The Effect of Dominant Negative MKK Expression on pH-induced Increases in PEPCK mRNA Levels

Morgan O'Hayre, Lynn Taylor, and Norman P. Curthoys
Colorado State University Department of Biochemistry and Molecular Biology

Abstract:

Acid-base homeostasis is essential for survival. When metabolic acidosis is induced by factors such as prolonged starvation, severe shock, high protein diet, or uncontrolled type I diabetes, the kidneys act to compensate for the decreasing pH. Renal catabolism of glutamine, which is sustained through increased expression of phospho*enol*pyruvate carboxykinase (PEPCK) and glutaminase (GA), is activated during metabolic acidosis. Mitogen activated protein kinase kinases three and six (MKK3 and MKK6) are thought to play roles in the signal transduction pathway that lead to enhanced PEPCK and glutaminase activity. To examine the potential roles of MKK3 and MKK6, LLC-PK$_1$-FBPase$^+$ cells were stably transfected with dominant negative (dn) forms of either or both kinases. Expression of the transgenes was controlled by a Tetracycline-responsive promoter element (TRE). Doxycycline (dox) is used to inhibit transcription by preventing the tTA transcription factor from binding to the TRE. The absence of dox then enables transcription and turns on expression of the mutated kinase. Western blots were performed on extracts of clonal cell lines to determine the levels of the MKK isoforms as well as the levels of p38 and phosphorylated p38 in LLC-PK$_1$-FBPase$^+$ cells grown in both the absence and presence of dox. Northern blots were also performed to determine the effect of dnMKK expression on levels of PEPCK mRNA. Expression of both dominant negative kinases, but not the expression of either dnMKK3 or dnMKK6 alone, blocked the acid-induced increases in the levels of PEPCK mRNA and the Anisomycin stimulated increases in levels of phosphorylated p38.

Figure 1

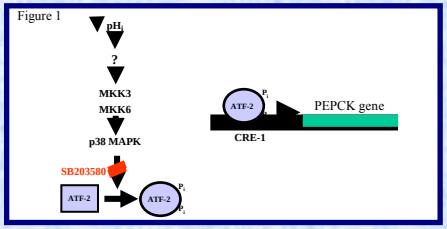

Fig. 1: The proposed signal transduction pathway results in an increase in transcription of the PEPCK gene in response to decreasing pH. MKK3 and MKK6 are thought to mediate the activation of p38 MAPK during metabolic acidosis, which then phosphorylates and activates ATF-2 so it can bind to the CRE-1 transcription element and turn on the transcription of the PEPCK gene. SB203580, a specific inhibitor of the p38 MAPK, was found to block the acid-induced increases in PEPCK mRNA[1].

Introduction:

Metabolic acidosis involves an abnormally high acid level and a low level of bicarbonate in the blood. Generation of bicarbonate ions by catabolism of α-ketoglutarate and production of ammonia normally help to balance decreases in pH[2]. During metabolic acidosis, renal catabolism of glutamine is activated to compensate for the acidosis and is enhanced by increased levels of phospho*enol*pyruvate carboxykinase (PEPCK) and glutaminase (GA)[3]. These two enzymes are regulated by different means: PEPCK is regulated at the level of gene transcription, while the activity of glutaminase is increased through stabilization of the mRNA by ζ-crystallin[3,4].

Mitogen activated protein kinases (MAPKs) function in regulating gene expression and metabolism. MAPKs are substrates for phosphorylation by highly selective MAPK kinases (MKKs)[5]. p38 MAPK is thought to play a key role in the enhanced transcription of PEPCK in response to acidosis. A decrease in pH activates p38 MAPK which then leads to the phosphorylation of the ATF-2 transcription factor in LLC-PK$_1$-FBPase$^+$ cells, a porcine renal proximal tubule-like cell line[1]. LLC-PK$_1$-FBPase$^+$ cells exhibit an increased ammonia synthesis from glutamine and adaptive increases in PEPCK and GA when incubated in acidic media (pH 6.9, 10 mM HCO_3^-). Thus they serve as a good model for *in vitro* studies involving metabolic acidosis[6].

The involvement of known MAPK activities (ERK1/2, SAPK/JNK, p38) in the associated signal transduction pathway was examined by determining the effects of specific MAPK activators and inhibitors on basal and acid-induced PEPCK mRNA levels[1]. Anisomycin (AI), a potent activator of the p38 kinase, led to increases in PEPCK mRNA to levels that are comparable to those observed with acid stimulation. Experiments also demonstrated that a specific inhibitor of the p38 MAPK, SB203580, blocked acid-induced increases in PEPCK mRNA levels, while selective inhibitors of the ERK1/2 and JNK had no effect[1]. MKK3 and MKK6 are thought to mediate the activation of p38 during acidosis, which then phosphorylates and activates ATF-2 so it can bind to the CRE-1 transcription element of the PEPCK promoter to turn on transcription of the PEPCK gene[1].

