FLJ23231    | 2.7         | M KK5, M KK1, M KK1, M KK1, M KK1 |
| interleukin 1, beta                                                      | IL1B        | 3.6         | M KK5, M KK1, M KK1, M KK1, M KK1 |
| tumor necrosis factor, alpha-induced protein 3                           | TNFAIP3     | 5.1         | M KK5, M KK1, M KK1, M KK1, M KK1 |
Table S1. PCR Primers used in this study

| Gene Symbol | PCR Primers | Product size |
|-------------|-------------|--------------|
| MMP1        | 5'-GGAGATCATCGGGACAACACTC-3' | 164 bp |
|             | 5'-AGTTCATGAGCTGCAACACG-3'    |        |
| SERPINB2    | 5'-TCAAACCAAAGGCAAAATCC-3'    | 81 bp  |
|             | 5'-ACAGCATCCACCAGGACCAT-3'    |        |
| EFNA1       | 5'-ACATCTCCAAACCCATCCAC-3'    | 149 bp |
|             | 5'-ATGTAGAACCCGCACCTCTTG-3'   |        |
| ICAM        | 5'-CACCTATGGCAACGACTCCT-3'    | 146 bp |
|             | 5'-CCGGAAAGCTGTAGATGGTC-3'    |        |
| DDIT4*      | 5'-GTGGAAGGTGGTTGTGTATAC-3'   | 150 bp |
|             | 5'-CACCCCTTGCTACTCTTTAC-3'    |        |
| ACTB        | 5'-CACCCAGCAATGAAGATC-3'      | 120 bp |
|             | 5'-CCTGCTTGCTGATCCACATC-3'    |        |
| TBP         | 5'-TATAATCCCAAGCGTTTGC-3'     | 143 bp |
|             | 5'-CTCCTGTGCACACCATTTC-3'     |        |

*primers were designed against the Affymetrix probe sequence using GenAct primer design program ([http://enhancer.colorado.edu:6400/~hudakg/home.html](http://enhancer.colorado.edu:6400/~hudakg/home.html)).

Table S2. Confirmation of changes induced by ERK5 and ERK1/2

| Gene Symbol | Affymetrix Fold Change | RTQ RT-PCR |
|-------------|------------------------|------------|
|             | MKK5 | MKK1 | MKK5 | MKK1 |
| MMP1        | 1.2  | 16.3 | 2.3  | 195  |
| SERPINB2 2  | 1.2  | 14   | 3    | 24   |
| EFNA1 1     | 5.7  | 2.8  | 1.38 | 2.93 |
| ICAM 1      | 3.3, 2.5 | 2.1  | 5.4  | 1.2  |
| DDIT4       | 5.2  | 1.9  | 1.62 | 2.16 |

1 normalized to β-actin (ACTB)
2 normalized to TATA binding protein (TBP)
A

Schwepp et al. Fig. 1

CA-MKK1/ERK2/5

ppERK5

ppERK1/2

ERK2

B

EGF

Control

0 0.3 1 3 10 μM U0126

ppERK5

ppERK1/2

C

% Inhibition

0 20 40 60 80 100

0 0.3 1 3 10

U0126 [μM]

ERK5

ERK1/2
Schweppe et al Fig. 2

ERK2

CA-MKK5β

β

CA-MKK1

β

CA-MKK5

β

CA-MKK1

ERK5

Fold Activation

0

1

2

3

4

5

6

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0

1

2

3

4

5

6

CA-MKK5β

CA-MKK1

CA-MKK5β + CA-MKK1
Schwepp et al Fig. 4

ERK2

MKK5

α

MKK5

β

+ +−− −− − −

+ +−− −− − −

ERK5

Fold Activation

0.5
1
1.5
2
2.5
3
3.5
4
4.5

MKK1

MKK5

α

MKK5

β

−

−

+ 

−

−

+ 

MKK1

MKK5

α +

MKK1

MKK5

β +

MKK1

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Schweppe et al. Fig. 5

CA-MKK5
β/ERK5
CA-MKK5α/ERK5
CA-MKK1/ERK2
CA-MKK5β/MKK1/ERK2/5
CoCl2
CoCl2 + U0126

HIF-1α

115 kDa
| Control | EGF | 1 h | 5 h | 24 h | EGF | 1 h | 5 h | 24 h |
|---------|-----|-----|-----|------|-----|-----|-----|------|
|         | -   | +   | -   | +    |     | -   | +   | -    |
|         |     |     |     |      |     |     |     |      |
|         |     |     |     |      |     |     |     |      |

**Normoxic**

**Hypoxic**

![Image of protein expression levels](image_url)

- **ppERK5**
- **ERK5**
- **ppERK1/2**
- **HIF-1α**
- **ERK2**

**Scheppe et al Fig. 6**
Global gene expression analysis of ERK5 and ERK1/2 signaling reveals a role for HIF-1 in ERK5-mediated responses
Rebecca E. Schweppes, Tom Hiu Cheung and Natalie G. Ahn

J. Biol. Chem. published online May 30, 2006

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