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Hypertonicity stimulates PGE_2 signaling in the renal medulla by promoting EP3 and EP4 receptor expression

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Hypertonicity in the renal medulla stimulates local cyclooxygenase 2 expression, leading to abundant PGE_2 production. Here we found that mRNA expression by the PGE_2-activated G-protein-coupled receptors, EP3 and EP4 in the renal medulla was decreased by furosemide treatment, a procedure that reduces medullary hypertonicity. When HepG2 cells were cultured in hypertonic conditions by addition of salt or sorbitol, EP3 expression was induced. A specific EP3 agonist inhibited cAMP production, indicating receptor functionality, and this led to a substantial increase in cell survival in hypertonic media. Survival was independent of the SLC5A3 inositol transporter and aldose reductase expression, suggesting that EP3 promoted cell survival under hypertonic conditions independent of cellular organic osmolyte accumulation. Reduced cAMP production did not contribute to increased survival. EP4 expression was stimulated by hypertonicity in MDCK and HepG2 cells, which was associated with increased cAMP production in response to an EP4 agonist. Our study shows that local hypertonicity promotes PGE_2 signaling in the renal medulla by stimulating cognate receptor and cyclooxygenase 2 expression that likely regulates local hemodynamics and tubular transport.

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Operation of the urinary concentrating mechanism produces hyperosmolality in the interstitium of the renal medulla due to the accumulation of salt and urea. Although the interstitial osmolality fluctuates depending on physiological and pathophysiological conditions, it stays hyperosmotic even when hypoosmotic urine is produced.[1] Thus, the cells in the renal medulla are continuously bathed in hyperosmolality under most conditions.

Salt and urea exert fundamentally different effects on cells. Hyperosmotic salt is hypertonic, that is, causes net efflux of water across the plasma membrane raising the cellular ionic strength. Exposure to hypertonicity results in double stranded DNA breaks[2] and, depending on the degree of hypertonicity, cell cycle arrest or cell death.[3] Cells adapt to the hypertonic insults by activating several pathways. (1) Repair of the DNA breaks takes place over the course of several hours[2] as much of the DNA damage signaling remains active in hypertonic conditions.[4] (2) Normal cellular ionic strength is restored as the ions are osmotically replaced by the massive accumulation of organic osmolytes. Stimulation of transcription by the transcription factor TonEBP is a key event in the accumulation of organic osmolytes. This is because expression of the active plasma membrane transporters and biosynthetic enzymes involved in the cellular accumulation of organic osmolytes is transcriptionally stimulated by TonEBP.[5] (3) Cyclooxygenase 2 (COX-2) is constitutively expressed in the renal medulla because of transcriptional stimulation by local hypertonicity in a manner dependent on nuclear factor-κB[6] but independent of TonEBP.[7] The high COX-2 activity leads to abundant production of prostaglandin E_2 (PGE_2) which promotes cell survival against hypertonic stress in a manner independent of organic osmolyte accumulation.[8]

Urea is highly permeable to the plasma membrane and, therefore, a hyperosmotic fluid made by addition of urea is not hypertonic. Nonetheless, over 300 mM urea, which is routinely found in the inner medullae of mammal kidneys, causes oxidative DNA damages[9] and cell death.[3] Heat-shock protein 70 (HSP70) which is highly expressed in the renal medulla contributes to the protection from the deleterious effects of urea.[10] Both TonEBP and COX-2 are involved in the high expression of HSP70. TonEBP binds directly to the

promoter of the *HSP70* gene and stimulates its transcription.[7] One of the prostaglandin metabolites found in the renal medulla, Δ^{12}-PGJ_2, directly stimulates heat-shock factor 1 and as a result the transcription of HSP70.[11] It is interesting to note that cellular responses to hypertonicity, namely stimulation of TonEBP and expression of COX-2, are critical in protecting the renal medulla not only from the deleterious effects of hypertonicity itself, but also from the harmful effects of urea.

In this study we found that expression of the G-protein-coupled receptors for PGE_2 in the renal medulla was transcriptionally stimulated in response to ambient hypertonicity. The receptors mediated the pro-survival signal and are known to modulate local hemodynamics and tubular transport processes. Thus, we have found yet another cellular response to hypertonicity which is critical to the function of the renal medulla.