Stable 8C LLC-PK$_1$-FBPase$^+$ kidney cells were transfected with plasmids that encode dominant negative forms of MKK3, MKK6, and both kinases (MKK3/6). A tet-off system was used to regulate expression of the dominant negative and constitutively active constructs. The dominant negative and constitutively active clonal lines were screened for FLAG expression using Western blots, and lines that showed induced expression upon removal of dox and thus expression of the construct were selected for studies. Western blots were used to analyze the effect of dnMKK expression on levels of phosphorylated p38 in cells grown at acidic and normal pH conditions and under anisomycin stimulation. Northern blot analysis was also performed to examine the effect of dnMKK expression on levels of PEPCK mRNA made in cells grown in both acidic and normal pH media.

Figure 2

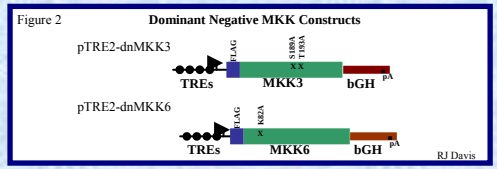

Figure 2: The dnMKK3 construct contains mutations in the residues that are phosphorylated when MKK3 is activated: serine 189 to alanine and threonine 193 to alanine. The dnMKK6 construct was created by mutation of lysine 82 to alanine within the ATP binding site[8]. An N-terminal FLAG-tag (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Asp-Lys-Cys) was incorporated into the constructs and the four constructs were then cloned into a pTRE-2 plasmid, a vector in which the promoter contains multiple tetracycline-responsive elements (TREs).

Figure 3

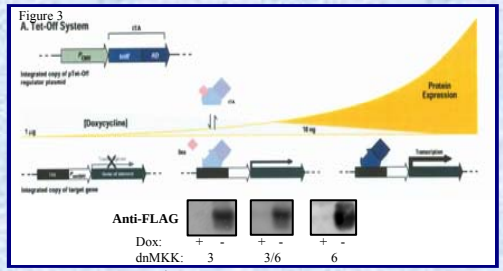

Figure 3: LLC-PK$_1$-FBPase$^+$ cell lines expressing dominant negative forms of MKK3 and MKK6 from a tet-responsive promoter were developed. In the absence of Doxacycline (Dox), transcription is strongly activated since the tTA proteins are able to bind the TRE. In the presence of Dox (>25ng/ml), the tTA protein is unable to bind the TRE and transcription is turned off. Western blots using FLAG antibody indicate that the dnMKK constructs are induced following the removal of dox.

Figure 4

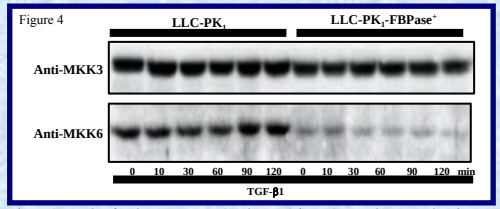

Figure 4: Levels of endogenous MKK6 and MKK3 in LLC-PK$_1$, the parental and non-gluconeogenic line of the FBPase+ cells, and LLC-PK$_1$-FBPase$^+$ cells were quantified by western blots. The results indicate the presence of high endogenous levels of MKK3 but only low levels of MKK6 in LLC-PK$_1$-FBPase$^+$ cells (Andratsch, Manfred).

Figure 5

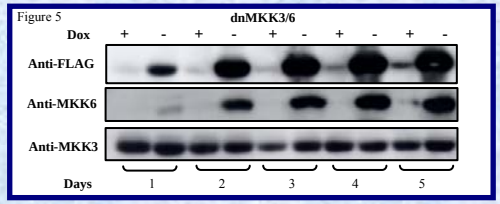

Figure 5: Time course of expression of dnMKK3/6 in cells grown with (+) and without (-) dox. Western blots were prepared from extracts of dnMKK3/6 cells grown for 1 to 5 days with or without dox and then probed with anti-FLAG, anti-MKK6, and anti-MKK3 antibodies.

Figure 6

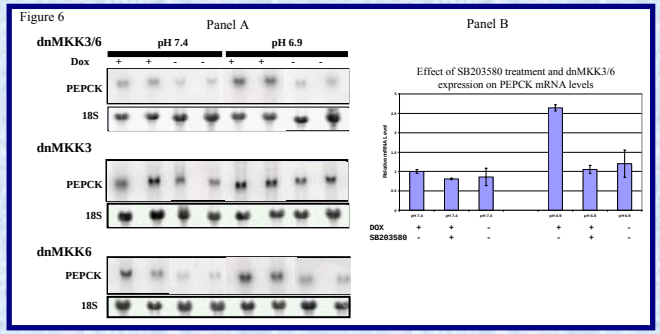

Figure 6: Panel A: Effect of dnMKK expression on levels of PEPCK mRNA. dnMKK cells were grown to full confluency for 12 days. 24 hours prior to harvest, cells were treated with pH 7.4 or 6.9 media. RNA was isolated from the cells and Northern blots were performed probing for PEPCK mRNA. The blots were stripped and standardized with 18S RNA. Panel B: Comparison of the effect of dnMKK3/6 expression and SB203580 inhibitor use on acid-induced increases in PEPCK mRNA. dnMKK3/6 cells were grown to full confluency for 12 days. 24 hours prior to harvest, cells were treated with pH 7.4 or 6.9 media and 10 μM of SB203580 inhibitor was added to cells growth with dox. Northern blots were performed on dnMKK3/6 cells and the effect of dnMKK3/6 expression was compared to implementation of SB203580 on cells grown in pH 7.4 and 6.9 media.

Figure 7

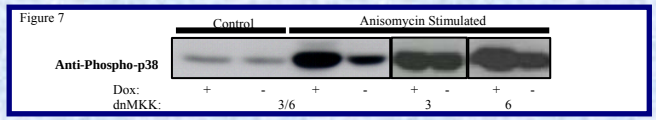

Figure 7: Effect of dnMKK expression on anisomycin stimulated levels of phosphorylated p38. Extracts were prepared from cells that had been grown 6 days with (+) or without (-) dox. Control samples were treated with pH 7.4 (25 mM HCO_3^-) media. Anisomycin stimulation involved treatment of cells for 30 minutes with 5μM anisomycin (AI). Extracts were then probed with anti-phospho p38 antibody.

Conclusions and Future Directions:

-Selected dnMKK cell lines exhibited a large difference in FLAG expression when grown with and without dox, thus demonstrating control of construct expression

-Maximal induction in FLAG-tag is observed 5 days following removal of dox

-The dnMKK3/6 construct exhibits a strong induction in MKK6 levels when the construct is expressed, however only slight increases in MKK3 are observed, possibly due to the high endogenous levels of MKK3

-Expression of both dnMKK3 and dnMKK6 constructs blocks acid-induced increases in PEPCK mRNA

-However, expression of the dominant kinases individually is not sufficient to completely block the acid-induced increases in PEPCK mRNA

-Expression of dnMKK3/6 significantly reduces anisomycin stimulated increases in levels of phosphorylated p38, while expression of dnMKK3 and dnMKK6 individually have reduced abilities to block the anisomycin induced phosphorylation of p38

-Although LLC-PK$_1$-FBPase$^+$ cells express high levels of MKK3 but only low levels of MKK6, it appears that the MKK6 is involved in the signal transduction pathway leading to increases in PEPCK mRNA in response to treatment with acidic media.

-Either MKK3 and MKK6 are both required for signal transduction that leads to increases in PEPCK mRNA in response to growth in acidic media or they serve in parallel, redundant paths, possibly with MKK6 as the preferred kinase

-Further experiments will be performed to determine when the pH response is observed in phosphorylated p38 levels and quantify the relative amounts of the phosphorylated form by normalizing to the total levels of p38.

-Studies involving constitutively active MKKs should help clarify whether the MKK3 and MKK6 act cooperatively or independently and should provide another tool for investigating the role of the p38 MAPK pathway in the acid-induced increases in PEPCK mRNA

-Future experiments will also involve investigation of the effect of dominant negative MKK expression on glutaminase mRNA levels in order to determine if MKK3 and MKK6 are also involved in stabilization of GA mRNA in response to acidosis

References:

1. Feifel, Elisabeth et al. *Am J Physiol Renal Physiol* 283: F678-688, 2002.
2. Gstraunthaler, Gerhard, et al. *Am J Physiol Renal Physiol* 278: F227-F237, 2000.
3. Hwang J-J and Curthoys NP. *J Biol Chem* 266: 9392-9396, 1991.
4. Curthoys, Norman P. and Gerhard Gstraunthaler. *Am J Physiol Renal Physiol* 281:F381-F390, 2001.
5. Johnson, Gary L. and Razvan Lapadat. *J. Am. Soc. Nephrology*. 14:xxx, 2003.
6. Gstraunthaler G and Handler JS. *Am J Physiol Cell Physiol* 252: C232-C238, 1987.
7. Loflin PT, Chen CY, Xu N, Shyu AB. *Methods* 17(1):11-20, 1999.

Acknowledgements:

Norman P. Curthoys, Lynn Taylor, Gerhard Gstraunthaler, and Manfred Andratsch for their time and guidance.

All Curthoys lab members.