RESULTS

Reduced expression of EP3 and EP4 mRNA in the renal medulla in response to furosemide treatment

We were interested in those genes whose expression in the renal medulla was promoted by the local hyperosmolality. To find these genes, we decided to look for downregulated genes in response to washout of the hyperosmolality in the renal medulla using the diuretic furosemide as described in 'Materials and Methods'. Functional genomic screening of RNA samples revealed many genes downregulated in response to furosemide treatment including the PGE_2 receptors EP3 and EP4 (data not shown). To confirm the downregulation of EP3 and EP4 mRNA, we performed quantitative reverse transcriptase (RT)-PCR on a separate set of RNA samples. As shown in Figure 1, the abundance of EP3 mRNA decreased more than 60% in the outer medulla and inner medulla in response to furosemide. Because of large variations in vehicle groups and small sample numbers ($n = 4$), statistical significance was reached only in the outer medulla after 1 day of treatment and in the inner medulla after 5 days. Significant reduction in the abundance of EP4 mRNA was also seen in the inner medulla after 5 days of treatment. These results confirm that expression of the EP3 and EP4 mRNA in the renal medulla is reduced by furosemide treatment raising the possibility that the expression of EP3 and EP4 is promoted by hyperosmolality.

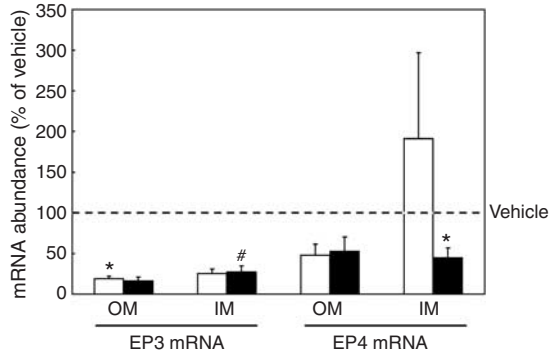

Figure 1 | Effects of furosemide on renal expression of EP3 and EP4 mRNA. Rats were administered vehicle or furosemide (40 mg/day) for 1 day (open bars) or 5 days (solid bars). mRNA for EP3 and EP4, and 18S rRNA were measured from outer medulla (OM) and inner medulla (IM) of the kidney using quantitative RT-PCR. The abundance of EP3 or EP4 mRNA was corrected for RNA loading using the abundance of 18S rRNA. Abundance of mRNA in furosmide-treated animals relative to vehicle treated animals is shown. Mean + s.e.m., $n = 4$. $^{*}P < 0.05$, $^{\#}P < 0.01$ compared to vehicle.

Induction of EP3 and EP4 by hypertonicity

We examined kidney-derived cell lines—Madin–Darby canine kidney (MDCK) and mIMCD3 cells—for expression of EP3 and EP4. RT–PCR analyses revealed that MDCK cells expressed EP2 and EP4 (Figure 2a), and mIMCD3 cells expressed EP1 and EP4 (not shown). In MDCK cells, the amount of the EP4 RT–PCR product increased in response to hypertonicity in correlation with increase EP4 protein expression (Figure 2b) demonstrating the induction of EP4 by hypertonicity. The amount of the EP2 RT–PCR product also increased in the hypertonic cells. However, we could not demonstrate increased EP2 expression because immunoblot analysis did not work. We screened a number of other cell lines to find cells that expressed both EP3 and EP4, but not EP1 and EP2. The lack of EP1 and EP2 would allow examination of the physiological role of EP3 or EP4 without the complications stemming from EP1 and EP2. We found that HepG2 cells satisfied these criteria (Figure 2a). Although HepG2 cells are not derived from the kidney, they provide an

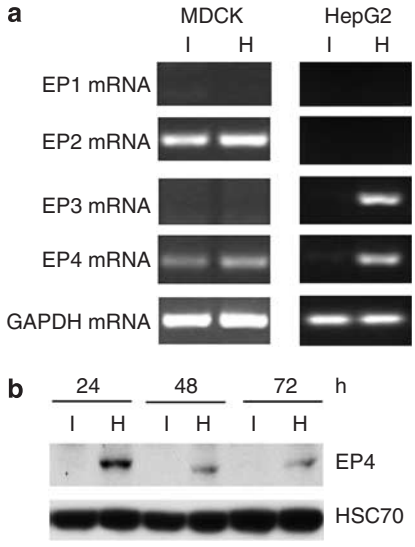

Figure 2 | Effects hypertonicity on expression of EP3 and EP4. (**a**) Confluent MDCK and HepG2 cells were cultured for 24 h in isotonic (I) or hypertonic medium (H, made by addition of 100 mM NaCl). RT–PCR was performed to detect mRNA for EP1, EP2, EP3, EP4, or GAPDH (glyceraldehyde 3-phosphate dehydrogenase). The amplified bands were confirmed by sequencing. A representative of three independent experiments is shown. (**b**) Confluent MDCK cells cultured as in **a** for 24–72 h were immunoblotted for EP4 and HSC70.